Toxicogenomics: a transcriptomics approach to assess the toxicity of 4-nitrophenol to *Saccharomyces cerevisiae*

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I hereby declare that this thesis is based on work conducted by myself and that the work has not been accepted in substance for any other degree. All references to ideas and work of other researchers has been specifically acknowledged.

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Abstract

Since the industrial revolution there has been a significant increase in the production, use and release of man-made chemicals (xenobiotics) into the environment. This is cause for concern because the toxicity of some xenobiotics are unknown, consequently there is an increased need for high throughput sensitive assays that can be used to detect and evaluate the toxicity of xenobiotics. The advent of transcriptomics has provided scientists with a sensitive, accurate high throughput method to measure gene expression in response to chemicals (toxicogenomics). The aim of this work was to investigate the effects of the widely distributed xenobiotic and model organic pollutant, 4-nitrophenol on gene expression in the model eukaryote *Saccharomyces cerevisiae*. This would assess if this chemical had more subtle effects on cells than previous traditional biochemistry studies revealed and to see if certain genes could be used to develop a specific microarray test to detect the presence of 4-nitrophenol in the environment.

Traditional growth inhibition tests were used to ascertain the toxicity of 4-nitrophenol to *S. cerevisiae*. Traditional tests were used to establish EC_{10} & EC_{50} concentrations in standard defined media (SDM). Subsequently *S. cerevisiae* were exposed to 10 & 39 mg/l 4-nitrophenol in SDM and samples taken for expression profiling when conditions were optimal, one, two and three hours after 4-nitrophenol exposure. qRT-PCR was used to validate the gene expression results.

Approximately 600 genes were increased in expression and ˜600 genes were decreased in expression at 10 & 39 4-nitrophenol. Genes associated with RNA processing, ribosome formation, mitochondrial biogenesis, and respiratory activity were differentially expressed. Time series analysis showed 4-nitrophenol caused damage to cell walls and membranes as inferred from increased expression of genes for cell wall and membrane synthesis (DCW1, GRE2). This resulted in hypo-osmotic stress (increased expression of SLN1, & AQY2) and decreased expression of genes involved in cell replication (MDY2, PAN3). At 39 mg/l 4-nitrophenol expression of additional drug resistance genes increased after one (PDR3, PDR15, PDR16), two (PDR3, PDR15) and three (PDR5) hour's exposure. After two hours cells had respiration deficiencies shown by; increased expression of RIM2 a mitochondrial carrier protein, which rescues respiration deficient cells, and decreased production of mitochondrial oxidoreductases. Fourteen iron homeostasis genes were increased in expression and iron requiring cytochromes and oxidoreductases were decreased in expression alongside glucose transporter encoding genes. The results showed respiration was reduced and implicated an increased requirement for iron. Expression of general Environmental Stress Response (ESR) genes initially decreased (one hour of exposure to 39 mg/l 4-nitrophenol). However, three hours after the addition of 4-nitrophenol expression of ESR genes increased. ESR genes are known to be repressed for up to two hours after chemical exposure, and are known to be involved in respiration. The results in this study show reduced respiration is temporary. Increased expression of genes involved in respiration and growth after three hours show that treated cells have adapted to 4-nitrophenol presence. Only two iron homeostasis genes were increased in expression after three hours exposure to 39 mg/l 4-nitrophenol showing iron concentrations inside the cell have stabilised.

Exposure *to* 4-nitrophenol resulted in hypo-osmotic stress, probably caused by membrane damage. This led to decreased intracellular iron concentrations and increased oxidative stress, iron availability directly controls expression of ESR genes and oxidoreductases and may explain the effects seen on mitochondrial respiratory activity and the general stress response observed. The study confirms biochemical results which have shown 4-nitrophenol damages cell membranes and reduces respiration, and implicates iron deficiency in playing a role in this process. It also shows that at sub lethal concentrations cells can adapt their respiration and growth to survive in the presence of 4-nitrophenol.

Chapter 1

1. Toxicogenomics: Transcriptomics of yeast exposed to 4-nitrophenol

1.1 General introduction

In recent years, there has been wide concern over the introduction of chemicals into the aquatic environment. Environmentalists, scientists, and regulators have studied the toxic effects of a wide variety of chemicals (including the widely distributed, 4-nitrophenol) using traditional toxicity testing methods (Bergauer *et al* 2004; Gemini *et al* 2005; Williamson *et al* 1995; Lei *et al* 2003; Lei *et al* 2003; Mitra and Vaidyanathan 1982). Physiological and biochemical investigations with 4-nitrophenol have found it damages mitochondria and inhibits energy metabolism (TenBrook *et al* 2003, Williamson *et al* 1995; Braunbeck *et al* 1988). Additionally sensitive assays (Hershberger & uterotrophic assay) with rats have identified that 4-nitrophenol concentrations as low as 10µg/l can affect the endocrine and reproductive system when repeated doses are given (Li *et al* 2006); this concentration is equivalent to the acceptable concentration in natural water set by the US EPA (Karim & Gupta 2003). Traditional toxicity methods are insensitive, the type of species affects the way results can be measured for example; microbial tests measure growth inhibition because microbes grow quickly, but tests with animals measure mobility inhibition and behaviour (Mitchell *et al* 2002; McKim *et al* 1987; Blaise *et al* 2000). Differences in the way studies are conducted make species comparisons difficult, but chemicals universally affect gene expression. The measure of gene expression (transcriptomics) is extremely sensitive so low chemical concentrations can be tested to identify subtle effects that cannot be identified in a traditional toxicity test (Heijne *et al* 2003). Additionally *in vitro* tests can be used with cell cultures to establish the No Transcriptional Effect Level (NOTEL) reducing or even eliminating the need for whole animal tests, expanding the range of cells and chemicals that can be tested, and significantly increasing the speed and sensitivity of toxicity tests (Poynton *et al* 2008; Labenhofer *et al* 2004). Proteomics has also been used to identify changes in the protein composition of the cell, but this is more difficult and time consuming than measuring transcription. Transcriptomics has previously been successfully used to study the interactions of chemicals with model organisms; this study adds to previous studies by using the model organism (*S. cerevisiae*) and a model pollutant (4-nitrophenol). The availability of toxicity information for 4-nitrophenol based on physiological and biochemical studies makes it an ideal candidate for a

transcriptomics study; additionally tools including commercially available standardised microarrays for *S. cerevisiae* are available. Traditional toxicity tests were used to establish the toxicity of 4-nitrophenol to *Saccharomyces cerevisiae*, and the effects of the EC_{10} & EC_{50} concentrations' on gene expression over a three-hour exposure period were investigated using microarrays.   The gene expression changes were used to ascertain the toxic effects of 4-nitrophenol on *S. cerevisiae* at high (significantly inhibitory) and low (not significantly inhibitory) concentrations. Low and high concentrations of chemicals can damage cells in different ways (Rudzok *et al* 2010), leading to different gene expression patterns that can be used to identify how the chemical causes damage to cells and how concentration affects toxicity. Most frequently, gene expression changes occurring in the first two hours of exposure to chemicals are measured, this study investigates changes occurring one, two and three hours after exposure. It provides a profile of gene expression changes during exposure to 4-nitrophenol, identifying the processes affected by treatment.

1.2   Pollution & toxicity testing

Pollution of water and aquatic toxicology

Industrial development has led to the widespread use of manmade chemicals (xenobiotics) (Valavinidis *et al* 2006) as well as the release of high concentrations of naturally occurring heavy metals in the environment (Bailey *et al* 1999). The presence of pollutants in water is of particular concern because water is an important natural resource. Legal restrictions have been put in place to prevent contamination of water (**Table 1.2.1**) (Park *et al* 2003; Niu *et al* 2009; McKim *et al* 1987).

Table 1.2.1 shows the legal restrictions set by the US EPA for a selection of pollutants.

Chemical	Legal restriction in water	Agency	Potential harm caused
Copper	1,300 µg/l	US EPA	Liver kidney damage
Arsenic	6 µg/l	US EPA	Increased risk of cancer
Cadmium	5 µg/l	US EPA	Kidney damage
Lead	0 µg/l	US EPA	Kidney problems
Bromate	0 µg/l	US EPA	Increased risk of cancer
Chorine	4 mg/l	US EPA	Eye nose irritation
4-nitrophenol	9 µg/l	US EPA	Suspected mutagen
Benzene	0 µg/l	US EPA	Increased risk of cancer
Chlorobenzene	100 µg/l	US EPA	Liver kidney problems
Dichlorobenzene	600 µg/l	US EPA	Liver kidney problems
Dioxin	0 µg/l	US EPA	Reproductive
Pentachlorophenol	0 µg/l	US EPA	Increased cancer risk

The legal restrictions for chemicals are set very low, especially for those that increase the risk of cancer when consumed. The restrictions are decided following aquatic toxicity testing which aims to study the effects of manufactured chemicals and other anthropogenic and natural materials on aquatic organisms (Rand *et al* 1995). Chemicals found in the aquatic environment include non-specific pollutants that act as narcotics and chemicals with a specific mode of action (reactive chemicals). Chemicals are described as narcotics when their toxicity is related directly to the quantity of the toxicant acting upon the cell; a large array of organic chemicals can act as narcotics (Mitchell *et al* 2002). Narcotics are non-reactive, whereas chemicals with specific toxic mechanisms are reactive and more potent (Ren 2003). Specific mechanisms include respiratory uncoupling, electrophilic reactivity, and central nervous system seizures in higher organisms. Specific reactions result from a chemical reaction with an enzyme, or inhibition of a metabolic pathway through the toxin binding to protein receptors. Specific reactions are dependent on both the specific structure of the toxin and the protein (Mitchell *et al* 2002); the diagram in *Figure 1.2.2* diagrammatically represents reactive and non-reactive toxic mechanisms.

1.2.2 Cellular effects of reactive and non-reactive toxins

Both reactive and non-reactive toxicity rely on cellular interactions with toxins for a cellular response to be elicited. The first point of contact for a toxin is the cells plasma membrane receptors there are approximately 20 different families of receptor proteins (Pollard & Earnshaw 2002; Burchett *et al* 2002). The cellular responses elicited by the chemical depend largely of its shape and properties, which determine which proteins it reacts with. In reactive toxicity, the shape and chemical properties of the toxin allow it to fit into the binding site of the receptor protein (*Figure 1.2.2*).
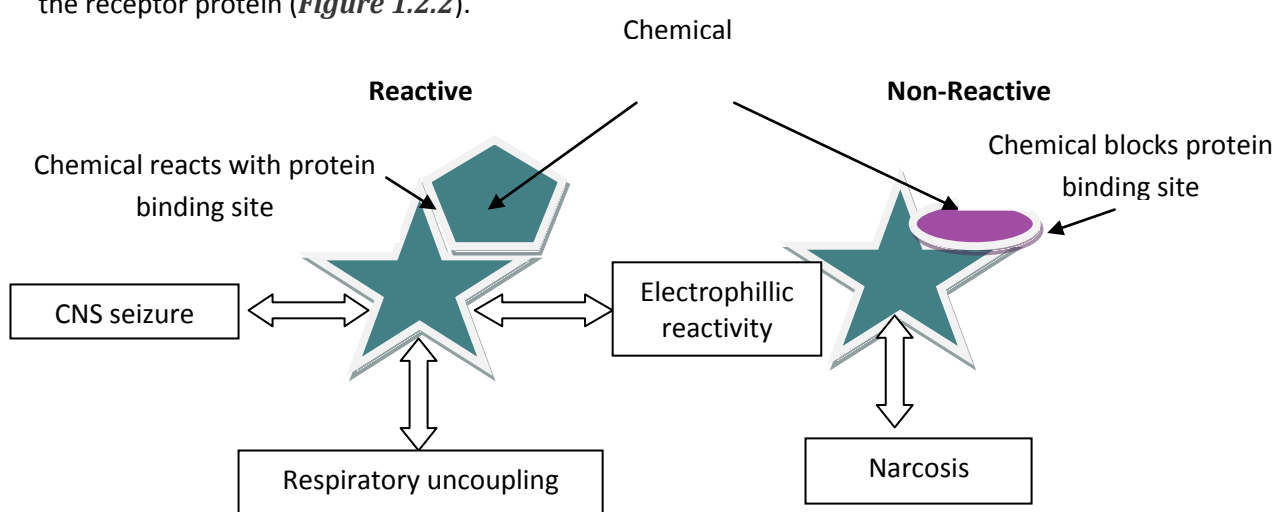
Figure **1.2.2** Reactive and non-reactive toxicity and their biological effects

The chemical reacts with the receptor protein sending signals that may cause the cell to react inappropriately to its environment. It also blocks the protein-binding site so that the correct signaling molecules cannot bind to it anymore. The chemical may have chemical or physical properties that result in permanent binding of the toxin, or structural changes to the proteins binding site, particularly if it has unbound electrons. This can render the receptor inactive in future thus preventing response of the cell to future environmental stimulus necessary for survival. In non-specific (non-reactive) toxicity caused mainly by organic molecules, the effect is related to the concentration of the toxin. This is probably due to membrane protein sites being blocked by the chemical or increased competition for protein binding sites by the toxin molecules. Thus as the concentration of the toxin increases the competition between toxicant and the correct signaling molecules increases and signal molecules become less likely to bind to their receptors (Chaisuksant & Connell 1999). Dysfunction of the cell results because of its inability to detect and respond to environmental stimulants that are important for its survival. Toxicity tests are carried out to determine damaging concentrations of pollutants and set restrictions on chemical concentrations. Sensitivity to chemicals is species dependent so Direct Toxicity Assessment is carried out using various organisms.

1.2.3   Direct Toxicity Assessment

Direct Toxicity Assessment (DTA) uses standardised test organisms and is conducted to establish whether chemicals are toxic. The assessment of toxicity (toxicology) traditionally uses chronic and acute toxicity tests. Chronic toxicity tests investigate the responses of live organisms to varying levels of toxicants over a long period. The results are used to identify an Effective Concentration, usually the concentration at which 50% of the organisms are affected (EC_{50}). DTA testing with microbes' measures growth inhibition and in multi-cellular organisms' movement inhibition and behaviour changes are measured. On the other hand acute toxicity tests have a lethality endpoint and are carried out quickly compared to chronic tests. An LC_{50} (Lethal Concentration) value is used to show the concentration at which 50% of the test organisms suffer mortality. Acute toxicity tests provide a basis for understanding the limiting effects of various chemicals on organisms; they have a discrete end and are easily and relatively cheaply conducted (Poston and Purdy 1985). However chronic toxicity tests are preferable because they can identify the effects of sub-lethal concentrations of pollutants but they take a long time to conduct, and they are expensive because they are restricted to laboratories and live organisms need to be continuously cultured (Mitchell *et al* 2002). The organisms chosen for toxicity tests are usually microorganisms, plants and animals (Blum and Speece 1990).

1.2.4 Toxicity testing using animals

Animal tests are considered to be inhumane however they are preferable to microbial tests because they provide information on long term ecological effects. Studying the accumulation of toxins in tissues of animals helps develop understanding of the passage of toxins from one organism to another (Abram 1993). For example Algae are the dominant primary producers in most aquatic food chains, but they have an affinity for positively charged ions, such as heavy metals. Heavy metals adsorb to cell surfaces increasing the availability of metals to organisms that consume algae, resulting in increased concentrations in successive members of the food chain (Mitchell *et al* 2002). The Fish Acute Toxicity Syndromes (FATS) are commonly used to asses toxicity, they are based on physiological-biochemical and behavioural measurements (McKim *et al* 1987). The fish used are either laboratory bred, farmed, or caught in the wild (Abram 1993). Species commonly used in monitoring of chemicals discharged into freshwaters include: The vertebrate *Pimephales promelas* (the fathead Minnow *Figure 1.2.4a*) and the invertebrate *Ceriodaphnia dubia* (the water flea *Figure 1.2.4b*) (Doherty 1996). Invertebrates are used alongside vertebrates to compensate for species selectivity which may occur (Doherty 1996).

Figure **1.2.4a** Fathead minnow   *Figure* **1.2.4b** water flea
(www.Environmentalhealthnews.org)   (www.nanopedia.case.edu)

The number of vertebrates used in aquatic toxicity tests need to be reduced because:

- Data generation is slow, limiting the number of samples that can be tested
- Large numbers of fish are required for tests making it expensive,
- Fish are not easily bred in the laboratory, and genetic diversity is limited
- Fish are protected animals in Britain thus studies using fish may be considered inhumane (Mitchell *et al* 2002; McKim *et al* 1987).

It has been proposed that the use of whole animal testing will eventually be replaced by the use of improved chemical, physical, biological and mathematical techniques. To this aim DTA (**1.2.3**) using *in vitro* cell culture methods (**1.2.5**) and microbes (**1.3**), can be used to assess the toxicity of chemicals (Fentern and Balls 1993).

1.2.5   *In vitro* cell culture toxicity testing

In vitro toxicity testing was introduced in 1989 in response to calls for reduced use of live animals. Cell culture methods and cell lines have been validated for toxicology studies; cell lines validated include hepatocytes, lymph nodes, and epidermal cells (Speilmann & Liebsch 2001; Baudoin *et al* 2007). Comparative studies have found the results of *in vitro* and *in vivo* studies are correlated and the use of *in vitro* tests has increased since their validation (Sayes *et al* 2007; Benassi *et al* 1999).

1.3   Microbial toxicity tests

1.3.1   Advantages of using microbes in toxicity testing

Variability in the results of toxicity tests using animals can occur, because of; Type of species, life stage, size of individual, nutritional status, health. In addition environmental (abiotic) factors such as; temperature, pH, dissolved oxygen content, salinity or hardness of water can also affect results. Individual differences such as; interclonal and genetic differentiation, differences in sequestration and elimination of contaminants, prior history of exposure, adaptation and different lifestyles can also affect the results of a toxicity test (Mitchell *et al* 2002).   One of the major advantages of using microbes is their ability to overcome many of the factors, which can affect the result of a toxicity test. Millions of microbes can be cultured in a small amount of defined media and sampled during mid-logarithmic growth, i.e. when the majority of cells are in a similar life stage and of a similar size. In addition, conditions can be kept constant, genetic clones can be used and exposure history of laboratory strains is known, all of which decreases variation in results.

1.3.2   Microbial tests for monitoring toxicity

The number of chemicals and environmental samples has grown enhancing the need for quick, cost effective testing. Microbial toxicity tests are quick, easy to use, and cost effective requiring few resources and little space (Blaise *et al* 2000). Microorganisms grow rapidly and are often used by man in combating toxicity in waste systems. They have properties, which make them particularly useful for this role such as, being versatile, having a wide range of metabolic activities, high reproduction and mutation rates, which allow them to adapt to new chemicals under a wide range of environmental conditions. Microorganisms are however, sensitive to environmental changes and chemicals can reduce their growth rates, cause the inhibition of metabolic pathways resulting in their death, devastating biological systems (Painter 1993). However, these properties also make them extremely useful for measuring the toxicity of chemicals.

1.3.3 Microorganism growth regulation in response to a changing external environment

The direct contact of microbes with the external environment means microbes have developed mechanisms to respond to variations in external conditions. Alterations in growth rates occur in response to toxins and changes in morphology and physiology accompany those (Rolland *et al* 2002). The term growth describes two distinct processes: an increase in cell mass, and cell proliferation (an increase in cell number); cell numbers are usually measured in microbial toxicity studies (Schmelze & Hall 2000). Cells contain internal and external receptor proteins, which allow recognition, and binding of their signaling molecules. The quality of the external environment (i.e. nutrient availability, toxin presence) is conveyed to cells via receptor proteins so they can alter growth processes accordingly (Schneper *et al* 2004)

1.3.4 Microbial growth inhibition assays

Growth/multiplication inhibition tests are used widely in microbiology to estimate the effects of differing environmental conditions. In toxicity tests the growth inhibition of the microorganisms to varying levels of toxicants are investigated. Observations made during the experiment can be used to generate a dose/concentration response curve such as the one in *Figure* **1.3.4** (Mitchell *et al* 2002).
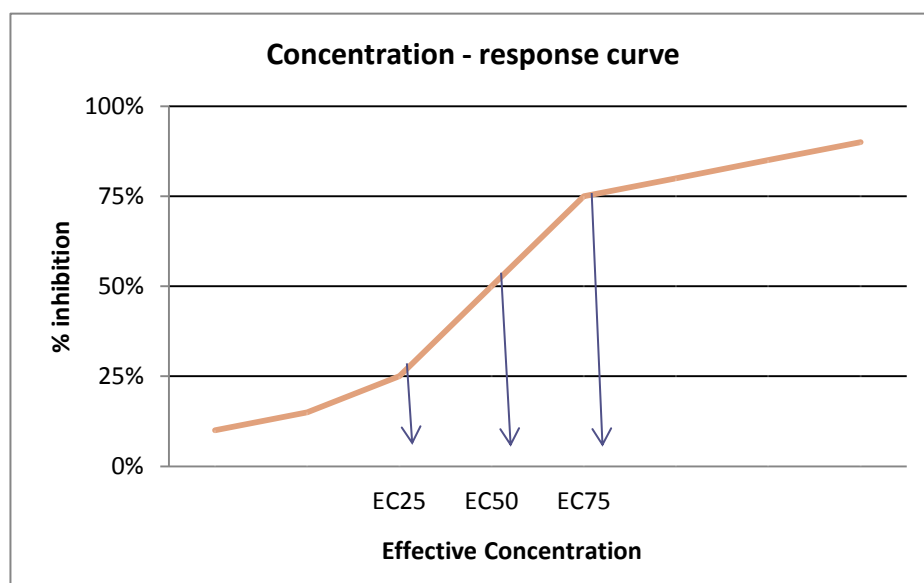
Figure **1.3.4** An example of a concentration-response curve calculated from chronic toxicity testing

Chronic toxicity tests are used to compare different species; organisms differ genetically and physiologically making the sensitivities and responses very different. For this reason

comparisons are often made by comparing the responses of organisms at the same Effective Concentration (e.g. for microbes 50% growth inhibition is the EC_{50}).

1.3.5 Microbial toxicity tests

As previously discussed exposure to toxins, causes decreased growth rates in microorganisms and this can be measured to assess toxicity. The Microtox test uses luminescent bacteria; *Vibrio fischeri* decreased luminescence is related to a decreased cell number and is a means of detecting toxicants. Microtox can be used in aquatic environments and was designed in response to new regulations in the petrochemical industry as a quick screening test for acute toxicity (Isenberg 1993). Microtox (**Figure** 1.3.5) is conducted in a temperature-controlled photometer; the luminescent data is transferred to a PC and analyzed by a software package, decreased light output gives a quantifiable indication of toxicity.

Increasing toxin concentration
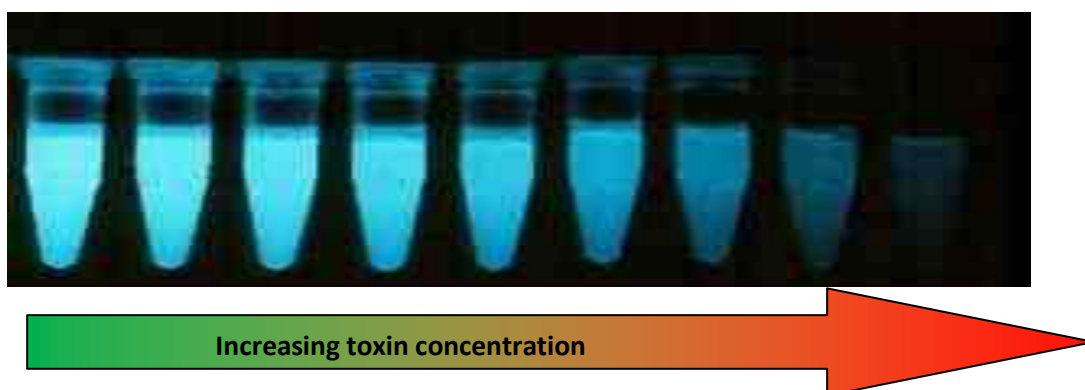
Figure 1.3.5 Decreased luminescence of *Vibrio fischeri* in response to increasing toxin concentrations (image from http://myweb.scu.edu.tw/~94134001/2.files/image009.jpg)

Only a small sample is required, and few laboratory supplies and space are required for the test. Since the development of Microtox numerous toxicity tests utilising living organisms have been devised, all of which are useful but the sensitivities of them vary. In a study conducted by Manusadzianas *et al* (2003) the sensitivity of Microtox was compared with several other microbial toxicity tests, a description of each test and the results of comparisons are shown in **Table 1.3.5**.

Table 1.3.5 shows the sensitivities of microbiotests to urban waste in the investigation carried out by Manusadzianas *et al* (2003).

Test type	Microorganism	Measurement	Detection capacity	Quantifying capacity
Daphtoxkit	*Daphnia magna*	Change in fluorescence	43%	33%
Thamnotoxkit	*Thamnocephalus platyurus*	24 hour LC_{50} calculated	81%	62%
Charatox	*Nitellopsis obtuse*	After 45 mins at EC_{50} cell membrane, depolarisation measured.	90%	71%
Microtox	*Vibrio fischeri*	Change in luminescence	57%	33%

The comparison in **Table 1.3.5** shows that the Charatox test has the greatest toxin detection and quantification capacity followed closely by Thamnotoxkit. This demonstrates that tests based on 50% lethality (LC_{50}) and effective (EC_{50}) concentrations can outperform luminescence and fluorescence based assays in both detection and quantification. However, decreased growth is an effect seen at a relatively high chemical concentration but subtle changes such as DNA damage can occur because of exposure to low toxin concentrations. To address this issue several microbial tests capable of detecting DNA damage have been developed.

1.3.6   The SOS chromotest for genotoxicity

The SOS chromotest takes advantage of *Escherichia coli's* capacity to respond to DNA-damaging events via the SOS response pathway; it is used to determine the genotoxic status of chemicals (Blaise *et al* 2000). A specific *sul*A *lac z* gene fusion resulting in the production of β-galactosidase, *sul*A is inducible by DNA damaging agents and a colimetric assay can be used to detect increased β-galactosidase (Blaise *et al* 2000) (*Figure* **1.3.6**).   Acute toxicity results in no colour, DNA damage can be seen as dark blue and the background colour (Blank) can be seen in column one.
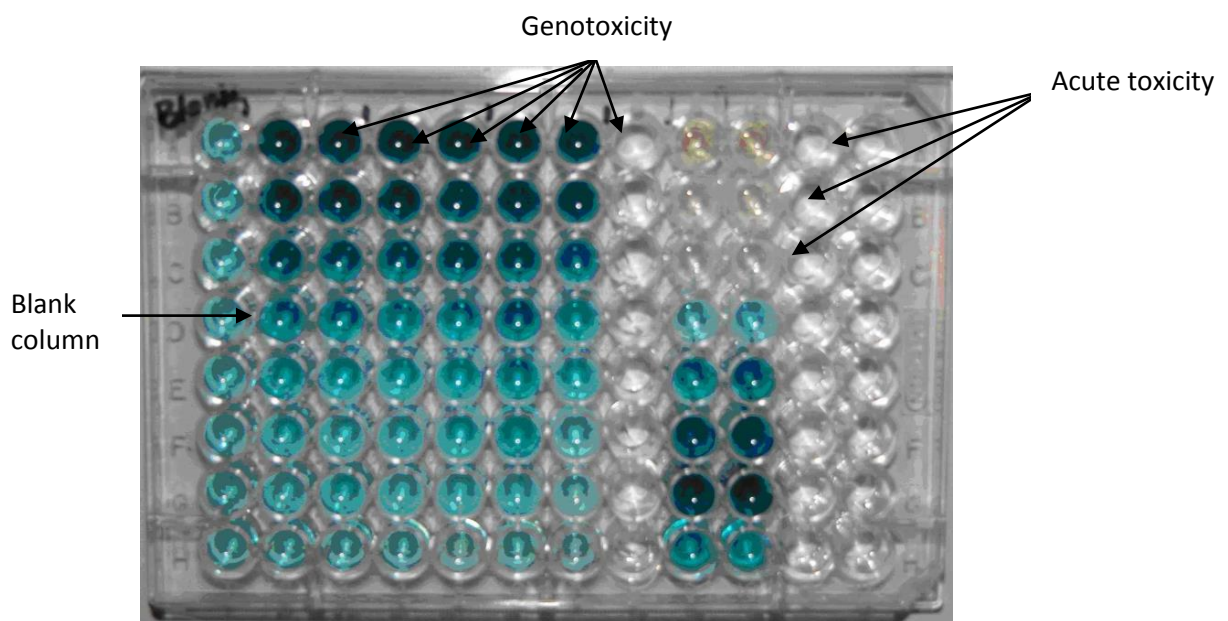
Figure 1.3.6 The results of an SOS chromotest, showing genotoxicity and acute toxicity
(http://www.ebpi-kits.com/images/SOS-Chromo.jpg)

Bioassays such as the SOS chromotest are advantageous because they are rapid, reliable and can be used without prior knowledge of the contaminant (Chen & White 2004). However only mutagens that interact with the DNA chemically induce the SOS response in *E. coli*, and the SOS response pathway is only used when DNA mutations can be accurately repaired. An

alternative unrelated translesion DNA synthesis pathway exists.   The SOS chromotest can only detect some types of DNA damage. Consequently, the SOS chromotest is often used alongside other genotoxicity tests such as the Ames test (Vasilieva 2002).

1.3.7   The Ames test for genotoxicity

Other bacterial tests for genotoxicity have been developed; the Ames test uses *Salmonella typhimurium* it is used to screen chemicals for genotoxicity, it has been used to detect mutagenicity in ground and surface waters in over 3000 locations (Kim *et al* 1999; Sasaki *et al* 2000; Siddiqui & Ahmad 2003). The Ames test uses histidine auxotrophs grown and exposed to chemicals on/in minimal media with a limiting amount of histidine. Mutations result in the ability to make histidine and cells exposed to mutagens grow (Margolin *et al* 1981). The results from Ames tests have proved interesting because the genotoxicity of compounds has been found to decrease at high chemical concentrations (Kim *et al* 1999). Margolin *et al* (1981) attributed the decreased genotoxicity at high concentrations to acute toxicity of chemicals inhibiting the growth of *S. typhimurium.* Prokaryotes are used in both the SOS chromotest, and Ames test, however biological assays that measure mutagenicity in eukaryotes are also needed to account for chromosome damage and double strand breaks (Lui *et al* 2008).

1.3.8   Eukaryotic genotoxicity tests

The eukaryotic *Saccharomyces cerevisiae* has been widely used for toxicity testing and for the detection of gene mutations in culture systems. It is a single celled conserved model eukaryote with known genetics (Weiss *et al* 2004). DNA damage results in the expression of RAD genes, which have been fused with Green Fluorescent Protein (GFP), so that increased fluorescence is indicative of DNA damage.   In addition luminescent luciferase genes have been used as DNA damage reporters (Liu *et al* 2008). *S. cerevisiae* is ideal for studying DNA damage because a wealth of information is available about its genetic regulatory mechanisms (DeRisi *et al* 1997). Additionally genetic studies have shown that *S. cerevisiae* genes are similar and perform similar functions to those of mammals and the control of its genes follows similar mechanisms. Making *S. cerevisiae* an ideal eukaryote for studying whether pollutants cause genetic mutations and the potential effects of mutations in eukaryotes (Weiss *et al* 2004).

1.3.9   The use of *S. cerevisiae* in genomics

Relatively few organisms have had their genomes sequenced, however the genome for *S. cerevisiae* was sequenced in 1996 and it was the first eukaryotic genome to be sequenced (Baxevanis & Oullette 2001). In addition a complete set of gene deletion strains (~6000 genes) are available providing a unique resource for assessing the functional role of individual genes (Giaever *et al* 2002; Weiss *et al* 2004). Recent advances in technology have allowed for

measurement of the way in which DNA sequences give rise to functional units (i.e. proteins) in cells (Phelps *et al* 2002), termed transcriptomics (**1.4.1**) (Aardema & MacGregor 2001). *S. cerevisiae* is an ideal microorganism for studying how pollutants affect cell growth and proliferation as well as gene expression (transcriptomics) (Weiss *et al* 2004).

1.4   The emergence of toxicogenomics: Combining genomics and toxicology

1.4.1   Transcriptomics

Cellular responses to changing environmental conditions rely on transcription of genes (gene expression) (Pollard & Earnshaw 2002), the study of which is termed transcriptomics (Phelps *et al* 2002). During transcription, sequences of genes are converted by enzymes to messenger ribonucleic acid (mRNA), which is used as a template for protein synthesis (Pollard & Earnshaw 2002). The mRNA can be extracted and measured to determine which genes are being transcribed (expressed) to produce functional proteins (transcriptomics) (Baldi & Hatfield 2002; Baxevanis & Oullette 2001). However, cells contain thousand of mRNA sequences so to determine which genes are differentially expressed array technologies are used.

1.4.2   The use of array technology for monitoring gene expression

Array technologies use libraries of molecular probes (single stranded DNA of known sequence) to monitor changes in gene expression. There are two main forms of array, macroarrays and the more advanced microarrays; both of which utilise an organized arrangement of multiple cDNA probes fixed on an immobilized surface (Baldi & Hatfield 2002; Afshari *et al* 1999). Microarrays are smaller and are constructed by one of two methods; either by spotting prefabricated oligonucleotides on a glass support or by direct in *situ* oligo synthesis on a glass surface by photoligraphy (Nuwaysir *et al* 1999). Thousands of nucleotides are arranged in a grid on a glass microscope slide (microarrays) or nylon filters (macroarrays); molecular probes (DNA gene sequences) are labelled with fluorophores (microarrays) or radioactively labelled (macroarrays) (Pollard & Earnshaw 2002) (**Table 1.4.2**).

Table 1.4.2 Differences between types of expression array

Array type	Support material	Labeling method
Microarray	Glass slide	Fluorophores
Macroarray	Nylon filter	Radioactive isotopes

When labelled samples are hybridised with probes on the array, those bound to the probe produce a signal and the intensity of fluorescence / radioactivity is proportional to the amount of labelled sample allowing an estimation of the amount of mRNA. The mRNA goes on to

produce proteins and the amount of mRNA is proportional to the amount of protein produced so the results can be related back to changes in protein production (Nuwaysir *et al* 1999).

1.4.3 Using transcriptomics and model organisms in toxicology

Chemicals interact with receptor proteins and enzymes, which affects gene expression, protein production and metabolism (Pollard and Earnshaw 2002; Heijne *et al* 2003; Nuwaysir *et al* 1999; Aardema & MacGregor 2001). Transcriptomics can be integrated with traditional toxicity testing methods to assess the effects of chemicals at a molecular level identifying subtle metabolic changes resulting from chemical exposure (Heijne *et al* 2003). It is used with model organisms that have had their genomes sequenced, and the availability of DNA microarrays and gene deletion strains for many model organisms have allowed functional characterization of genes (Neumann *et al* 2002). The functions of most genes in model organisms have been established providing a platform for a function-based approach for drug and chemical safety evaluation.

1.4.4 Toxicogenomics: Aiding cross species comparisons

It is difficult to make species toxicity comparisons because different species have different sensitivities to chemicals. To counteract this EC_{50} values are used to compare species accounting for differences in sensitivity. Differences in the way the EC_{50} is derived (i.e. growth inhibition (in microbes) vs. immobility (in vertebrates) can also complicate comparisons. It has been proposed that genetic similarities will allow comparisons based on gene expression at the EC_{50} concentration. This idea has been further developed and it is thought that obtaining a No Observed Transcriptional Effect Level (NOTEL) from toxicogenomic studies with individual tissues and cells will allow safe concentrations of chemicals to be identified (Poynton *et al* 2008; Labenhofer *et al* 2004).

1.4.5 Potential classification of chemicals using gene expression profiles

Several studies have reported that toxic compounds produce specific gene expression patterns that relate to their mechanisms of toxicity (Kier *et al* 2003; Amin *et al* 2002; Bouton *et al* 2001; Burczynski *et al* 2000; Meydan *et al* 2008; Hamadeh *et al* 2002). As a result, chemicals have been classified into toxicological classes using gene expression profiles (Thomas *et al* 2001; Suter *et al* 2004; Ellinger-Ziegelbauer *et al* 2008; Falciani *et al* 2008). Chemical exposure can result in the production of general and specific stress response proteins in cells (Heijne *et al* 2003). Toxicogenomics could eventually allow signature patterns to be recognized, and attributed to chemicals acting by specific mechanisms (Burczynski *et al* 2000; Aardema & MacGregor 2001; Pennie 2000; Nuwaysir *et al* 1999).

1.4.6   Applying toxicogenomics to genotoxicity

Based on the assumption that compounds with similar mechanisms of toxic action cause specific gene expression changes the toxicity of structurally related compounds can be predicted from gene expression data (Thybaud *et al* 2007). The sensitivity of toxicogenomics makes it an ideal technology for identifying potentially genotoxic compounds. As previously discussed (**1.3.7**) gene mutations cause changes in the expression of DNA damage repair genes. Toxicogenomics can thus be used to identify DNA damage response genes expressed in response to toxins to identify mutagens. *S. cerevisiae* is a useful model for assessing toxicity, and genotoxicity, and the effects of chemicals on gene expression in *S. cerevisiae* and mammalian cells are similar (John *et al* 2005).

1.4.7.   Response to stress in *S. cerevisiae*

Gene expression microarray analyses of *S. cerevisiae* have already shown ordered global environmental stress response pathways act to protect cells from chemicals (Weiss *et al* 2004; Gasch & Werner-Washburne 2002). The transcription of around 200 genes constitutes the general stress response in *S. cerevisiae*. This response is mediated by a number of stresses, including; oxidative, heat, osmotic, pH, glucose, and chemical stresses. The general stress response genes known as STress Response Elements (STRE) are transcribed under stressful conditions. They contain the STRE sequence, (5'CCCCT) in several of their regulatory regions and are activated by two structurally related zinc finger proteins Msn2 and Msn4 (Bose *et al* 2005; Neumann *et al* 2002; Weiss *et al* 2004).

1.5   Genomic technologies; history, developments and standardisation

1.5.1 Expression technology: The emergence of macroarrays

Macroarrays were developed from the Northern blot method for detecting specific RNAs in agarose gel. During Northern blot, RNA is separated in an agarose gel and attached to diazobenzyloxymethyl (DBM) groups on Whatman filter paper. Radioactively labelled DNA probes are hybridised to the RNA and visualised with autoradiography. The Northern blot method is still used today; however, macroarrays are advantageous because they can measure hundreds of genes on a single array. As previously mentioned (**1.4.2**) macroarrays were the first global method of monitoring gene expression and use hundreds of cDNA clones spotted on nylon / nitrocellulose membranes to compare gene expression profiles. The first use of this approach used a nylon membrane filter (8 x 12 cm) and the technology was referred to as high-density filter analysis (HDCFA) (Brown 2005).

1.5.2 Moving from macroarrays to microarrays

Macroarray technology has advanced, and smaller glass/silicon microscope slides, called microarrays are now used. Macro and microarrays utilise the same concept, but microarrays are smaller, contain thousands of probes, and use fluorescence to detect ESTs. The miniaturisation of the macroarrays was initially aimed at standardising array methodologies (Brown 2005). Obtaining consistent high quality data from microarrays is difficult because of the complex nature of a microarray experiment and the many sources of variability.

1.5.3 Sources of variation in microarray experiments

Time series analysis considers gene expression profiles of samples taken at different time points, and biological differences arise from changes in gene expression over time (Lloyd and Murray 2006; Rustici *et al* 2004).   Biological differences are not considered to be a problem in gene expression studies because they reflect true variation among individual samples (Zakharkin *et al* 2005). If biological differences account for the variation in gene expression patterns observed they can provide important information that can aid data analysis. Sample-handling procedures used in a microarray experiment can also affect the reproducibility and quality of data. Variability in the results of toxicogenomic experiments can be reduced by using standardised procedures, the impact of biological variation can be reduced by using experimental repeats (Novak *et al* 2001; Zakharkin *et al* 2005). To reduce variation and improve the reliability of microarray experiments guidelines (1.5.4) have been laid out for conducting microarray experiments and recording information.

1.5.4 MIAME guidelines

The Microarray Gene Expression Database Society (MGED - http://www.mged.org) was founded in 1999 and adopts standards for microarray experiments. Standards aim to improve gene annotation, data representation, experimental controls and data normalisation methods used when recording and reporting array data. MGED published documents stating the Minimum Information About a Microarray Experiment (MIAME) that should be submitted to public databases (http://www.mged.org/Workgroups/MIAME/miame.html). The MIAME standards outline the minimum information required to interpret unambiguously, and potentially reproduce and verify array based gene expression profiling experiments. MIAME/Tox is specific to toxicogenomics and defines the information that is common to toxicogenomic studies (Brown 2005; mged.org 2009). Guidelines minimise biological variation but the complex nature of experiments means technical variation occurs in array studies (1.5.5).

1.5.5   Sources of technical variation in microarray experiments

Technical variability can occur because of sample quality, labelling protocol, hybridisation conditions, scanning instruments, image acquisition, processing of images. Microarray technologies have been developed and standardised to limit variation derived from the technology, and microarray experiments are carefully planned and conducted according to MIAME guidelines (1.5.4). Technical variation can detrimentally affect statistical analysis and subsequent data analysis (Baldi & Hatfield 2002). Technical variation can be introduced by conducting different parts of the experiment in different laboratories, doing RNA extractions, RT-PCR, and labelling protocols separately as well as using different batches of microarrays and a different microarray scanner.   To reduce technical variation in microarray experiments, replication and standardised procedures are used in both sampling for expression profiling, preparing samples for microarray hybridisation, and hybridisation procedures (1.5.6).

1.5.6   Standardised microarray experimental procedures

The production of expression microarrays is a multi-step procedure, and to conduct a successful toxicogenomic study precision and attention to detail are required.   Variation that arises from equipment and reagents needs to be carefully controlled. A large number of factors need to be considered including but not limited to those highlighted in **Table 1.5.6**.

Table 1.5.6 Technical causes of variation in microarray experiments and ways to minimise their impact upon microarray results.

Cause of	Factor causing variation	Ways to minimise impact
Reagents	Batch to batch differences	Use reagents from the same batch
Microarray	Batch to batch differences	Use microarrays from the same batch in experiments.
Microarray	Differences in signal intensities	Normalise data so that the mean of signal intensities is used in statistical tests.
Microarray	Fluorescence of organic molecules causing background fluorescence (noise)	Filter data so that signals below the background noise threshold are not considered.
Microarray	Hybridisation efficiency	Do hybridisations together
Scanner	Signal measurement sensitivity	Scan all microarrays on the same scanner, use spike in controls for calibration.

Baldi & Hatfield 2002, Novak *et al* (2001), Zakharkin *et al* (2005)

As well as meticulously planning a microarray experiment to consider all of the variables in **Table 1.5.6** various standardisation techniques have been employed to improve the reliability of results. Current approaches for standardisation use universal reference samples, which work by generating a uniform fluorescent signal across the probes on an array. This provides a base level against which the abundance of transcripts from test samples can be compared. Additionally known RNA samples are spiked into the hybridisation mixture which can be used a

references to quantify RNA (Anderson & Foy 2005). Mathematics is then used to scale (normalise) the data to compensate for errors arising from sample handling or technical variation, and prevent these factors affecting the results (Baldi & Hatfield 2002).

Normalisation: theory, and importance in expression analysis

The normalisation process ensures that data sets are comparable (Baldi & Hatfield 2002); there are two normalisation procedures, local and global normalisation. The data is globally normalised before being locally normalised and the cut-off values used (i.e. 0.01 signal intensity values, to remove background noise) are the same in both local and global normalisations.

Global normalisation compares the gene expression profiles of samples across different microarrays by calculating a normalisation factor from the signal intensities of probes on the microarray. The signal intensities for each probe on the microarray are corrected using a normalisation factor calculated from distribution patterns, equalising the median intensities across the microarrays. Global normalisation methods take into account batch-to-batch variation, and potential differences in hybridization efficiency (Agilent 2008).

Affymetrix microarrays have multiple probes per gene 'probe sets', local normalisation transforms the data set to the median per gene, removing non interesting biological differences, such as differing amounts of RNA. This method scales the expression data so that correlations seen are as a result of experimental baseline, and not because of differences in the way that the data was scaled (Agilent 2008). Global and local normalization are incorporated into probe summarisation algorithms in GeneSpring GX 10^{TM} (**1.5.8**), that perform in order, background noise correction, global normalisation, local normalisation and probe summarisation (Agilent 2008).

1.5.8   Probe summarisation

Probe summarisation algorithms make expression signal values comparable by correcting for background fluorescence (noise), calibrating signal intensity values to spike-in and housekeeping probe sets (global normalisation), and converting probe level values to probe set expression values (local normalisation).

Robust Multi-array Analysis (RMA) is a probe summarisation method that can remove errors due to technical variation (i.e. degraded cRNA) making bad data look better. RMA removes background noise and globally (**1.5.7**) normalises data. Signal values reported from Perfect Match probes have exponential distribution, whereas background noise and non-specific binding to Mis Match probes are normally distributed. Corrected signal intensities are calculated by applying a positive distribution to the Perfect Match signal values (eliminates

negative values) and subtracting the difference between normally distributed and exponentially distributed signal values (averaged across microarrays) from the exponentially distributed values (global normalization) (Bolstad 2002; Irazarry *et al* 2003; Agilent 2008). An alternative is Guanine Cytosine Robust Multi-array Analysis (GCRMA, **1.5.8**) which retains some technical variation in the data, allowing problem samples (i.e. samples with degraded cRNA) to be removed after probe summarisation (Irazarry *et al* 2003).

GCRMA is identical to RMA except that the background correction step corrects for probe base sequences. The Guanine Cytosine contents of probes are related to their potential to participate in non-specific hybridization to Perfect Match and Mis-Match probes. GCRMA uses the Guanine Cytosine content of individual probes (Perfect Match and Mis-Match) and subsequent potential for non-specific hybridization to calculate a background correction factor for each probe. Probe signal values are multiplied by their respective Guanine Cytosine content background correction factor, and the resulting corrected probe signal values proceed to quantile normalisation. GCRMA can compensate for genetic differences between individual cells, and provides more statistically significant data than RMA. GCRMA retains some of the technical problems in the data allowing post summarisation analysis to remove any problematic samples (i.e. arrays with degraded cRNA) that can detrimentally affect statistical analysis (Wu *et al* 2004).

1.5.9   Checking the quality of microarray data

Following probe summarization quality checks are used to assess the quality of the data produced from a microarray experiment. Quality checking methods can identify degraded cRNA samples with signal intensity values that do not match the rest of the data (outliers). Outliers are defined as observations whose values do not fall within the values expected given correlations within the dataset (Lalor and Zhang 2001). Removing outliers can increase the statistical significance of results obtained (Hoo *et al* 2002). Two methods are used to view summarised gene expression profiles and assess data quality after probe summarisation, they are; Principal Component Analysis (**1.6.1**), Box Whisker plots (**1.6.2**). Following quality checks on the data, the results can be analysed and interpreted in a number of different ways (**1.6.3**)

1.6   Confirming microarray results using Real Time PCR

1.6.1   Development of Real Time PCR and Quantitative PCR

The first demonstration of RT-PCR was shown by Higuchi in the early 1990s at Roche Molecular Systems, where ethidium bromide was used in a PCR reaction run under UV light and a video camera was used to record the accumulation of DNA. Applied Biosystems first made RT-PCR instrumentation available in 1996, and many companies shortly followed. The benefit of

monitoring the amount of DNA in a logarithmic linear PCR reaction over each amplification cycle is that it gives the researcher the ability to calculate the starting amount of DNA. Quantitative PCR is the process by which the amount of starting DNA is calculated, but real time PCR is the method of choice for the quantification of DNA. Real time PCR amplifies a specific target sequence in a sample and then monitors the amplification process using fluorescence technologies. Because the reaction is linear, the amount of DNA can be measured after each amplification round and used to calculate the starting amount of DNA (Valasek & Repa 2005). Real time PCR testing platforms have equivalent sensitivity and specificity as conventional PCR combined with southern blot analysis (Espy *et al* 2006). Reactions are characterised by PCR cycle where the target amplification is first detected, consequently the greater the quantity of target DNA in the starting material the faster a significant increase in fluorescent signal will appear (Wong & Medrano 2005).

1.6.2   Benefits of Real Time PCR

The advantages of using RT-PCR to quantify gene expression are it can produce data within an accurate dynamic range of 7 to 8 log orders of magnitude, and does not require post-amplification manipulation. In addition, RT-PCR is 10,000 – 100,000 time more sensitive than RNase protection assays, and 1000 fold more sensitive than dot blot hybridisation, RT-PCR can detect a single copy of a transcript. It can reliably detect gene expression differences as small as 23% between samples and have lower variation coefficients. It can discriminate between mRNAs with almost identical sequences and requires much less RNA template than other gene expression analysis methods (Wong & Medrano 2005).

1.6.3   How Real Time PCR works

In real-time PCR, the reaction is set as in a normal PCR with the exception that a detection dye is added to the mixture for the detection of amplification products. Primers specific to the genes being investigated are used so that only those genes are amplified and measured.

Four major phases of PCR occur:

1. The linear ground phase: Fluorescence at background levels
2. Early exponential phase: Fluorescence representative of starting RNA copy number
3. Log linear (exponential phase): Optimal amplification, fluorescence increases linearly with amplification
4. Plateau phase: Reaction components become limited, fluorescence data no longer useful

1.6.4 Requirements for successful RT-PCR

Successful performance of RT-PCR depends on clear understanding of the primary aim of the assay, with specificity, sensitivity and reproducibility being the most important qualitative characteristics. RNA cannot be used as templates for PCR so the first step is to reverse transcribe it to cDNA. If the two procedures are separated the cDNA sample can serve as samples that can be stored indefinitely (Bustin 2000). The RT step can be primed using specific primers, random hexamers or oligo-dT primers. Where mRNA specific primers decrease background priming, the use of oligo-dT primers maximises the number of mRNA molecules that can be analysed from a small sample of RNA (Bustin 2000). The most commonly used enzyme for RT-PCR is Taq DNA polymerase, the only problem with using this enzyme is it does not have 3'-5' proofreading exonuclease activity. If fidelity is a major concern proofreading exonucleases such as Vent and Deep Vent or Pfu should be used. However, proofreading can lead to primer degradation and longer primers are degraded faster. Buffers containing Mg2+ affect enzyme activity and imbalanced dNTP mixtures will reduce polymerase fidelity. In addition to this Mg2+ increases the melting temperature of double stranded DNA. High concentrations of dNTPs interfere with polymerase activity and affect primer annealing by reducing free Mg2+ (Bustin 2000). For these reasons, it is important that the correct amounts and concentrations of reagents used.

1.6.5 Quantitative and semi quantitative RT-PCR without instrumentation

Semi-quantitative RT-PCR results are generated from frequent sampling of the RT-PCR mixture followed by dot-blot analysis. However, semi-quantitative RT-PCR is difficult to automate and results are only semi-quantitative. Quantitative RT-PCR can use competitive or non-competitive techniques. Non-competitive co-amplifies the target with a second spike in known quantity RNA that shares no similarity to the target. The resulting amount of each RNA is used to calculate the original amount using the standard to calibrate the target. However, this process can be unreliable as the efficiency of RT-PCR on different RNA samples may be different. In competitive PCR, known amounts of RT-PCR amplifiable competitors are spiked into the RNA before RT. The internal standard shares the same primer recognitions sites as the target leading to competition for reagents, and because they are virtually identical both should be amplified with the same efficiency. A series of PCR tubes containing the target are spiked with known copy numbers of the internal standard, with greater concentrations of the standard resulting in greater chance that the primers will bind and amplify it rather than the target. The resulting DNA can then be stained visualised and quantified on an ethidium bromide gel. However this process may not lead to exact quantification because stable differences between amplification efficiency of target and competitor remain undetected (Bustin 2000).

1.6.6 Normalisation of qPCR

Quantitative PCR is one of the best methods available for estimating gene expression due to its ability to detect and accurately quantify genes with low expression levels. However, normalization procedures are needed due to non-specific variation between samples. The various stages involved in the procedure mean that variation can occur at any stage, and the nature of qPCR means variation is amplified. The most commonly used normalization strategy is the inclusion of reference genes in PCR reactions (Lee *et al* 2010). Selected genes are usually housekeeping genes whose expression is usually constant.

1.6.7 Methods for calculating differential expression

Real time PCR results need to be processed by an absolute or relative method to obtain gene expression levels. In order to establish absolute quantification of the gene the cycle at which fluorescence is detected is compared to a standard curve plotted from dilutions of a standard template. Whereas relative methods provide results as a fold, change difference compared to a reference gene. Absolute quantification is more difficult to achieve and some relative methods can be affected by amplification efficiency and result in misleading results (Skrzypski 2007). The comparative quantitative PCR (qPCR) (Pfaffl) is regularly used to compare mRNA levels in expression analysis. It is capable of accounting for less than 100% efficiency in amplification, and is cheaper than other absolute methods (O'Connor & Runquist 2008; Skrzypski 2007). In the comparative qPCR method, the concentration of mRNA from genes of interest is normalised to the concentration of reference genes whose expression levels remain constant in the cell. PCR of the reference gene occurs alongside the PCR for the gene of interest. This way all conditions remain the same and the reference gene provides a control for RNA quality, yields and the efficiency of the reaction (O'Connor & Runquist 2008). The method then calculates the expression ratio between the gene of interest and the reference gene, for which the amount of original mRNA needs to be estimated. This is achieved by calculating the original amount using the equation below (R_0 (original amount of transcript) * $(1 + E)^{ct}$ where E is the efficiency of PCR, and ct is the cycle time for the gene).

$$\frac{R_0\ ^{(target)}}{R_0\ ^{(reference)}} = \frac{(1 + E)^{Ct(reference)}}{(1 + E)^{Ct(target)}} = r$$

The comparative ct (Pfaffl) method requires the use of reference genes, which are usually housekeeping genes that have constant expression levels.

1.6.8 How many genes need to be confirmed to validate a microarray experiment?

Corroboration of all microarray data by alternate methods is impractical because of the large amount of data produced. Confirmatory studies are thus conducted on only a small number of

genes from the data set (Rockett & Hellmann 2004). With regard to publishing the results of a microarray experiment, the confirmation of expression levels of genes is important with many publishers refusing to publish data without confirmatory results (Rockett & Hellmann 2004). However, publishers do not provide guidelines on which genes should be confirmed so the questions remain, how many genes expression levels should be confirmed? Which genes should be picked for confirmation? In order to establish how many genes would need to be confirmed by qRT-PCR a survey of the literature was conducted. The results of the survey are recorded in **Table 1.6.8,** they show the number of probes on the array used, and the number of genes confirmed using qRT-PCR and basis for selection of genes.

Table 1.6.8 The number of genes from microarray studies confirmed by qRT-PCR

Author Name	Number of qRT PCR genes tested	Number of genes on array	Basis for selection
Peatman *et al* 2008	6	28,518	2 fold change
Zhao *et al* 2007	8	14,500	Large fold change
Baisden *et al* 2007	2	14,000	Upregulated
Eyster *et al* 2007	7	10,000	Passed statistical screening
Yang *et al* 2007	6	2,743	Differentially expressed
Sung *et al* 2007	18	39,000	Hypoxia responsive

The results in **Table 1.6.8** shows that between two and eighteen genes are confirmed using qRT-PCR. Studies with fewer than 15,000 genes on the microarray generally use ~six genes, and genes are selected because they are differentially expressed. In this study, 5,744 *S. cerevisiae* genes were measured using microarrays, so approximately six genes need to be confirmed using qRT-PCR.

1.6.9   Selecting genes for confirmation using qRT-PCR

The expression changes of genes confirmed using qRT-PCR need to be normalized against reference genes whose expression does not change under the experimental conditions. Genes usually chosen as reference genes are housekeeping genes, however microarray data is often used to confirm that the expression of these genes do not change. It is imperative that the reference genes have unchanged expression across treatments and gene expression data from the microarray experiments provides valuable data to aid their selection (Bohle *et al* 2007). To confirm the results of a microarray experiment the genes most frequently confirmed using qRT-PCR are those shown to have a high fold change in expression, picking such genes allows expression data to be easily confirmed (Rockett & Hellmann 2004). However, selecting genes that are all differentially expressed four fold, for example, only confirms that microarrays provide accurate results for genes altered four fold. To ensure equal representation of genes, genes with different magnitudes of change can be used for qRT-PCR.

1.7　Analysing gene expression data

1.7.1　Assessing data quality using Principal Component Analysis (PCA)

Signal intensity values for each gene can vary from every gene on every microarray therefore the data can be very complex (Yeung and Ruzzo 2001). Principal Component Analysis (PCA) is a multivariate statistical technique that analyses the covariance structure of data (Han and Wang 2009) it is used to simplify complex data sets allowing exploratory analysis. PCA can be used to cluster genes based on expression information and can determine whether experiments contain independent or correlated information (Moller *et al* 2005; Raychaudhuri *et al* 2000). PCA has been used to identify factors affecting gene expression in a range of studies, as well as in the identification of outliers (Hilsenbeck *et al* 1999). PCA checks replicate integrity, by using an algorithm that assesses the constant variables in the data such as the number of microarrays, and the number of genes represented by signal values on each microarray (Pierce *et al* 2005). It works by identifying variables affecting the data most first, and moves to variables that are having less and less of an effect on the data. Ratios that describe the variables affecting signal intensity values are assigned to the data, the ratios describe how associated the signal intensity values are to the variables (components) assigned. PCA can determine both positive correlations and whether data is negatively correlated (Yeung and Ruzzo 2001). Consequently, samples with similar/correlated gene expression profiles are plotted on graphs in clusters. PCA has been widely used in cluster analysis both within (Madi *et al* 2008; Hirode *et al* 2008) and outside of gene expression data analysis (Carvalho *et al* 2004; Pasqualoto *et al* 2007; Pierce *et al* 2005). PCA is a valuable tool that can be used to identify poor quality samples, and describe relationships in the data; using box whisker plots alongside PCA ensure stringency in quality checking.

1.7.2　Assessing data quality using Box Whisker plots

Box Whisker plots visually display the descriptive statistics for the data and can be used to remove fluctuating data. They are particularly useful for quality checking gene expression data because they do not make assumptions about the distribution of data. Data that has been hybridised and normalized successfully has medians that follow a straight line across the middle of the Inter Quantile Range (IQR). Biological differences between treatment groups are identified if the medians for all of the replicates within a treatment are lower or higher than those in other treatment conditions. Problems with hybridization such as degraded cRNA are identified if the median of one of the normalized replicates within a treatment differs from the medians of the rest of the replicates in that treatment (Ludbrook 2007). Upon completion of quality checks, differentially expressed genes can be identified.

1.7.3 Identifying differentially expressed genes

Currently several methods for identifying differentially expressed genes are used, the most simple of which is filtering the data on fold change and identifying genes which are two fold or more differentially expressed in test samples compared to controls. Statistical tests such as One-Way ANOVA can identify whether treatment has a significant effect on the expression of genes, and Two-Way ANOVA can identify interactions between experimental parameters (i.e. treatment and time). Both two fold differential expression and significant differential expression are published in gene expression results from microarray experiments, but two fold differentially expressed genes are not always significantly differentially expressed, and vice versa. The literature on gene expression data contains work, which is analysed in different ways. Some authors (Yin *et al* 2007; Chen *et al* 2007; Zhang *et al* 2003) publish results on genes that are both two fold differentially expressed as well as being significantly differentially expressed. Others (Gielchinsky *et al* 2008; Radeke *et al* 2007; Yang *et al* 2007; Ding & Raghavarao 2008; Garcia *et al* 2004) report on only significantly differentially expressed genes. But some authors publish genes that are two fold or more differentially expressed and do not report on their significance (Semjidsuren *et al* 2008; Wang *et al* 2008; Meng *et al* 2005; Alexandre *et al* 2001; Kim *et al* 2006). A thorough survey of the literature reveals that fold change in expression is usually used to identify differentially expressed genes in toxicogenomic studies with *S. cerevisiae*. However, the fold change in expression used to identify differentially expressed genes varies from one study to another, to illustrate this point an outline of toxicogenomic investigations with *S. cerevisiae* is provided in **Table 1.7.3.**

Table 1.7.3 differentially expressed genes identified in toxicogenomic studies with *S. cerevisiae*

Authors	Chemical (conc^n mg/l)	Exposure (hours)	Statistical test	p-value	MTC	Fold change	Genes changed in expression
Zhang *et al* (2003)	DMSO (0 & 22.8 mg/l)	2	Not defined	0.005	Not defined	2.5	1,338 (680 up, 658 down)
John *et al* (2005)	Mixture (N/A)	2	None	None	None	2.0	302 (90 up, 212 down)
Alexandre *et al* (2001)	Ethanol (0, & 887 mg/l)	0.5	None	None	None	3.0	279 (194 up, 85 down)
Kim *et al* (2006)	Mixture (N/A)	2	None	None	None	2.0	91 (63 up, 28 down)
Hirasawa *et al* (2007)	Ethanol (0 & 633 mg/l)	0.25, 0.5, 1, 2, & 3	N/A	N/A	N/A	2.0	Not reported
Pandey *et al* (2007)	NaCl (0, 14,610, 29,220, 43,830, 58,440 mg/l)	0.25, 0.5, 0.75, 1, & 2	N/A	N/A	N/A	2.0	Not reported

Overall toxicogenomic studies sample at a single exposure time and differentially expressed genes are identified based on fold change in expression using no statistical analysis. Hirasawa *et al* (2007) and Pandey *et al* (2007) sampled at more than one exposure time because the purpose of their studies were to identify genes involved in resistance to ethanol and salt stress. Despite toxicogenomic studies with *S. cerevisiae* considering genes two fold or more differentially expressed, toxicogenomic studies with multicellular eukaryotic organisms have considered genes that are significantly differentially expressed and sometimes two fold and significantly differentially expressed.

1.7.4 Advantages of significant differential expression over fold changes in expression

The strengths of array based technologies in toxicology lie the number of transcripts that can be measured simultaneously (Snape *et al* 2004). The sensitivity helps characterize toxicity and expression profiling has been used to identify subtle effects of low concentrations of genotoxic chemicals (Thybaud *et al* 2007; Harries *et al* 2001). Statistical analysis generally identifies a large number of differentially expressed genes so researchers often use fold changes in expression to reduce the number of genes going into subsequent analysis. Large lists of differentially expressed genes can be dealt with by categorizing genes into functional groups providing useful overviews of effects. Such as in the study by Klaper *et al* (2008) who used one-way ANOVA ($p<0.05$) to identify 650 genes differentially expressed in the liver of the fathead minnow exposed to $2.0\mu g\ g^{-1}$ methylmercury, genes were classified using their functions (Gene Ontology). The results provided researchers with an overview of effects that could be investigated in more detail. One of the problems with statistical analysis for gene expression data is the cut off used for significance is usually $p<0.05$, but *S. cerevisiae* has 5,710 protein coding genes, using a p-value of 0.05 means 5% (285) genes can pass statistical tests by chance (Leung & Cavalieri 2003). Multiple Testing Correction methods (1.6.5) can be used to eliminate genes expected to pass statistical analysis by chance.

1.7.5 Multiple Testing Correction

Multiple Testing Correction (MTC) methods used in identifying differentially expressed genes should control for two types of error that can occur when identifying significantly differentially expressed genes. Type I errors identify genes significantly differentially expressed when they are not (false positives), and type II errors fail to identify differentially expressed genes (false negatives) (Dudoit *et al* 2003). MTC methods with different stringencies have been developed and the stringency of the MTC methods used determines whether type I or type II errors are made. Ideally, a method that reduces type I and type II errors should be chosen to identify differentially expressed genes. Multiple MTC works by multiplying the p-value by a correction

factor that increases the p-value in a manner that accounts for the number of genes tested in the analysis.

1.7.6 Types of MTC

There are several types of MTC; for example, Benjamini-Hochberg false discovery rate is a medium stringency MTC, it works by ranking the p-values in order from smallest to largest. The largest p-value is multiplied by the number of genes under test and n/n-1 (n=number of genes) for the rest of the genes in the list. If the p-values are below the specified p-value, (0.05 is usually used for significance) then the gene passes the filter. It gives the researcher enough stringency to reduce the number of false positives (genes identified as differentially expressed when they are not) without being too stringent.

Bonferroni-Holm is the most stringent of the MTCs; in this algorithm, the p-values for each gene are ranked from smallest to largest. The first p-value is multiplied by the number of genes being assessed and if the end p-value is less than 0.05 (or the specified p-value) the gene is significantly differentially expressed. The second gene in the list is multiplied by n-1 (n = number of genes), and the third n-2 etc, the sequence is followed until no genes are found to be significant.

1.7.7 Categorisation of genes using Gene Ontology

Toxicogenomic studies can identify large numbers of differentially expressed genes; Zhang *et al* (2003) reported 1,338 genes to be differentially expressed in *S. cerevisiae* exposed to dimethyl sulfoxide (DMSO). Due to the high number of differentially expressed genes, individual genes were not studied; instead, an overview of results was obtained by classifying genes into metabolic functional classes. This allowed the researchers to compare the effect of DMSO with general responses to environmental stress and identify that 938 of the differentially expressed metabolic genes were not responsive to general environmental stress. Detailed analysis of the functional groups affected by DMSO identified that among other results genes in the lipid biosynthetic pathways were significantly decreased in expression by DMSO (Zhang *et al* 2003). Overviews of this nature are useful because they can identify patterns in large data sets directing detailed analysis (Dillman & Phillips 2005). Microarrays and gene expression profiling are relatively new, and the functions of all genes are yet to be elucidated, so additional methods of analysing the data include clustering (**1.6.8**) which can identify genes with related functions, and pathway analysis (**1.6.9**) that can be used to identify and illustrate relationships between differentially expressed genes.

1.7.8 Clustering of gene expression data

GeneSpring GX10^{TM} provides three clustering methods (self-organising maps, hierarchical and k-means). Clustering methods have been used in metabolic and toxicogenomics studies (Tanaka-Tsuno *et al* 2007; Guo *et al* 2008), to identify regulatory networks (Han & Zhu 2007; Gupta & Ibrahim 2007), network interactions (Pu *et al* 2008), and in identification of transcriptional networks (Wei & Pan 2008; Yan *et al* 2007; Liu *et al* 2007). Pearson's correlation is most frequently used in calculating correlations in data (Sobel & Mishra 2008). Pearson's correlation has been employed in a wide array of studies including but not limited to; toxicogenomic studies (Van Delft *et al* 2005; Ryan *et al* 2007), identifying genes involved in tumour formation (Leung *et al* 2006; Yang *et al* 2007; Talbot *et al* 2005; Tilton *et al* 2008; Avci *et al* 2008), stress response (Yee *et al* 2008; Krasnov *et al* 2005) and time series analysis (He & Zeng 2006; Little *et al* 2005). Clustering methods can identify relationships between differentially expressed genes, and genes involved in similar pathways (**1.6.9**) often cluster together.

1.7.9 Pathway analysis

Traditional analysis of gene expression data involves applying statistical analysis to identify genes that are differentially expressed between the experimental conditions. However, it is difficult to extract a biological effect from a list of individual genes obtained from statistical analysis. Pathway analysis puts statistically significant findings into a biological context. In a study by Cusick *et al* 2009 Gene Ontology and pathway analysis helped to reveal that exposure of *S. cerevisiae* to saxotoxin resulted in copper deficiency and increased glutathione biosynthesis. Showing that pathway analysis a useful method for identifying significant effects of chemicals. In this study statistical analysis with MTC will be used to generate large lists of differentially expressed genes which will then be categorised using Gene Ontology and pathway analysis. Increased statistical stringency will be used alone or alongside fold changes in expression where appropriate / required to decrease the number of genes analysed in detail.

1.8   4-nitrophenol: A model toxicity testing compound

1.8.1   Phenolics are high priority pollutants

Industrial development has led to the widespread use and distribution of phenolics (Niu *et al* 2009). Phenolics are a high priority xenobiotic because they are harmful to life at low concentrations and drinking water contaminated with such compounds has a bad smell and taste even at concentrations as low as 1.8 n*M* (Ngah & Fatinathan 2006). Phenolics are highly

stable in water (Niu *et al* 2009) as well as being suspected human carcinogens due their acceleration of tumour formation (Ngah & Fatinathan 2006) so even low phenolic concentrations in water deem it unfit for human consumption.   Phenolics can be classified according to their mechanism of toxic action: polar narcotics, pro-electrophiles, electrophiles, respiratory uncouplers (Niu *et al* 2009).

1.8.2   4-nitrophenol as a model organic pollutant

Nitrophenols have been listed as priority pollutants by the United States Environmental Protection Agency (US EPA) (Ngah & Fatinathan 2006). 4-nitrophenol (also known as *Para*-nitrophenol, *p*-nitrophenol and abbreviated to PNP *Figure* **1.8.2**) is a respiratory uncoupler (Bhatti *et al* 2001). 4-nitrophenol is water-soluble (TenBrook *et al* 2005) and weakly acidic (Lei *et al* 2003; Gemini *et al* 2005) with a pKa of 7.2 (Sterner 1999). It is considered important for metabolic studies of toxic responses because it is less toxic than many other nitrophenolic compounds, but the metabolic responses of organisms are considered similar to those invoked by more toxic phenolics (TenBrook *et al* 2003, Williamson *et al* 1995). 4-nitrophenol is used as a model organic pollutant in many toxicity assays because it is representative of aqueous organic pollutants (Sabio *et al* 2004) and can be found in significant concentrations in the environment (Gemini *et al* 2004).
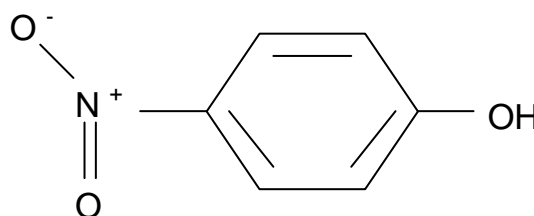
Figure **1.8.2** 4-nitrophenol is a nitro aromatic compound, whose main structural feature is the aromatic benzene ring.

1.8.3   Sources of 4-nitrophenol in the environment

Organophosphate pesticides (parathion, ethyl-parathion and methyl-parathion) are transformed into 4-nitrophenol in soil (Heitkamp *et al* 1990). The hydrolysis of pesticides and herbicides can result in high 4-nitrophenol concentrations in soil (Shimazu *et al* 2001; Takeo *et al* 2002; Cornett 2003; Gemini *et al* 2005). 4-nitrophenol is highly water soluble (TenBrook *et al* 2005), it can leach from soil into groundwater and has been found in drinking water (Kuscu & Sponza 2005). The US EPA has imposed a 0.07μM (10μg/l) restriction on 4-nitrophenol concentrations in natural waters (Karim & Gupta 2003) but in urban run-off catchments concentrations have been found to be almost double this concentration (0.13μM) (TenBrook *et al* 2003). Due to its wide distribution it is important to use a sensitive assay to ascertain the toxicity of 4-nitrophenol and assess its potential impact on organisms.

1.8.4   Toxicity of 4-nitrophenol

Due to its use as a model organic pollutant the toxicity of 4-nitrophenol has been studied in a wide range of organisms, including plants, animals and microbes. The main structural component of 4-nitrophenol is a phenol ring; phenolic compounds are known to cause mitochondrial membranes to become permeable (Bakkali *et al* 2008). Biochemical investigations have shown that 4-nitrophenol uncouples mitochondrial oxidative phosphorylation (TenBrook *et al* 2005) by migrating across inner mitochondrial membranes and discharging the proton gradient created by electron transport (Williamson *et al* 1995). The toxicity of 4-nitrophenol is thought to arise from mitochondrial malfunction. Braunbeck *et al* (1988) studied the physical effects of 4-nitrophenol on mitochondria, they found that 4-nitrophenol caused partial lysis, swelling, reduction of cristae, and disruption of the inner membrane.   However whole organism toxicity tests have also been conducted as detailed in the following sections.

1.8.5   Toxicity of 4-nitrophenol to microbes

Considering the ease, cost effectiveness and importance of microbial growth inhibition tests, investigations into the toxicity of 4-nitrophenol towards microorganisms are relatively scarce in the literature. In a study conducted by Brown (2005), the toxicity of 4-nitrophenol to *Escherichia coli* was investigated using growth inhibition tests. The EC_{50} for *E. coli* exposed to 4-nitrophenol under these conditions was found to be 402.6 μM *(*56 mg/l*)*. However a separate investigation carried out by Bergauer *et al* (2004) found the growth inhibitory toxicity of 4-nitrophenol (EC_{50}) to yeast was 230μM (32 mg/l). This would appear to show that 4-nitrophenol is more toxic to eukaryotic yeast than to prokaryotic *E. coli*.

1.8.6   Toxicity of 4-nitrophenol to invertebrates

Gemini *et al* (2005) investigated the toxicity of 4-nitrophenol using mobility inhibition assays with the model aquatic toxicity testing organism *Daphnia magna*. A concentration of 10 μM caused 50% inhibition of mobility in the population under test. Additionally Williamson *et al* (1995) investigated the toxicokinetics and biotransformation of 4-nitrophenol by the black turban snail (*Tegula funebralis*). The *T. funebralis* were exposed to 4-nitrophenol at varying concentrations to establish sub lethal concentrations for future experiments; the toxic endpoint was the inability of snails to adhere to flask surfaces. The tests revealed that the No Observed Effect Concentration (NOEC) was 71.9 μM (10 mg/l); further investigation showed that at 71.9 μM 4-nitrophenol was absorbed exponentially. These toxicity studies demonstrate that measured environmental concentrations of 1.37μM, and the recommended concentration in natural waters 0.07μM may be below harmful concentrations for microbes and invertebrates

1.8.7 Toxicity of 4-nitrophenol to terrestrial vertebrates

4-nitrophenol is a metabolite of pesticides such as parathion and methyl-parathion which are converted to 4-nitrophenol in the liver (Bhatti *et al* 2002). Studies in animals suggest that exposure to 4-nitrophenol may cause the blood disorder methaemoglobinaemia, liver and kidney damage as well as skin and eye irritation and systemic poisoning (Bhatti *et al* 2002). 4-nitrophenol has been shown to be carcinogenic, mutagenic cytotoxic and embryotoxic in mammals and is generally lethally toxic to mammals at concentrations higher than 0.36 *M* (Lei *et al* 2003). It is poorly biodegradable and is known to accumulate in both plant and animal tissues (Sabio *et al* 2004; Kuscu & Sponza 2005). In an investigation conducted by Li *et al* (2006) the rodent uterotrophic and Hershberber assays (OECD validated assays for assessing the estrogenic and androgenic properties of endocrine disrupting chemicals), were used to assess the effects of 4-nitrophenol. They found that daily injections of 4-nitrophenol equal to 0.072, 0.72, and 7.2 μ*M* over a 7-day period in female rats did not lead to decreases in body weight or the weights of vital organs. However, treatment did lead to a significant increase in uterine weight. After 5 days of injecting 0.072, 0.72, and 7.2 μ*M*, (10, 100, & 1000 μg/l) male rats showed significant decreases in liver and kidney weight. The weights of androgen dependent reproduction glands were also measured and significant decreases were seen in all but one of the reproductive glands assessed. In male rats it was also found that the plasma concentrations of Lutenising Hormone (LH) and Follicle Stimulating Hormone (FSH) were increased. Li *et al* (2006) therefore concluded that 4-nitrophenol shows estrogen like effects on female rats and anti-androgen like effects on male rats. At the acceptable concentration in natural waters (10 μg/l) subtle effects are seen in the endocrine and reproductive systems of mammals if repeated doses are given.

1.8.8 Toxicity of 4-nitrophenol to aquatic vertebrates

In a study conducted by TenBrook *et al* (2005) the toxicity of 4-nitrophenol to white sturgeon (*Acipenser transmontanus*) was investigated from both toxicokinetic and biotransformation standpoints. *A. transmontanus* was exposed to *p*-nitrophenol at concentrations of 0, 7.2, 36 and 72 μ*M* for up to 24 hours; inactivity of the fish was used as the endpoint for the experiments. The No Observed Effect Concentration (NOEC) on the activity of *A. transmontanus* was seen at 7.2 μM (1 mg/l), and at this concentration, 4-nitrophenol was taken up from water within 24 hours, and depurated from tissues within 48 hours.

1.8.9 Toxicity of 4-nitrophenol to plants

Mitra and Vaidyanathan (1982) investigated the effect of 4-nitrophenol on the growth of cucumber seedlings. In their investigation, seedlings were exposed to 4-nitrophenol at 0.72, 1.44, and 2.16 μM for 96 hours. The number of seeds germinating and the shoot lengths of

seedlings were measured. They found that at 0.72 μ*M* no toxic effects were seen. However, at concentrations of 1.44 μM (33μg/l) and above, significantly less root and shoot growth occurred.

For comparative purposes the toxicities of 4-nitrophenol to the organisms discussed are shown in **Table 1.8.9.**

<p align="center">Table 1.8.9 Published toxicity of 4-nitrophenol to microorganisms, invertebrates and vertebrates.</p>

Organism name	Toxic concentration	Author
Escherichia coli	(EC_{50}) (56 mg/l)	Brown (2005)
Yeast	(EC_{50}) (32 mg/l)	Bergauer *et al* (2004)
Daphnia magna	(EC_{50}) (1.4 mg/l)	Gemini *et al* (2005)
T. funebralis	NOEC (10 mg/l)	Williamson *et al* (1995)
A. transmontanus	NOEC (1 mg/l)	TenBrook *et al* (2005)
Cucumis sativus	Shoot and root length decreased (0.33 mg/l)	Bhatti *et al* (2002)
Rattus rattus	Endocrine and reproduction affected (10μg/l)	Li *et al* (2006)

All of the toxicity information currently available for 4-nitrophenol is based on traditional toxicity tests, biochemical and physiological data. There have been no transcriptomic studies with 4-nitrophenol, and the mechanisms by which it is toxic to living organisms is not fully understood. Transcriptomics is extremely sensitive and can detect gene expression changes occurring at low chemical concentrations, identifying the NOTEL in microbes and *in vitro* cells can establish safe concentrations of 4-nitrophenol for all organisms and can reduce reliability on expensive and time consuming animal tests. This study uses the model eukaryote (*S. cerevisiae*) to investigate the effects of 4-nitrophenol on gene expression at high concentrations (EC_{50}) and the NOEC for *T. funebralis* (10 mg/l).

1.9 Summary

Toxicology uses model organisms and standardised tests to assess the impacts of pollutants on organisms. Over the years many toxicity tests have been developed and validated for use in toxicology. The types of test range from microbial, to whole organism tests, however because a large number of chemicals need to be tested using whole animal tests is expensive, inhumane, and time consuming. Recent developments have allowed for *in vitro* toxicity testing using cells and tissues and it has been suggested that *in vitro* methods and microbial cell culture methods will replace whole animal tests. Further developments in the field of genomics and particularly the advent of microarrays have allowed for global measurement of gene expression.   Microarrays can be used to monitor changes in global gene expression. They can be used to study changes in gene expression in response to many different

physiological stimuli and alterations in gene expression patterns caused by *null* mutants can help identify the function of unknown genes. Expressed genes ultimately give rise to proteins which are responsible for all processes in cells, making transcriptomics an extremely powerful technique. The use of transcriptomics in toxicology has been termed toxicogenomics. Both *S. cerevisiae* and 4-nitrophenol are well studied models for toxicity testing and microarrays are available for *S. cerevisiae*. 4-nitrophenol is a suspected mutagen and *S. cerevisiae* has previously been shown to express RAD genes in response to DNA damage. A toxicogenomic approach to studying 4-nitrophenol toxicity could identify molecular processes that lead to DNA damage and reveal other toxic responses not previously observed in physiological and biochemical studies. 4-nitrophenol was chosen as the investigative test chemical to obtain a greater understanding of the biological effects of chemically-induced toxicity. In the context of this project, microarrays will be used to analyse cellular processes invoked by exposure of the model organism *S. cerevisiae* to the model toxicity testing compound 4-nitrophenol.

Aims of this study

The main aim of the project is to assess how gene expression in *S. cerevisiae* changes in response to 4-nitrophenol exposure over time. This aim will be achieved by the following individual objectives

1. Establish the concentrations of 4-nitrophenol to be used in subsequent microarray experiments with *S. cerevisiae* by use of chronic toxicity tests (cell growth experiments)
2. Use microarrays to measure gene expression of *S. cerevisiae* exposed to pre-selected amounts of 4-nitrophenol
3. Provide an overview of the effects of 4-nitrophenol on gene expression in *S. cerevisiae*: Use Gene Ontology to identify the effects of 4-nitrophenol
4. Examine gene expression changes over time and analyse data using GeneSpring
5. Select a subset of differentially expressed 'signature' genes that could be used to identify unsafe 4-nitrophenol concentrations in water.

Chapter 2

Determining the effects of 4-nitrophenol on growth of *Saccharomyces cerevisiae* in different media: Identifying experimental conditions for subsequent toxicogenomic studies.

2.1 Introduction

2.1.1 Growth inhibition to determine EC$_{50}$ values

Microbial growth inhibition assays are commonly used to determine the EC$_{50}$ of chemicals (the concentration of a chemical at which population growth is inhibited by 50%) (Mitchell *et al* 2002). Chemicals that have low EC$_{50}$ values are more toxic than those with high EC$_{50}$ values because a low EC$_{50}$ value means adverse effects on the organisms are seen at a low concentration. The EC$_{50}$ of a chemical can be used to prioritise the selection of chemicals that should be tested *in vivo,* decreasing the time it takes to evaluate chemicals for toxicity (Schmitt *et al* 2004). They are well-established, vital components of drug, chemical and environmental risk assessment (Weiss *et al* 2004). The advantages of comparing EC$_{50}$ values are that different species of microorganisms have different sensitivities so it makes cross species comparisons possible.

2.1.2 *Saccharomyces cerevisiae* as a model organism for toxicity testing

Wild type (BY4741) *Saccharomyces cerevisiae* has been widely studied because it is quick and easy to grow; in addition, the cell cycle is simple and very similar to that of human cells (Rodrigues *et al* 2006; Rojas *et al* 2008). As a result, *S. cerevisiae* is a useful model eukaryotic organism for studying cellular functions and the toxic effects of chemicals. It has been widely used in growth inhibition studies; for example, the effects of acids (Nielsen *et al* 2007; Narendranath *et al* 2001), para-nonylphenol (Okai *et al* 2000), molybdenum-hydroxylamido (Bennett *et al* 1999), chlorinated phenoxyacetic acid herbicides (Cabral *et al* 2003), on *S. cerevisiae* have all been investigated using growth inhibition studies. The *S. cerevisiae* genome is fully sequenced and standardised commercially available microarrays and protein chips for *S. cerevisiae* allow complicated cellular responses to environmental conditions to be extrapolated (Weiss *et al* 2004). Microarrays have been used to investigate the effects of 5-Fluorocytosine (Zhang *et al* 2002), ethanol, 1-pentanol, and 1-octanol (Fujita *et al* 2004), high carbon dioxide concentrations (Nagahisa *et al* 2005), sulfometuron methyl (Jia *et al* 2000), and DMSO (Zhang *et al* 2003) on gene expression in *S. cerevisiae*.

2.1.3　4-nitrophenol: a model organic pollutant

4-nitrophenol is known to be toxic to plants animals and some microorganisms, and is a high priority pollutant for environmental protection agencies due to its mutagenicity and carcinogenicity (Kuscu & Sponza 2005). 4-nitrophenol is used as model pollutant in many toxicity assays because it is both representative of aqueous organic pollutants, and environmentally relevant (Sabio *et al* 2004). Biochemical studies have identified the mitochondrion as a target of 4-nitrophenol and exposure has been reported to depolarize the mitochondrial inner membrane decreasing ATP production (Williamson *et al* 1999). However, nothing is known about the effects of 4-nitrophenol on gene expression and 4-nitrophenol may have other more subtle effects on cells. Increasing demand to maximise the knowledge output from toxicity studies means there is a need to use traditional toxicity tests in conjunction with more advanced methods for toxicity assessment like microarrays (Snape *et al* 2004). Microarrays are extremely sensitive and many factors including the type of growth media used, and nutrient concentrations can affect the results of a gene expression study (Brewster *et al* 1994).

2.1.4　Growth media used for microarray experiments with *S. cerevisiae*

S. cerevisiae has been widely used in microarray experiments due to its status as a model eukaryote. Three media types have been used in microarray experiments: Yeast Extract Peptone Dextrose (YEPD), Synthetic Defined (SD) and Synthetic Complete (SC).

Table 2.1.4 Types of media used in transcriptomic studies with *S. cerevisiae.*

Author	Study type	Media type
Feng *et al* (2007)	DNA function	SC
Peppel *et al* (2005)	Expression profiling in epistasis	SC
Schoondermark-Stolk *et al* (2005)	Gene function	SD
Parveen *et al* (2004)	Toxicogenomics	YEPD
Sirisattha *et al* (2003)	Toxicogenomics	YEPD
Iwashashi *et al* (2006)	Toxicogenomics	YEPD
Kim *et al* (2006)	Toxicogenomics	YEPD
Kitagawa *et al* (2002)	Toxicogenomics	YEPD
Murata *et al* (2006)	Evaluating environmental waters	YEPD
Erasmus *et al* (2003)	High sugar stress	YEPD
Matsuoka *et al* (2005)	High pressure stress	YEPD
Matsumoto *et al* (2005)	Heat stress	YEPD
Fujita *et al* (2004)	Response to alcohol	YEPD
Mercier *et al* (2001)	Stress response	YEPD
Andrau *et al* (2005)	Stationary phase gene expression	YEPD
Daran-Lapujade (2003)	Strain comparison	YEPD
Pan *et al* (2006)	Genome interactions	YEPD
Mizukami *et al* (2004)	Microarray standardisation	YEPD
Belenkiy *et al* (2000)	Mitochondrial transport proteins	YEPD

The summary of transcriptomic studies conducted in *S. cerevisiae* (**Table 2.1.4**) indicates that the most commonly used growth medium for studies with *S. cerevisiae* is YEPD. YEPD is advantageous because it is easy and cheap to make and cultures grow quickly in this medium. However, for microarray experiments where reproducibility is paramount, it is not clear that an undefined medium such as YEPD made with yeast extract is most suitable.

2.1.5 MIAME guidelines aim to increase microarray reproducibility

MIAME (Minimal Information About a Microarray Experiment) guidelines (also see section **1.5.3**) were developed to increase the reproducibility of microarray experiments and allow valid comparisons between studies conducted in different laboratories to be made. Nevertheless, the components of growth media or even the type of growth media used do not need to be specified for MIAME compliance and any of the three media listed in **Table 2.1.4** could be used for a microarray experiment.

2.1.6 Expression microarray reproducibility

Expression microarrays are a powerful and sensitive tool for the assessment of environmental impacts on cells (Burgoon *et al* 2005). Experimental variables that can alter gene expression must be considered if high quality reliable results are to be obtained. Differences in gene expression over time in all cells depends on the cell cycle (Rustici *et al* 2004), synchronous cultures can provide more reliable gene expression results by compensating for cell cycle gene expression oscillations (Lloyd & Murray 2006). However, chemical treatment alters cell cycles (Lloyd & Murray 2006), so maintaining synchronous cultures in toxicogenomic studies is difficult. Alternatively, toxicogenomic researchers treat and sample when cells in a particular growth phase (during logarithmic growth) thereby partially compensating for cell cycle dependent gene expression changes. Additionally external conditions (i.e. glucose concentration (Rolland *et al* 2002), & pH (Lamb *et al* 2000; Blanchin-Roland 2008)) can alter gene expression, so external conditions need to be monitored (to assess their potential impacts on) or controlled (to prevent them altering) the results of toxicogenomic studies.

2.1.7 Obtaining high quality RNA

In addition to cell growth conditions, the quality of RNA samples extracted from exposed cells can alter the reproducibility of microarray expression results (Naderi *et al* 2004). High quality RNA is required to produce reproducible high quality gene expression data, and there is a strong relationship between the quality of RNA and quality of gene expression data produced (Copois *et al* 2007). Degradation of RNA can result in the loss of primer binding sites and shorten templates which can affect the Reverse Transcriptase PCR reaction and its products (Cox *et al* 2007). There are numerous methods and kits available for the extraction of RNA, with so much choice researchers often have several options open to them. Many of the

commercially available kits claim to be fast and effective providing the user with high concentrations of pure, high quality RNA. However, this comes at a cost, as some of the RNA extraction kits cost in excess of £350. There are cheaper alternatives for the extraction of RNA such as the hot phenol/chloroform method (http://www.microarrays.org/pdfs/Total_RNA_from_Ecoli.pdf), as well as the TRIZOL method (Chomczynski & Sacchi 1987). An important part of a microarray experiment is extracting enough high quality RNA. RNA extraction methods should be explored to find the most effective for the user.

2.1.8   Assessing RNA quality

Traditional techniques for estimating RNA quality involve separating ethidium bromide stained rRNA on an agarose gel and visualising it, intact RNA produces clear bands of the correct molecular weight. However, such techniques are laborious and RNA quality can be affected by RNase contamination during the assay (Ricicova & Palkova 2003). Alternatively spectrophotometery can be used to quantify RNA and 260/280 ratios can be used to describe the quality of RNA; however degraded RNA cannot be reliably distinguished by this method (Ricicova & Palkova 2003). Due to the importance of having intact RNA for gene expression studies (Naderi *et al* 2004) more advanced methods for the assessment of RNA quality have been developed. The Agilent Bioanalyser RNA 6000 assay uses the Agilent 2100 Bioanalyser ® (a minaturised electrophoresis system for nucleic acid separation). The RNA 6000 assay kits supplied by Agilent consist of RNase free cartridges containing electrophoresis channels (*Figure* **2.1.8)**, a gel dye mix, gel matrix mix (Agarose gel equivalent), and standards to check performance. Results are reported on a graph showing 18s and 28s rRNA peaks, and a gel image is produced. If RNA degradation is detected the software generates a RNA Integrity Number (RIN) that indicates the extent of degradation. Smaller samples are required for Bioanalyser analysis making analysis cheaper, and certified RNase free consumables increase reliability (Agilent 2008).
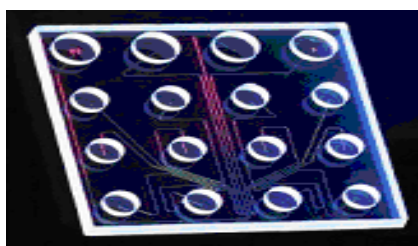
Figure 2.1.8 Agilent Bioanalyser Cartridge showing electrophoresis channels
www.Agilent.com

Aims of Chapter 2

Aim 1 Establish growth media to be used in subsequent gene expression studies to achieve the most reproducible and reliable results.

Objectives to meet Aim 1

    1. Determine the effect of 4-nitrophenol on growth in Synthetic Defined (SD), Synthetic Complete (SC) and Yeast Extract Peptone Dextrose (YEPD).

    2. Establish the EC_{50} of 4-nitrophenol exposed *S. cerevisiae* in each of the three media

    3. Measure variables known to alter gene expression in *S. cerevisiae* (pH, glucose concentrations)

    4. Use results to determine the best media, 4-nitrophenol concentration, and times at which to sample in a microarray experiment

Aim 2 Extract high quality total RNA from *S. cerevisiae* exposed to 4-nitrophenol for expression profiling

Objective to meet Aim 2

    1. Expose *S. cerevisiae* to 4-nitrophenol in determined conditions (Aim 1), extract total RNA and assess quality using an Agilent Bioanalyser RNA 600 assay

2.2 Materials and Methods

2.2. Growth of *S. cerevisiae* treated with 4-nitrophenol in SD, SC and YEPD media

2.2.1 Culture of Saccharomyces cerevisiae

Saccharomyces cerevisiae wild type strain BY4741 growing on YEPD agar, was acquired from Simon Avery (Nottingham University). Cultures were streaked out on fresh Synthetic Defined medium (SD), Synthetic Complete (SC) and Yeast Extract Peptone Dextrose (YEPD) agar plates (**Appendix I, Table 2.1 – 2.3**). The plates were incubated at 30°C for two days and colonies were transferred from each plate to a fresh plate up to 10 times to adapt cells to each of the different media.

2.2.2 Growth characteristics of *S. cerevisiae* in SD, SC and YEPD medium to establish mid log phase

A single yeast colony from either a SD, SC or YEPD plate (incubated for 24 hours) was used to inoculate conical flasks (250 ml) containing 50 ml of the corresponding sterile aqueous media (SD, SC or YEPD) pre-warmed to 30°C (Zhang *et al* 2003). This starter culture flask was shaken overnight at 150rpm (BSI 1992) and 30°C (Kungolos *et al* 1999, Weiss *et al* 2003, Zhang *et al* 2003). The culture was sampled and the OD_{600} measured using a Libra S12 spectrophotometer. When the culture reached an OD_{600} value of 1.0, 1 ml of the starting culture was removed from the corresponding flask and used to inoculate 49 ml in each of 3 test flasks (triplicate flasks for each medium). The OD_{600} of each flask was recorded immediately after inoculation and then hourly. The samples were diluted when the OD_{600} value exceeded 0.5 so that they were between 0.1 and 0.5 (the linear range of the spectrophotometer used), and the corresponding OD_{600} was calculated at the end of the experiment (Avery 2004). The measured OD_{600} of the samples were then plotted against time and used to determine the OD_{600} corresponding to middle logarithmic growth (Weiss *et al* 2003).

2.2.3 Preparation of stock samples of 4-nitrophenol

An 11 mg/ml (79.07 mM) stock solution of 4-nitrophenol was prepared by dissolving 4-nitrophenol in de-ionised water. The stock solution was filter sterilised using a 0.2μm Nalgene® PTFE filter and diluted using sterile de-ionised water so that the total volume of 4-nitrophenol added to each flask was 1 ml and corresponded to test concentrations of (10, 20, 32 & 64 mg/l) (**Appendix I, Table 2.4**).

2.2.4 Using a standardised *S. cerevisiae* inoculum to establish the EC_{50} concentration for *S. cerevisiae* exposed to 4-nitrophenol

To calculate the concentration of 4-nitrophenol causing 50% inhibition of growth by comparison with controls (EC_{50}), growth inhibition tests were conducted using a standardised inoculum. This consisted of the addition of 1ml of cells from a starting culture which had reached middle logarithmic growth phase, corresponding to an OD_{600} of 0.36 (SC & YEPD) 0.28 (SD) (**Appendix II, *Figure* 2.5**), to corresponding flasks containing 48 ml of fresh, pre-warmed growth medium.

2.2.5 Exposing *S. cerevisiae* to 4-nitrophenol

Flasks were prepared in triplicate, containing 48 ml SD, SC, or YEPD medium for each treatment (4-nitrophenol concentration). The standardised inoculum (**2.2.4**) was added to each pre-warmed medium at $30^{o}C$ and 150rpm. The 4-nitrophenol (0, 10, 20 32, & 64 mg/l) was added when the cells reached middle logarithmic phase (**2.2.4**).

2.2.6 Determining the EC_{50} values for *S. cerevisiae* exposed to 4-nitrophenol in middle logarithmic growth

After the addition of 4-nitrophenol (**2.2.5**) the OD_{600} was measured every hour until the control flasks reached stationary phase (***Figure* 2.3.1, *Figure* 2.3.2, & *Figure* 2.3.3**). In addition, pH was measured every 3 hours during the incubation period, using a glass electrode (FiveEasy FE20), (***Figure 2.3.5***). To monitor media composition changes the concentration of glucose in the media was measured before and after the addition of 4-nitrophenol (**2.2.9**).

2.2.7 Calculating the EC_{50} for *S. cerevisiae* exposed to 4-nitrophenol in middle logarithmic growth

The end of experiment (control flask OD_{600} no longer increasing) OD_{600} values were used to calculate the percentage inhibition caused by 4-nitrophenol using the formula (1-(OD_{600} of Test / OD_{600} of Control))*100. The resulting percentage inhibitions were then plotted for a selection (10 & 32mg/l) of concentrations tested (***Figure 2.3.4***).

2.2.8 Identifying conditions for subsequent transcriptomic analysis

The growth inhibition results (***Figure* 2.3.4**) were used in conjunction with pH change results (***Figure* 2.3.5**) to select the type of media, 4-nitrophenol concentrations, and exposure times for analysis using transcriptomics. For reasons discussed in section **2.4.7** Synthetic Complete (SC) medium was chosen for subsequent microarray experiments. The 4-nitrophenol concentrations used for transcriptomic analysis were 10 & 39 mg/l (71.9μM & 280μM) (EC_{10} & EC_{50} respectively (**2.4.8**) for calculations see **Appendix II, Table 2.6**). *S. cerevisiae* cultures were exposed to 0, 10 & 39mg/l 4-nitrophenol during middle logarithmic growth phase (as in

section **2.2.5**), samples for transcriptomic analysis were taken after one, two and three hours (**2.4.9**) and RNA extracted using the methods in sections **2.2.12 - 2.2.17** .

2.2.9   Sampling to measure glucose concentrations in 4 –nitrophenol treated and untreated *S. cerevisiae* cultures

S. cerevisiae was cultured in SC media using the standard culturing and exposure procedures (**2.2.4, 2.2.5**). Samples were taken just after inoculation, and every three hours until 4-nitrophenol (0, 10 and 39mg/l) was added (11 hours). Samples (1.5ml) (taken hourly after 4-nitrophenol addition) were centrifuged at 15 x g for 10 minutes. The supernatant (expended media) passed through a 0.2µm Nalgene PTFE filter and frozen at -20^oC for storage until analysis.

2.2.10 Preparation of a glucose concentration standard curve

Methods described in the glucose assay kit (Sigma Aldrich) were followed as described in **2.2.11**. These briefly comprised, setting up a glucose standard curve and preparing samples as shown in **Appendix II Table 2.7.**

2.2.11 Measuring glucose concentrations in expended SC media

Media obtained in **2.2.9** was defrosted and 0.5ml of assay reagent added to each sample and mixed. Samples were incubated at 37^oC for 30 minutes before the reactions were stopped by adding 0.5ml of 12N H_2SO_4 and mixing. The absorbance of samples was measured in a Libra S12 Spectrophotometer at 540nm. The glucose concentration was calculated by comparison with the absorbance of samples on the standard curve in **Appendix I**, **Table 2.5**. The glucose concentrations of SC media incubated with 4-nitrophenol at (0, 10, & 39 mg/l) and *S. cerevisiae* can be found in *Figure 2.3.4*.

2.2.12 Preparation required for RNA harvesting

Glass beads (0.45-0.55mm) were soaked in nitric acid, washed with DEPC treated water, soaked in RNaseZAP®, rinsed with DEPC treated water, and dried in a baking oven. One milliliter of cleaned glass beads were aseptically transferred to screw cap centrifuge tubes (2ml) sealed and pre-cooled in liquid nitrogen. All pipettes, work areas and utensils were thoroughly cleaned with RNaseZAP® and all consumables were molecular biology grade.

2.2.13 Cell harvesting for transcriptomic analysis

Cultures treated with 4-nitrophenol (**2.2.8**) for exactly one, two and three hours were harvested by centrifugation at 700 x g for 5 minutes, the supernatant decanted, and the cell pellet re-suspended in the media remaining in the tube. The samples were pipetted as individual drops into pre-cooled (2ml) screw cap centrifuge tubes (**2.2.12**), filled with liquid nitrogen and sealed.

2.2.14 Cell disruption

Frozen cell samples in screw cap centrifuge tubes (**2.2.13**) were Ribolyzed (Fast Prep ^{TM}) and shaken at 6ms^{-1} for three 45 second intervals with liquid nitrogen cooling between each run.

2.2.15 total RNA extraction

TRIZOL (1.5 ml) was mixed with the frozen samples (**2.2.13**), supernatant was transferred to clean 2ml centrifuge tubes, and homogenised by vortexing. Chloroform (0.4ml) was added to the supernatant, shaken vigorously by hand for 15s and left at room temperature for 10 minutes. Samples were centrifuged at 12000 x g for 5 minutes, the upper aqueous total RNA containing phase transferred to a fresh centrifuge tube (2ml) and 0.5ml of IsoPropAnol (IPA) was added to precipitate the total RNA. Samples were left at room temperature for 15 minutes (Chomczynski & Sacchi 1987).

2.2.16 total RNA purification

RNA preparations (**2.2.15**) were centrifuged at 12000 x g for 10 minutes, the supernatant was removed and the pellet washed twice, vortexing it with 70% ethanol for 1 minute and centrifuging at 12,000 x g for 10 minutes. The ethanol was removed and the pellet air dried at room temperature for 5 minutes. DEPC treated distilled water (500µl) and 500µl LiCl buffer were added, and the samples frozen at -20°C overnight. Defrosted samples were centrifuged at 15 x g for 30 minutes, the LiCl buffer and water were removed and the pellet washed twice with ethanol (70%). The pellet was air dried at room temperature and dissolved in 100µl of DEPC treated RNase free water and stored at -70°C (Chomczynski & Sacchi 1987).

2.2.17 total RNA quantification and quality determination with the NanoDrop 1000 and Agilent 2100 Bioanalyser ®

Dilutions (10^{-1}) of RNA samples (**2.2.16**) were quantified using a NanoDrop 1000 (Thermo Scientific), sufficient total RNA was acquired, 260/280 ratios indicated no RNA samples were degraded, and 260/230 ratios showed all samples were free from chemical contamination (Agilent 2008). The conditions of rRNA bands were investigated using an Agilent 2100 Bioanalyser (*Figure* 2.3.7).

2.2.18 Statistical analysis

One way ANOVA $p < 0.05$ (SPSS 15) with LSD (Least Significant Difference) post hoc test was used to identify statistically significant results

2.3 Results

The OD_{600} of Control and Treated cultures measured using the methods in section **2.2.5 & 2.2.6** for each media type are shown in *Figure* **2.3.1** (YEPD) *Figure* **2.3.2** (SC) & *Figure* **2.3.3**(SD).

2.3.1 Determining the toxicity of 4-nitrophenol to *S. cerevisiae* growing in YEPD medium

In YEPD medium, the addition of 10 mg/l 4-nitrophenol/l caused no significant inhibition of *S. cerevisiae* growth: a slight but non-significant decrease in growth is observed later on in the growth phase. When exposed to 32 mg/l 4-nitrophenol the cells continued to grow at the same rate as the control for one hour, but significant inhibition was seen after two hours exposure (p = 0.035) and by 21 hours growth was about half that seen in the control (*Figure* **2.3.1**).
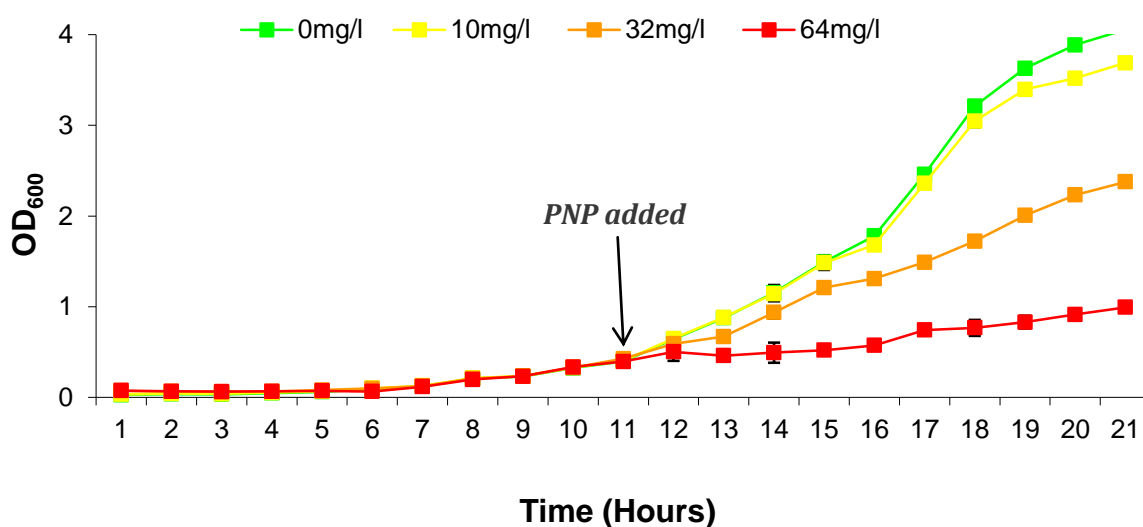
Figure **2.3.1** Growth of *S. cerevisiae* in YEPD medium, treated with 0, 10, 32 and 64 mg/l 4-nitrophenol (PNP) after 11 hours of incubation. The addition of 4-nitrophenol was made during mid logarithmic growth (after 11 hours of growth in YEPD media). Error bars (not visible) show standard deviation.

When exposed to 64 mg/l 4-nitrophenol a significant (p = 0.022) decrease in growth rate occurred immediately after the addition of 4-nitrophenol. The EC_{50} for *S. cerevisiae* exposed to 4-nitrophenol in YEPD medium was calculated as shown in **Appendix II 2.6 - 2.9** and was found to be 39mg/l.

2.3.2 Determining the toxicity of 4-nitrophenol to *S. cerevisiae* growing in SC medium

The growth curves in *Figure* **2.3.2** show in SC medium the toxicity of 4-nitrophenol is similar to that in YEPD (*Figure* **2.3.1**). At 10 mg/l 4-nitrophenol slight inhibition of growth occurred and this was apparent after 3 hours of exposure to 4-nitrophenol. When exposed to 32 mg/l 4-nitrophenol, cell growth was about half that of the control. After the addition of 64 mg/l, 4-nitrophenol the growth of *S. cerevisiae* was immediately affected. The EC_{50} for *S. cerevisiae* exposed to 4-nitrophenol during logarithmic growth was calculated as shown in **Appendix II** and was found to be 39 mg/l.
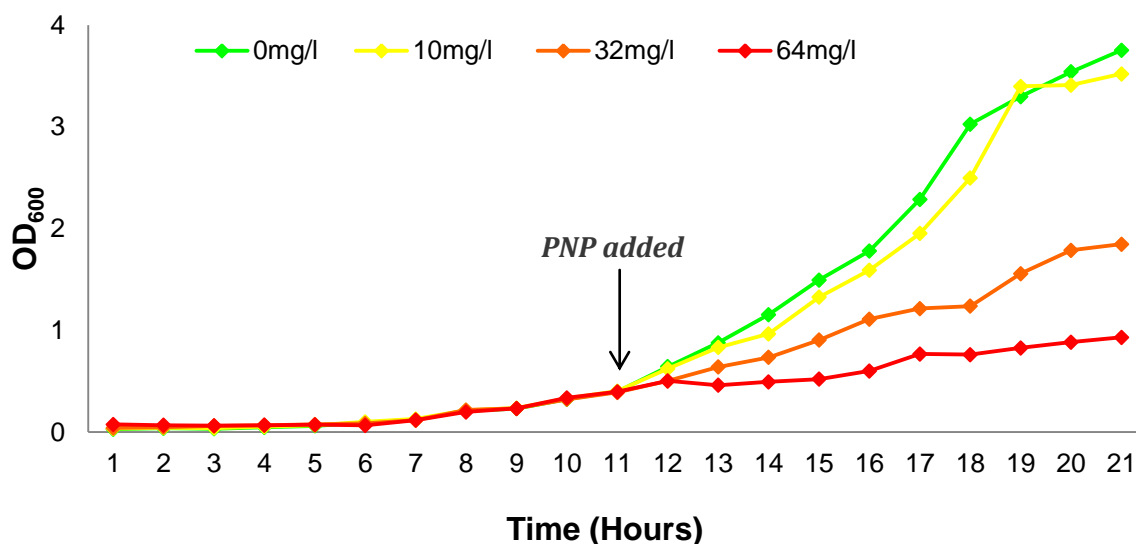
Figure **2.3.2** Growth of *S. cerevisiae* in SC medium, treated with 0, 10, 32 and 64 mg/l 4-nitrophenol after 11 hours of incubation. The addition of 4-nitrophenol was made during mid logarithmic growth which was after 11 hours of growth in SC media. Error bars (not visible) show standard deviation

In SC media at 10 mg/l 4-nitrophenol no significant inhibition of growth occurred, after 1 hours exposure to 32mg/l 4-nitrophenol cell growth was significantly (p = 0.035) inhibited. One hour after the addition of 64 mg/l 4-nitrophenol the growth of *S. cerevisiae* was significantly inhibited (p = 0.022, *Figure* **2.3.2**).

2.3.3 Determining the toxicity of 4-nitrophenol to *S. cerevisiae* growing in SD medium

The growth curves in *Figure* **2.3.3** show that in SD minimal medium, the final OD_{600} reached by *S. cerevisiae* is lower than in the other media, and the toxicity of 4-nitrophenol is increased.

At 10 mg/l 4-nitrophenol, growth was initially inhibited yet the cultures reach a higher final OD_{600}. Following exposure to 20 mg/l 4-nitrophenol, the OD_{600} continued to rise, but very slowly, and growth in these flasks was severely inhibited. Exposure to 32 mg/l 4-nitrophenol resulted in complete growth inhibition. The percentage inhibition caused by 4-nitrophenol in each of the three media was calculated from the growth curves using the formula in methods section **2.2.7**. The EC_{50} for *S. cerevisiae* exposed to 4-nitrophenol during logarithmic growth is 16mg/l.
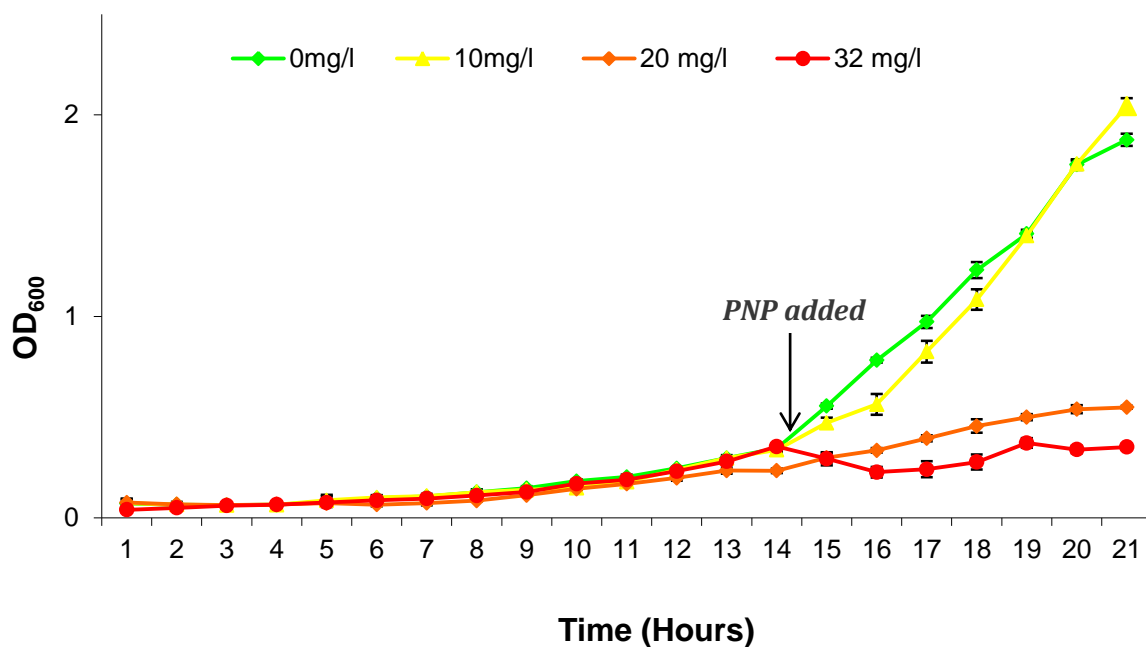
Figure **2.3.3** Growth of *S. cerevisiae* in SD minimal media treated with 0, 10, 20 and 32mg/l 4-nitrophenol. The addition of 4-nitrophenol was made during mid logarithmic growth which was after 14 hours of growth in SD media. Error bars show standard deviation.

In SD media at 10 mg/l 4-nitrophenol, growth was initially inhibited (p = 0.002 after 2 hours exposure) yet after 8 hours exposure the cultures reach a higher final OD_{600} than controls (p = 0.001). Exposure to 32 mg/l 4-nitrophenol resulted in complete growth inhibition after 1 hour of exposure (p = 0.009, *Figure* **2.3.3**).

2.3.4 Percentage inhibition of *S. cerevisiae* exposed to 4-nitrophenol at 10 & 32 mg/l

The percentage inhibition of *S. cerevisiae* exposed to 4-nitrophenol at 10 & 32 mg/l was calculated using the dose response curves (*Figures* **2.3.1, 2.3.2, & 2.3.3** and the formula in section **2.2.7**, the percentage inhibition for each media type are shown in *Figure* **2.3.4.**
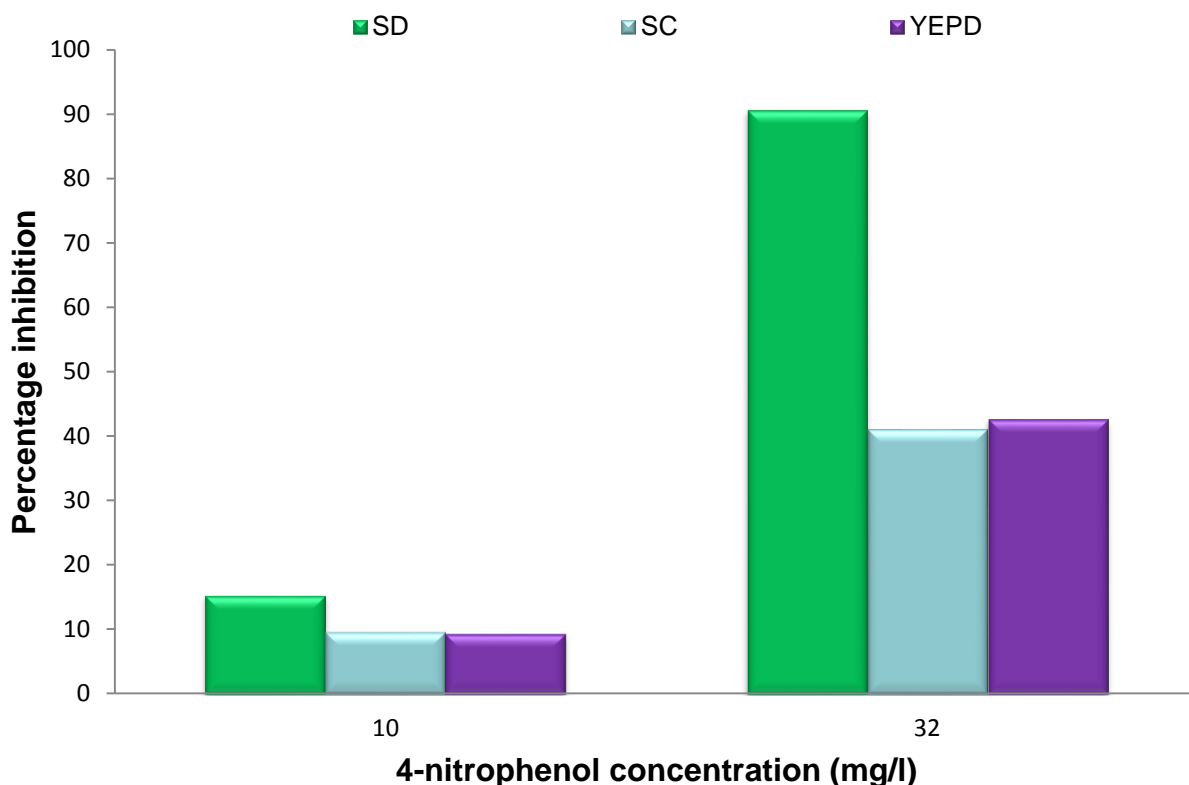
Figure **2.3.4** The percentage growth inhibition caused by 4-nitrophenol added during middle logarithmic growth of *S. cerevisiae* at 10 & 32 mg/l in SD, SC and YEPD media.

The bar chart in *Figure* **2.3.4** shows that 4-nitrophenol was more toxic in SD minimal media than in SC or YEPD. *S. cerevisiae* is more sensitive to 4-nitrophenol in SD media (EC_{50} 16mg/l). In YEPD and SC, media the EC_{50} value for 4-nitrophenol is higher (39 mg/l).

2.3.5 Changes in medium pH during 4-nitrophenol exposure

Each media type was investigated for its ability to resist changes in pH during the growth of *S. cerevisiae* (**2.2.6**), because changes in gene expression are known to accompany changes in pH

(**2.1.6**) this was considered an important factor for choosing the correct medium for microarray experiments.
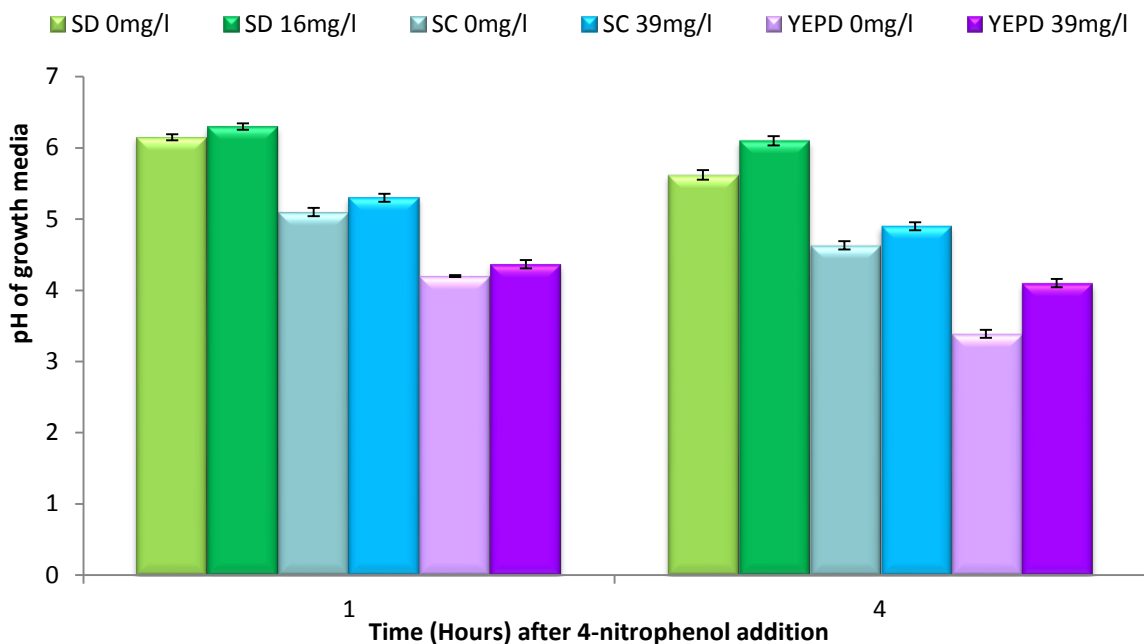
Figure 2.3.5 Changes in pH of each *S. cerevisiae* growth medium (SD, SC & YEPD) 1 and 4 hours after the addition of 4-nitrophenol at 0mg/l and the EC_{50} (16 mg/l SD, & 39 mg/l SC and YEPD) concentration. Error bars show standard deviation*.*

The bar chart in *Figure 2.3.5* shows the pH of treated (EC_{50}) and untreated (0 mg/l) *S. cerevisiae* growth media 1 & 4 hours after 4-nitrophenol addition. In SD media the pH was significantly ($p= 0.02$) higher in treated cultures after 4 hours but not 1 hour after addition. In SC, media there were no significant differences in the pH of media. In YEPD the pH was significantly higher ($p<0.00$) in treated cultures than untreated cultures 4 hours after 4-nitrophenol addition but not 1 hour after addition.

The results in **2.3.1 – 2.3.5** identified SC media as the best media for the gene expression study as the pH value between test and controls did not change. The concentrations chosen for further investigation were 10 and 39 mg/l 4-nitrophenol, and times for expression profiling were 1, 2 & 3 hours exposure to 4-nitrophenol. Therefore, the subsequent experiment to determine the quality of RNA extracted from yeast was conducted with the previously mentioned chosen conditions and in addition, glucose concentrations monitored (**2.3.6**) and total RNA extracted and quality assessed (**2.3.7**).

2.3.6 The effect of varying 4-nitrophenol concentration (0, 10 & 39mg/l 4-nitrophenol) in SC media on glucose levels over time.

S. cerevisiae cultures in SC media exposed to 39 mg/l 4-nitrophenol have significantly higher (p=0.00) glucose concentrations than cultures exposed to 0 mg/l 4-nitrophenol 2 & 3 hours after 4-nitrophenol addition. Cultures exposed to 10 mg/l 4-nitrophenol have glucose concentrations that are not significantly higher than cultures exposed to 0 mg/l 4-nitrophenol.
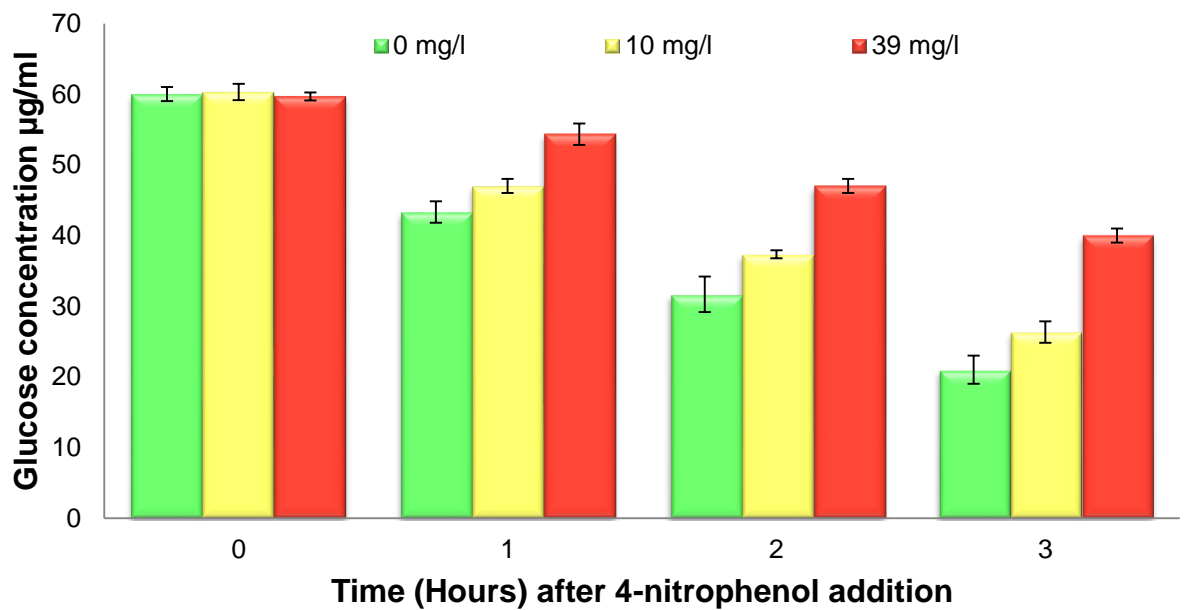
Figure 2.3.6 Changes in glucose concentrations (µg/ml) in SC medium throughout the growth of *S. cerevisiae* in untreated control cultures (0mg/l) and cultures treated with the EC_{10} (10 mg/l) & EC_{50} (39 mg/l) of 4-nitrophenol. Error bars show standard deviation*.

2.3.7 Agilent 2100 Bioanalyser image of selected total RNA samples extracted from 4-nitrophenol exposed yeast during growth in SC media.

The results in *Figure* 2.3.7 shows a gel picture of representative experimental total RNA samples produced by the Agilent 2100 Bioanalyser ®
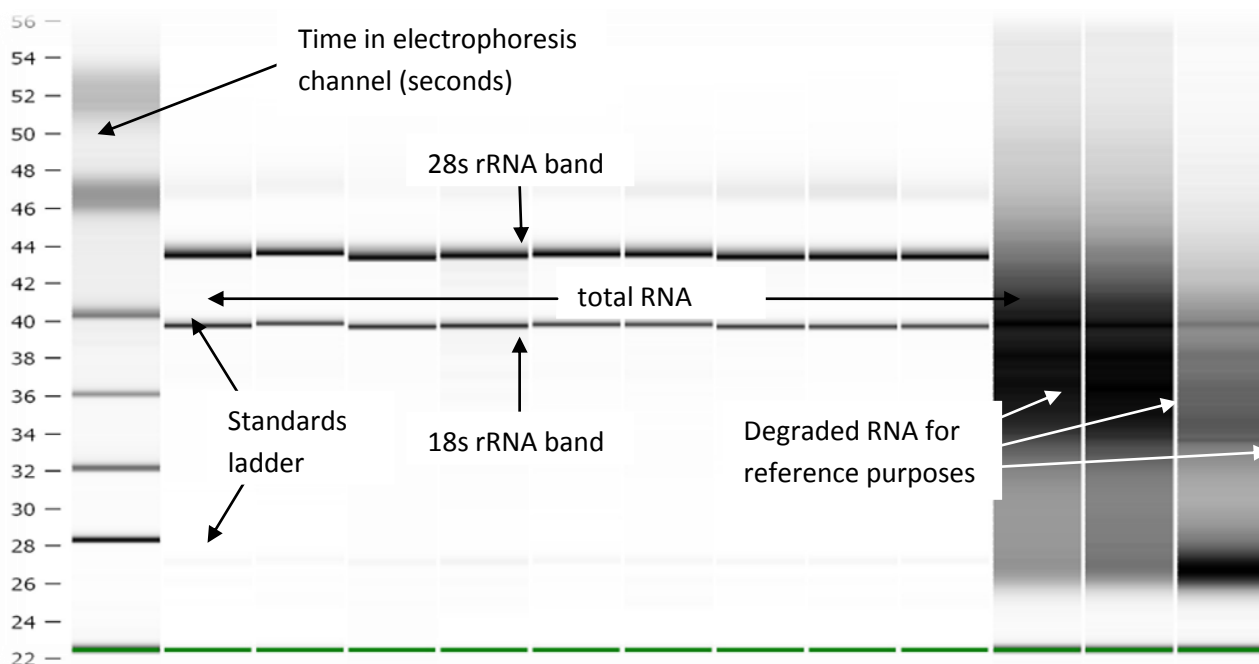
Figure 2.3.7 A labelled gel picture created by the Bioanalyser® for all samples (n=9) from 2 hours 4-nitrophenol exposure.

The clear sharp 18s & 28s rRNA bands (*Figure 2.3.7*) of the RNA samples extracted from *S. cerevisiae* exposed to 4-nitrophenol show that the total RNA samples are not degraded using the method described in **2.2**. Because the rRNA is the most abundant and thus can be inspected and quantified the 18s & 28s rRNA quality is related to overall RNA quality and is thus used to ascertain the quality of all RNA. High yields of high quality RNA were obtained using the Trizol extraction method.

2.4 Discussion

2.4.1 Toxicity of 4-nitrophenol to *S. cerevisiae*

This study assessed the toxicity of 4-nitrophenol to *S. cerevisiae* using growth inhibition tests. As a known uncoupler of mitochondrial oxidative phosphorylation (TenBrook *et al* 2005) 4-nitrophenol migrates across inner mitochondrial membranes and discharges the proton gradient created by electron transport; disrupting ATP synthesis (Williamson *et al* 1995). Growth inhibition (toxicity) of 4-nitrophenol is thought to arise from interference in ATP synthesis.

2.4.2 Toxicity of 4-nitrophenol to *S. cerevisiae* in different media

Three media (SD, SC, and YEPD) were compared for their ability to support the growth of 4-nitrophenol exposed *S. cerevisiae*; 4-nitrophenol was more toxic in minimal media (SD) than in YEPD or SC. This varying toxicity could be due to a number of possible factors. In a study by Barbey *et al* (2005) *S. cerevisiae* were more sensitive to cadmium in minimal media than in rich media. In *S. cerevisiae* glutathione chelates and detoxifies cadmium (Barbey *et al* 2005) and xenobiotics (Wheeler *et al* 2003). The protein Met4 that regulates glutathione production in *S. cerevisiae* is degraded when glutathione is required by cells. Barbey *et al* (2005) investigated the relationship between the degradation of the protein Met4 and cellular glutathione concentrations in rich and minimal media. In minimal media degradation of Met4 is impaired, decreasing cellular glutathione concentrations. In rich medium Met4 was degraded resulting in higher glutathione concentrations in cells (Barbery *et al* 2005). High cellular glutathione concentrations in *S. cerevisiae* stimulate cell division (Lloyd 2005; Lloyd & Murray 2006) and depletion of glutathione leads to increased sensitivity to oxidants and xenobiotics (Wheeler *et al* 2003). Therefore it is possible that inhibition of *S. cerevisiae* exposed to 4-nitrophenol in SD minimal media may have been higher due to depletion of cellular glutathione.

The bioavailability of cadmium in three media types was investigate by Hoffman *et al* (2005), they found that cadmium bioavailability was highest in minimal media. Ritchie & Bhattcharyya (2002) reported that Ca, Mg and Sulphates decrease availability of heavy metals for chelation clearly illustrating that difference in media composition could affect complexation and chelation of toxins, and therefore their bioavailability. Complexation and/or chelation of 4-nitrophenol in complex SC and YEPD media may occur reducing its availability and subsequent toxicity to *S. cerevisiae*.

Another measured variable in the experiment was pH; the pH results (*Figure 2.3.5*) show that one hour after 4-nitrophenol addition the pH of SC was 5, in YEPD it was 4 and SD was 6.5. The

pH of SD media is above the optimal pH range (4-6) for *S. cerevisiae* growth (Neelakantam *et al* 2004), and this may have contributed to the increased sensitivity of *S. cerevisiae* in SD media.

2.4.3 Comparing the effects of 4-nitrophenol on the growth of *S. cerevisiae* BY4741, other microorganisms and *Daphnia magna*.

2.4.3.1 *E. coli*

This study assessed the effects of 4-nitrophenol on the growth of *S. cerevisiae*, using growth inhibition. The EC_{50} for *S. cerevisiae* exposed to 4-nitrophenol (39 mg/l), is lower than the EC_{50} for *Escherichia coli* (56 mg/l) reported by Brown (2005). It is difficult to identify why species have different sensitivities because there are numerous potential reasons for differences in sensitivity between species including; physiology, cell size, how cells generate ATP, compartmentalisation and ultra structure, growth media differences and differences in the way toxicity was calculated.

2.4.3.2 *Alpine yeasts*

The toxicity of 4-nitrophenol on alpine yeasts was investigated by Bergauer *et al* (2004). They found that the EC_{50} for 4-nitrophenol exposed yeasts was 32 mg/l, which is close to 39 mg/l (the EC_{50} for *S. cerevisiae* exposed to 4-nitrophenol). A direct comparison of the EC_{50} concentration in the study by Bergauer *et al* (2004) and this study is not possible due to differences in culture and exposure conditions. However, the EC_{50} concentrations show that different species of yeast have similar sensitivities.

2.4.3.3 *Daphnia magna*

The toxicity of 4-nitrophenol to the model aquatic toxicity testing organism *Daphnia magna* was investigated by Gemini *et al* (2005) using mobility inhibition assays. A concentration of 1.4 mg/l caused inhibition of the mobility of 50% of the population under test and was thus calculated to be the EC_{50}. Growth inhibition assays are less sensitive than mobility inhibition assays, and the sensitivities of *S. cerevisiae* and *D. magna* cannot be directly compared. For comparative purposes a table of toxicity is provided (**Table 2.4.3.3**)

Table 2.4.3.3 Toxicity of 4-nitrophenol to three different microorganisms and the invertebrate *D. magna*

Species	EC_{50} (mg/l)	Author
S. cerevisiae	39	This study
E. coli	56	Brown (2005)
Alpine yeasts	32	Bergauer *et al* (2004)
D. magna	1.4	Gemini *et al* (2005)

2.4.4. Toxicity of 4-nitrophenol to terrestrial and aquatic vertebrates

2.4.4.1 *Aquatic vertebrates*

In a study conducted by TenBrook *et al* (2005) the toxicity of 4-nitrophenol to white sturgeon (*Acipenser transmontanus*) was investigated using inactivity of fish as an indicator of toxicity. *A. transmontanus* was found to be unresponsive to 1 mg/l 4-nitrophenol in water, the authors did not address the effect of higher concentrations.

2.4.4.2 *Terrestrial vertebrates*

In an investigation conducted by Li *et al* (2006) rodent uterotrophic and Hershberber assays were used for assessing the estrogenic and androgenic properties of 4-nitrophenol. In female rats' daily injections of 4-nitrophenol equal to 0.01, 0.1, and 1 mg/l over a 7-day period lead to a significant increase in uterine weight.

After 5 days of injecting 0.01, 0.1, and 1 mg/l, male rats showed significant decreases in liver and kidney weight. The weights of androgen dependent reproductive glands were also measured and significant decreases were seen in all but one of the glands assessed. In male rats it was also found that the plasma concentrations of Lutenising Hormone (LH) and Follicle Stimulating Hormone (FSH) were increased. Li *et al* (2006) therefore concluded that 4-nitrophenol shows estrogen like effects on female rats and anti-androgen like effects on male rats.

2.4.5 Comparing species sensitivities

The toxicity of 4-nitrophenol to test organisms are difficult to compare due to differences in the ways toxicity tests are conducted for each organism tested. Growth inhibition assays with microorganisms show they are the least sensitive to 4-nitrophenol however growth inhibition assays are themselves insensitive (**1.1.6**), but they can be used in conjunction with more sensitive assays. Gene expression analysis is extremely sensitive it can be used with model microorganisms such as *S. cerevisiae* and *E. coli* to assess the effects of 4-nitrophenol at low concentrations. Previous gene expression studies (Zhang *et al* 2003) have identified impacts of pollutants on microorganisms at concentrations lower than those required for observed growth inhibition. The lowest concentration tested in this study was 10 mg/l, at this concentration no significant growth inhibition was observed, subtle effects of low concentrations of 4-nitrophenol on gene expression will be investigated in *S. cerevisiae* using 10mg/l 4-nitrophenol.

2.4.6 Changes in pH of media during culturing

Growth of microorganisms results in pH changes due to metabolic activities and the production of acid metabolites (Snyder & James 1963). Changes in the pH of growth media

occur during the growth of cells, and pH changes cause changes in cell gene expression (Lamb *et al* 2000). Inhibiting the growth of cells by chemical treatment can slow pH changes in treated cultures by decreasing the rate of production of acid metabolites, leading to differing pH in control and treated cultures. The changes in pH of three media (SD YEPD and SC) in cultures of treated and untreated cells were compared to find the medium whose pH remained consistent in treated and untreated cultures for use in microarray experiments. The results show that the least variation in pH of media for treated and untreated cells occurred in SC media.

2.4.7 Choosing the best media for microarray experiments

Three *S. cerevisiae* growth media (YEPD, SC & SD) were compared to identify the best media for use in subsequent toxicogenomic studies. Calculating EC_{50} values for *S. cerevisiae* exposed to 4-nitrophenol in each media identified that *S. cerevisiae* is more sensitive to 4-nitrophenol in minimal Synthetic Defined (SD) media. Normal growth in SD media was restricted; SD had lower cell densities than in YEPD and SC. The pH of SD media is outside the optimal growth pH for *S. cerevisiae,* and treated cultures of *S. cerevisiae* in SD media have significantly higher pH than untreated cultures after four hours 4-nitrophenol treatment*.* Synthetic Defined (SD) media was eliminated from consideration as increased sensitivity and large pH differences between treated and untreated cultures were deemed to make it unsuitable for subsequent microarray experiment.

YEPD medium is produced using yeast extract and peptone, of which is neither defined nor standardised. It is unsuitable for this microarray experiment because it contains non-standardised ingredients and there are significant pH differences in treated and untreated *S. cerevisiae* cultures during growth in YEPD medium.

SC medium was chosen for use as a growth media in microarray experiments as there are no significant differences in the pH of treated and untreated *S. cerevisiae* cultures after four hours exposure to 4-nitrophenol. In addition SC is a defined synthetic medium whose exact composition is known, and it does not limit *S. cerevisiae* growth, making it ideal for a toxicogenomic study.

2.4.8 Changes in glucose concentrations of SC media during culturing

Glucose concentrations in untreated control cultures of *S. cerevisiae* in SC media were significantly lower than those treated with 39 mg/l 4-nitrophenol. Cultures treated with 4-nitrophenol were inhibited and used glucose reserves slower. Glucose is an important primary messenger, which signals to the cellular machinery when growth conditions are optimal (Rolland *et al* 2002). Changes in glucose concentrations trigger major metabolic shifts that can

result in the gene expression levels of up to 30% of genes being altered (Schneper *et al* 2004; Rolland *et al* 2002). One way of controlling for this is to use a chemostat called a gradostat, which maintains constant nutrient and solute concentrations (Hoskisson & Hobbs 2005), but chemostat culturing methods are expensive compared to batch culture methods. The purpose of this study is to develop a quick, relatively cheap, easy to use, sensitive assay, that can be used with microarrays to determine un-safe concentrations of 4-nitrophenol (**1.1.1**). *S. cerevisiae* responds to decreased glucose concentrations by producing ethanol an industrially and commercially important compound. The desire to gain an understanding of the processes leading to ethanol production in *S. cerevisiae* has lead to numerous investigations into the effects of reduced glucose concentration on gene expression in *S. cerevisiae* (Nisamendtinov *et al* 2008; Gibson *et al* 2008; Bertilsson *et al* 2008; Gibson *et al* 2008; Klockow *et al* 2008; Rintala *et al* 2008). As a result genes and metabolic pathways altered by glucose concentrations have been identified and well characterized, and genes that could potentially be differentially expressed in response to lower glucose concentrations in control cultures and pathways affected can be easily identified. Additionally in the study by Gibson *et al* (2008) gene expression responses to glucose depletion were investigated, changes in gene expression were not detected until glucose concentrations were below 20µg/ml, equal to the concentration of cultures exposed to 0mg/l 4-nitrophenol for three hours. These results suggest that glucose concentrations in control cultures after three hours are not low enough to alter glucose depletion responsive genes. Therefore to reduce the cost and complexity of the experiment and to meet the aims of the work i.e. to develop a simple, inexpensive yeast toxicity test, batch cultures were used in all further experiments.

2.4.9   Choosing 4-nitrophenol concentrations for microarray experiments

In toxicogenomic studies the EC_{50} concentrations are frequently used to assess the effects of chemicals (Schmitt *et al* 2004; Zhang *et al* 2003). This project aims to look at the effects of 4-nitrophenol on gene expression in *S. cerevisiae,* investigating the effects of 4-nitrophenol at the EC_{50} concentration will allow comparisons with other studies to be made. Gene expression data for *E. coli* exposed to 10 mg/l 4-nitrophenol is available (Brown, 2005) and 10 mg/l 4-nitrophenol does not cause significant inhibition of *S. cerevisiae*, despite this growth inhibition results show that 10% growth inhibition occurs at 10mg/l. Therefore it was decided that gene expression responses of *S. cerevisiae* exposed to the EC_{10} concentration of 10 mg/l would be assessed along with the EC_{50} value. This will allow the effects of the same concentration on each organism to be compared as well as allowing the effects of low concentrations of 4-nitrophenol on gene expression to be assessed.

2.4.10 Choosing time-points for expression profiling

Sampling times for microarray experiments are usually hourly, most frequently two hours after the addition of a toxicant. As in the studies by John *et al* (2005), Kim *et al* (2006) and Hirasawa *et al* (2007) where the effects of two hours exposure to cigarette smoke extracts, Textile Mill Effluents (TME's) and 5 % ethanol on gene expression in *S. cerevisiae* were investigated. However, in some studies more than one sampling time is chosen to measure the expression of genes involved in resistance, and these samples are usually taken in the first three hours of exposure (**1.6.4**). In this investigation samples will be taken 1, 2 and 3 hours after 4-nitrophenol exposure to gain a picture of time dependent cellular changes resulting from chemical treatment, and allow comparisons with other gene expression studies in *S. cerevisiae* and other organisms to be made.

2.4.11 total RNA Quality

Total RNA sample quality was assessed by looking at nanodrop™ readings and Agilent Bioanalyser results. The results showed that enough high quality (un-degraded, pure) total RNA was obtained to proceed with RT-PCR (Chapter 3).

2.5 Summary & Conclusions

The sensitivities of microorganisms to 4-nitrophenol vary and differences in the sensitivities of organisms to 4-nitrophenol may result from a variety of factors. Direct comparisons between the organisms are not possible due to differences in culturing conditions, ATP production sites (cell wall versus mitochondria), physiological differences (cell size, cell wall composition / membrane structures), and differences in the type of test used i.e. growth inhibition versus mobility inhibition.

Growth inhibition studies with microorganisms have shown that high concentrations of 4-nitrophenol in growth media are required to inhibit growth. Growth inhibition studies are insensitive, however when used alongside gene expression analysis, they can identify the effects of chemicals at concentrations below those that cause cell inhibition. The main aim of this chapter was to identify suitable media, exposure concentrations and exposure times for subsequent gene expression analysis. The results identified SC medium as the most reliable for microarray experiments because it is standardised, cell growth is the same as in YEPD rich media), and pH differences between treated and untreated cultures are minimal. The concentrations of 4-nitrophenol chosen for investigation were 39 mg/l (the EC_{50} for *S. cerevisiae* exposed to 4-nitrophenol) and 10mg/l (the EC_{10} for *S. cerevisiae* exposed to 4-nitrophenol) because both concentrations allow comparisons with other organisms   Most

frequently toxicogenomic studies conduct expression profiling after two hours exposure to toxins. To allow comparisons with other toxicogenomic studies it was decided to carry out expression profiling at one, two and three hours after exposure to 4-nitrophenol. Sufficient high quality RNA for RT-PCR and subsequent expression profiling was extracted from *S. cerevisiae* exposed to 0, 10 & 39mg/l 4-nitrophenol for one, two & three hours in SC media.

Chapter 3

3. Normalisation of gene expression data and subsequent determination of differentially expressed genes in response to 4-nitrophenol treatment in *S. cerevisiae;* gene ontology studies

3.1. Introduction

3.1.1   Quality checking gene expression results

As previously discussed (**1.6.1 – 1.6.3**) it is important to check the quality of gene expression results because many factors can affect microarray experiments. There are two quality-checking procedures, PCA, and Box Whisker plots. PCA is used to cluster genes based on their expression profiles and identify trends in the data such as changes in expression over time, as well as in the identification of outliers and samples with degraded cRNA. Additionally problems with hybridization can be identified using Box Whisker plots, which are used to visualise relationships between gene expression profiles of replicates within and between treatments (Dillman & Phillips 2005).

3.1.2   Identifying differentially expressed genes

Toxicogenomics studies with *S. cerevisiae* have previously used two fold or more changes in expression to identify differentially expressed genes (**1.6.4**). However, statistical tests often identify more differentially expressed genes, so two fold changes in expression may be too stringent. Investigators usually publish lists of genes that are two fold or more differentially expressed because these lists are smaller (**Table 1.6.3**). However, it is highly likely that two fold differentially expressed genes only tell part of the story, and while they are good indicators of processes being altered by pollutant exposure, this chapter will be used to illustrate how identifying significantly differentially expressed genes can be beneficial.

Aims of Chapter 3

Aim 1 Assess the quality of gene expression data in yeast exposed to 4-nitrophenol generated via microarray analysis and identify and remove samples with poor quality cRNA

Objectives to meet Aim 1

1. Expose *S. cerevisiae* to 4-nitrophenol using methods identified in Chapter 2 and conduct gene expression analysis

2. Normalise gene expression data using a GC-RMA summarisation algorithm and quantile normalisation (described in **1.5.8**). Use PCA, Box plots and correlation plots to assess gene expression data quality, remove outliers (samples with poor quality cRNA)

Aim 2 Identify appropriate gene filtering methods to select genes differentially expressed in response to 4-nitrophenol treatment

Objectives to achieve Aim 2

1. Compare two way ANOVA and MTC methods to select a workable number of genes known to be differentially expressed as a result of 4-nitrophenol exposure

2. Identify pathways significantly affected by 4-nitrophenol exposure

3. Remove the confounding effect of time on gene expression in yeast i.e. selection of genes only affected by 4-nitrophenol exposure (used in gene ontology analysis – Aim 3 this chapter)

Aim 3 Provide an overview of effects of 4-nitrophenol on *S. cerevisiae*

Objective to achieve Aim 3

1. Identify pathways and Gene ontology categories affected by 4-nitrophenol treatment and use these to draw conclusions about the effects of 4-nitrophenol on *S. cerevisiae*

3.2 Methods

4-nitrophenol exposure of yeast :

S. cerevisiae was exposed to 10 & 39 mg/l (**2.4.9**) in SC media (**2.4.7**), samples for expression profiling were taken after 1,2 & 3 hours exposure to 4-nitrophenol (**2.4.10**) and total RNA extracted using TRIZOL (**2.2**)

3.2.1   RT-PCR and cRNA labelling

3.2.1.1 *Primer hybridisation*

1µl (100pmol) of T7-(dT)24 primer was added to 15µg of total RNA in 10µl DEPC treated water, and incubated at 70°C for 10 minutes. Once primer annealing had occurred the samples were spun in a microfuge and put on ice for the addition of 5x First strand cDNA buffer (4µl, 1x), 0.1M DTT (2µl, 10mM DTT), 10mM dNTP mix (1µl, 500µM of each), samples were mixed and incubated at 42°C for two minutes. Once the samples had cooled to 42°C, 2µl of Superscript II RT (200U/µl) was added, before being mixed and incubated at 42°C for 1 hour (Affymetrix 2008).

3.2.1.2 *Second strand cDNA synthesis*

After one hour incubation at 42°C, samples were placed on ice and the reagents in **Table 3.2.1.2** were added.

Table 3.2.1.2 Second strand cDNA synthesis reagents

Reagent	Amount (µl)	Concentration
DEPC water	91	
5x Second strand cDNA buffer	30	1x
10mM dNTP mix	3	200µM of each
10U/µl *E. coli* DNA ligase	1	10U
10U/µl *E. coli* DNA Pol. I	44	40U
10U/µl *E. coli* RNase H	1	2U
Final volume	150	

The samples were spun briefly to mix and incubated in a cooled shaking heat block at 16°C at 300rpm for two hours.

3.2.1.3 *Clean up of double stranded cDNA using the Affymetrix GeneChip® sample clean up module*

Samples (**3.2.1.2**) were placed on ice before addition of 10µl of 0.5M EDTA and 600µl of cDNA binding buffer added to the double stranded cDNA synthesis. This preparation was mixed by

vortexing for 3 seconds. Then 500µl of this sample was added to the cDNA cleanup spin column and centrifuged for one minute at 8 x g flow through was discarded and the spin column reloaded with the remaining mixture and centrifuged at 8 x g for one minute. The spin column was then transferred to another 2 ml collection tube, 750µl cDNA wash buffer was added to the spin column and centrifuged for one minute at 8 x g. After centrifugation, the flow through was discarded. The sample was then centrifuged again at 8 x g for five minutes, and the flow through collection tube was discarded. The spin column (containing cDNA) was transferred to a 1.5ml collection tube and 14µl of cDNA elution buffer was pipetted directly onto the spin column membrane. This was then left at room temperature for one minute; the samples were then centrifuged at maximum speed for one minute to elute the cDNA.

3.2.1.4 *Synthesis of biotin labelled cRNA (In Vitro Transcription (IVT)): Using Affymetrix commercial reagents*

The following reagents were added to samples in the order and quantities described; a 6µl aliquot of the template cDNA, 14µl dH$_2$O, 4µl 10x IVT labelling buffer, 12µl of IVT labelling NTP mix, and 4µl IVT labelling enzyme mix. The tubes were tapped to mix them and placed in an oscillating incubator at 37°C for 16 hours.

3.2.1.5 *Cleanup of labelled cRNA using GeneChip® sample clean up module (Affymetrix)*

Samples were removed from the oscillating incubator (**3.2.1.4**), 60µl of RNase free water added and the tubes were vortexed for three seconds. Then 350µl IVT cRNA binding buffer was added and the samples mixed again by vortexing for three seconds. Samples were amended with 250µl of 100% ethanol and mixed well by pipetting before transfer to the IVT cRNA cleanup spin column contained in a 2ml collection tube. Tubes were centrifuged for one minute at 8 x g, the flow through and collection tube being discarded. The spin column was transferred to another 2ml collection tube, 500µl of IVT cRNA wash buffer added to the spin column and centrifuged for one minute at 8 x g, the flow through being discarded. 500µl of 80% ethanol was pipetted onto the spin column and the column centrifuged for one minute at 8 x g, the flow through was discarded, and centrifugation repeated at maximum speed, the flow through and collection tube again discarded. The spin column was transferred to a 1.5ml collection tube and 11µl of RNase free water was pipetted directly onto the membrane of the spin column and centrifuged for one minute at maximum speed. This process was repeated with 10µl RNase free water to elute the labelled cRNA. The cRNA collected was quantified using the nanodrop 2000 (Thermo scientific).

3.2.2 Hybridisation of labelled cRNA

3.2.2.1 *cRNA fragmentation using Affymetrix kit*

The reagents in **Table 3.2.2.1** were added to a clean sterile RNase & DNase free PCR tube and incubated 94ºC for 35 minutes after which they were placed on ice.

Table 3.2.2.1 cRNA fragmentation reagents and quantities

Component	Volume µl
15µg cRNA	24
5X Fragmentation buffer	6

3.2.2.2 *Target Hybridisation*

The reagents in **Table 3.2.2.1** were then mixed in a 500µl PCR tube.

Table 3.2.2.2 Hybridisation cocktail reagents and quantities.

Component	Standard array	Final concentration
Fragmented cRNA (from experimental samples)	15µg	0.05µg/µl
Control Oligonucleotide B2 (3nm)	5µl	50pM
20X Eukaryotic Hyb, Controls (bioB, bioC, bioD, cre)	15µl	1.5, 5, 2.5 and 100pM
Herring Sperm DNA (10mg/ml)	3µl	0.1mg/ml
Acetylated BSA (50mg/ml)	3µl	0.5mg/ml
2X Hybridisation buffer	150µl	1X
100% DMSO	30µl	
Water	64µl	
Final volume	300µl	Fill array with 200µl

Hybridisation cocktails were frozen for storage at -20ᵒC

3.2.2.3 *Preparing stock solutions for hybridisation protocols*

Stock solutions and buffers for washes were prepared; the constituents of which can be found in **Appendix II.**

3.2.2.4 *Hybridising experimental samples with Affymetrix Yeast 2.0 microarrays*

The Yeast 2.0 probe arrays were removed from cold storage and left for 30 minutes to warm up to room temperature. The hybridisation cocktail was prepared for hybridisation by heating it to 99ᵒC for 5 minutes and then 45ᵒC for five minutes. The array was filled with 200µl of 1x hybridisation buffer and incubated at 45ᵒC for 10 minutes. The hybridisation cocktail was spun at 8 x g for five minutes to remove insoluble material. The hybridisation buffer was removed

from the probe array and the array re-filled with the hybridisation cocktail. The probe array
was then placed in a rotisserie box and rotated at 60rpm and 45ºC for 16 hours.

3.2.2.5 *Staining of Affymetrix Yeast 2.0 microarrays*

Staining and antibody solutions were then made up freshly as described in **Appendix II**
Tables 3.6 – 3.8 The arrays were removed from the hybridisation oven, hybridisation
cocktails were removed and stored at -20ºC in labelled centrifuge tubes. Each probe array was
then filled with 200µl of Non-Stringent wash buffer, the fluidics station was primed using the
PRIME_450 protocol, and the samples were run through the fluidics protocol using the
specified programme (Mini_euk2v3) for an 11µm, 169 format array. The steps carried out by
this fluidics programme can be found in **Appendix II Table 3.9**.

3.2.2.6 *Data acquisition and processing*

Once fluidics was complete, the arrays were removed from the fluidics station and
immediately scanned in the Affymetrix scanner.

3.2.3 Data processing

Experimental raw data files (.CEL) were imported into GeneSpring GX 10^{TM}, using a GCRMA
summarisation algorithm and Quantile normalisation, with baseline transformation to the
median of all samples. All gene signals (10928) were filtered to remove normalized signal
values below the 20th percentile. The resulting gene list was filtered on error to remove probe
signal values with a cumulative variance greater than 50%. The resulting list of 6,434 gene
signals that included Affymetrix spike in hybridisation controls, reporter genes, and *S.
cerevisiae* genes was saved.

3.2.4 Checking the quality of gene expression data

The gene signals were Quality Checked (QC) by comparing the Affymetrix hybridisation control
profiles with those expected.   In addition, Principal Component Analysis (PCA) was used to
identify outliers and assess variables affecting the data (*Figures 3.3.1 - 3.3.3*). The 3' 5' ratios
of hybridised cRNA were used to identify and remove samples with degraded cRNA. A Box
Whisker plot (*Figure 3.3.4*) was used to identify biological differences between treatment
groups and show relationships between experimental parameters.

3.2.5 Comparing two Multiple Testing Correction methods to identify differentially
expressed genes

There are no studies like this one in the literature, some authors (Zhang *et al* 2003; John *et al*
2005; Alexandre *et al* 2001; Kim *et al* 2006) have looked at genes differentially expressed after
specific exposure times, and used two fold change in expression to identify genes of interest.

Whereas some have looked at the effects of stressors over time and used fold changes in expression alongside clustering to identify genes in related pathways (Hirasawa *et al* 2007; Pandey *et al*(2007). Two experimental parameters (time and 4-nitrophenol concentration) could affect the gene expression profiles. To ensure the analysed data was relevant and for the reasons discussed in **1.7.3 – 1.7.6** Two Way ANOVA with asymptotic p-value computation and Benjamini-Hochberg, and Bonferroni-Holm Multiple Testing Correction were used to find significantly differentially expressed genes (p<0.01). The number of genes significantly differentially expressed at p<0.01 for both Multiple Testing Correction methods were compared and used to select the Multiple Testing Correction method that gave an acceptable number of genes to go onto further analysis (*Table* 3.3.6).

3.2.6   Selecting genes for subsequent analysis

Two Way ANOVA with asymptotic p-value computation (p<0.01) and Benjamini-Hochberg Multiple Testing correction were chosen to create the list of genes for further analysis, because they were not overly stringent and could be used to identify the effects of experimental parameters. The find significant pathways feature in GeneSpring GX 11 was used to identify pathways significantly (p<0.05) affected by experimental parameters. Pathway analysis revealed genes affected by time were related to cell cycle, which was reduced by treatment due to cell inhibition. Genes were overlapped in Venn diagrams and a parent list of all genes affected by 4-nitrophenol concentration and affected by an interaction between time and 4-nitrophenol concentration (1,357), were separated from genes affected by time only and saved (*Figure 3.3.8*).

3.2.7   Identifying Gene Ontology categories significantly affected by 4-nitrophenol treatment

Gene Ontology Analysis using all genes significantly affected by 4-nitrophenol (**3.2.6**) using Benjamini-Yekutieli corrected p-values (corrected to remove bias caused by multiple GO term testing and subsequent correlations) classified Genes. P-values below 0.05 were considered significant. The resulting Gene Ontology categories affected by 4-nitrophenol, and the number of genes in each category were recorded (see *Table* 3.3.9).

3.2.8   Selection of primers for qRT-PCR

Eight genes were chosen for confirmation of expression levels by qRT-PCR; these were GRE2, PDR16, PDR15, FIT2, HXT5, PGU1, PDA1 & HAC1.   Six genes with altered expression (GRE2, PDR16, PDR15, FIT2, HXT5, & HAC1) were chosen alongside two housekeeping PGU1 & PDA1 (genes with unchanged expression) for the reasons highlighted in **1.6.9**. Briefly, most studies confirm the expression levels of six genes or less, and genes with high fold changes in

expression are confirmed. The fold change of each of the selected genes was calculated from normalised expression values in GeneSpring GX 11. Genes with fold changes in expression between one and thirteen fold were selected from the data (**Table 3.2.8**), apart from two genes (PGU1 & PDA1) that act as required controls and do not have changes in expression (**1.6.9**). Literature searches were conducted to identify studies that successfully used primers for RT-PCR of these genes. It was decided that existing primer sequences with proven track records would be used for qRT-PCR. The primer sequences and authors of studies that have previously used these primers can be found in **Table 3.2.8**. Standard concentrations (25 nmole) of the primer sequences shown in **Table 3.2.8** were ordered from Invitrogen.

Table 3.2.8 Primer sequences for genes selected for confirmation by qRT-PCR

Name	Forward primer	Reverse primer	Author
HXT5	GGGCGTTGGTGGTATTACAG	ATGGTACCCTCCATTGGACA	Suyllekom *et al* 2007
HAC1	GAACAACAACTTATTTTTACAATGA	AATAGACAGATAGATATGACACAA	Kimata *et al* 2006
PDR16	GAGTGCAAGCATGCGATATATCTC	CAGACAATGAGCACAATATTGTGCA	Danila *et al* 2003
PDR15	GGAATTCCGCCCAGCCTTTTATACCT	CGGGATCCCTTGAGCTCGAGCTCCG	Decottignies *et al* 1998
PGU1	GTGCTTCGGGACATACCATT	CTGCAACGCCAACTTTACAA	Divol & Rensburg 2007
PDA1	GGTCAGGAGGCCATTGCTGT	GACCAGCAATTGGATCGTTCTTGG	Del Aguila *et al* 2003
FIT2	CATCAACCAACCTTTACACCAAG	ACTAGCCTTTTGAAGGAGAAAGACA	Kitagawa *et al* 2005
GRE2	CGATTTTCAAACAAACAGATAGCAG	AAAATGCGCAGAGATGTACTAGATG	Ema *et al* 2006

3.2.9 Methods for qRT-PCR

The Lightcycler FastStart DNA Master SYBR Green I kit was purchased from Roche and the hotstart PCR master mix was mixed as described in the protocol. All cDNA samples were diluted so that their final concentration was 80ng/µl and 2µl was added to each reaction tube. A dilution series of 1 in 3 and 1 in 6 was then made for each cDNA sample. The MgCl$_2$ stock solution was then added in the quantities indicated by the corresponding authors (**Table 7.2.1**) along with 1µl (25 nmole) of each of the forward and reverse primers (**Table 7.2.1**). Then 2µl of the FastStart DNA Master SYBR Green I mix was added and the solution made up to 18µl with PCR grade H$_2$O. The reactions were run on (Roche lightcycler 2.0®) qPCR conditions were used with an initial denaturation step of 15 minutes at 95 °C followed by 45 cycles of 94 °C for 20 s, 58 °C for 30 s and 72 °C for 45 s with a final extension step of 5 minutes at 72 °C. A melting curve analysis followed PCR amplification at 95°C to 65°C at a rate of 0.2°C/1s with continuous acquisition of fluorescence decline. Standard curves were generated

with the primer pairs PGU1, PDA1 using the serially diluted *S. cerevisiae* cDNAs by plotting threshold cycle (Ct) versus concentration of cDNA of *S. cerevisiae* (Chang *et al* 2007). The fold change in expression was calculated using the comparative ct method detailed in **1.6.7**. In order to increase the reliability of the method, expression levels were calculated with each reference gene separately, and the reported fold change in expression was calculated from the average (**Table 3.3.1**).

3.3 Results

3.3.1   Comparison of expression levels calculated from microarrays and qRT-PCR

To provide an overview of the results and allow comparisons of expression levels measured using microarrays and qRT-PCR the fold changes recorded using each method are documented alongside their pearsons correlation coefficient in **Table 3.3.1.** Fold changes in expression were calculated using PDA1 & PGU1 as reference genes using the calculation in 1.6.7.

Table 3.3.1 Correlation of average fold change in expression of selected genes using microarrays and qRT-PCR

Gene name	Time	Fold change (array data)		Fold change (qRT-PCR)		Pearson's Correlation (r^2)	
4-nitrophenol		10 mg/l	39 mg/l	10 mg/l	39 mg/l	10 mg/l	39 mg/l
FIT2	1	-1.1	6.3	1.1	3.9	0.95	0.98
	2	1.1	13.2	1	12.4		
	3	-1.2	5	1.1	4.1		
GRE2	1	1.3	4.4	1.1	3.8	0.61	1.00
	2	1.3	5.2	1.1	5		
	3	1.3	2.6	1.8	1.2		
HAC1	1	1.0	-2.5	0.8	-2.2	0.71	0.99
	2	1.5	1.6	2.4	3.25		
	3	3.6	12.7	2.5	11.7		
HXT5	1	-1.5	2.5	-0.7	1.4	0.99	0.99
	2	-4.2	-3.6	-3.6	-4.5		
	3	-12	-14	-9.5	-11		
PDR15	1	-1.8	3.5	-0.8	2.0	0.98	1.0
	2	1	3.7	-0.8	1.5		
	3	-1.8	3.3	-1.2	2.5		
PDR16	1	1.1	2.1	0.9	2	1.0	0.99
	2	1.1	2.6	0.9	2.3		
	3	1.8	3.5	1.5	2.8		

The results in **Table 3.3.1** show that most of the gene expression changes measured using microarrays were confirmed using qRT-PCR. One exception is FIT2, which has negative values for microarray recorded expression values after one, and three hours exposure to 10 mg/l, but

positive values resulted from qRT-PCR, this result is discussed in **3.4.1**. No significant correlation is seen in GRE2 in cells exposed to 10 mg/l 4-nitrophenol, and HAC1 at 10 mg/l are not correlated either. Overall, the expression levels of genes from microarrays and qRT-PCR are highly correlated.

3.3.2 Use of Principal Component Analysis to identify outlying samples

Principal Component Analysis of all quantile normalised signal values was used to identify samples whose expression profiles did not cluster with the rest of the data.
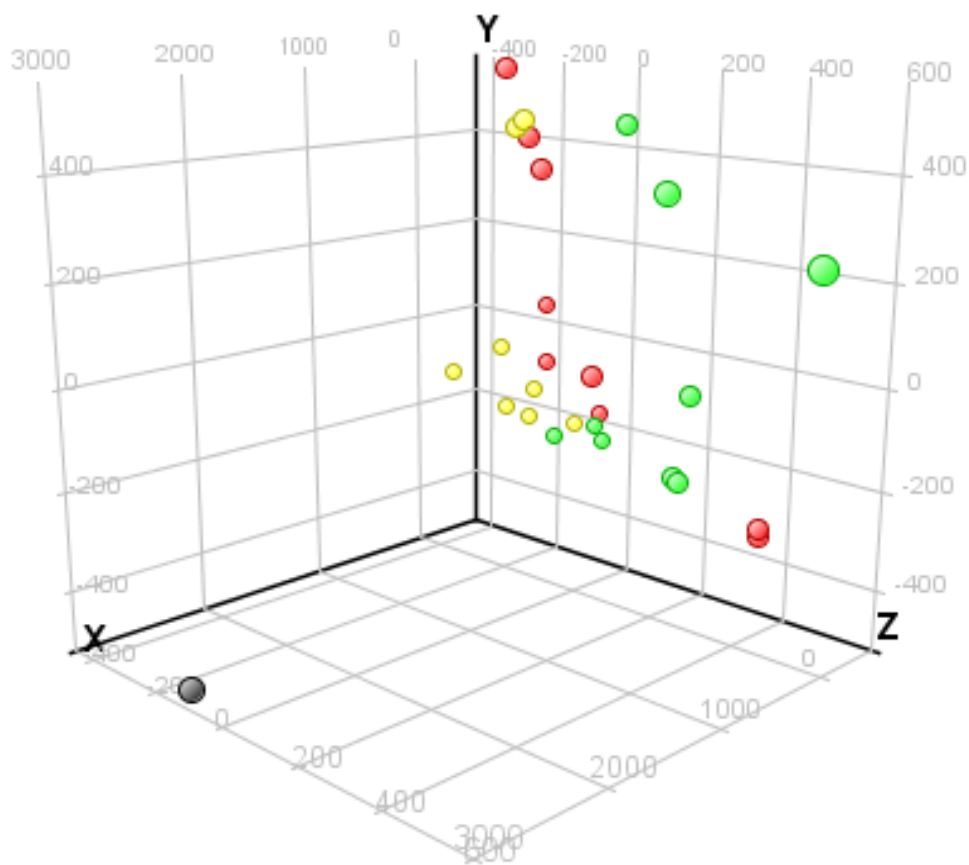
Figure **3.3.2** Principal Component Analysis of all quantile normalised samples coloured by 4-nitrophenol concentration. 0mg/l (green), 10mg/l (yellow), 39mg/l (red) the outlying sample (10r1h3 i.e. 10mg/l 4-nitrophenol, replicate 1, hour 3) is highlighted in black. The data on axis X, Y and Z are principal components 1, 2 and 3 respectively.

The Principal Component Analysis in *Figure* **3.3.2** shows that the expression profile of the highlighted sample (10r1h3, black) is affected by principal component 1 (cRNA quality) the sample is an outlier that does not group with the rest of the samples. The 3'5' ratios of the internal spike in controls (YER022W (71), YER148W (13), & YFL039C (1231) were above the

acceptable quality cut off (3) and this identified cRNA degradation so the sample was removed from the experiment for the reasons discussed in **3.4.1** and PCA repeated (*Figure* **3.3.3**)

3.3.3   Second round of Principal Component Analysis to identify outlying samples

A second round of Principal Component Analysis of all quantile normalised signal values (less 10r1h3) was used to identify additional samples whose expression profiles did not cluster with the rest of the data.
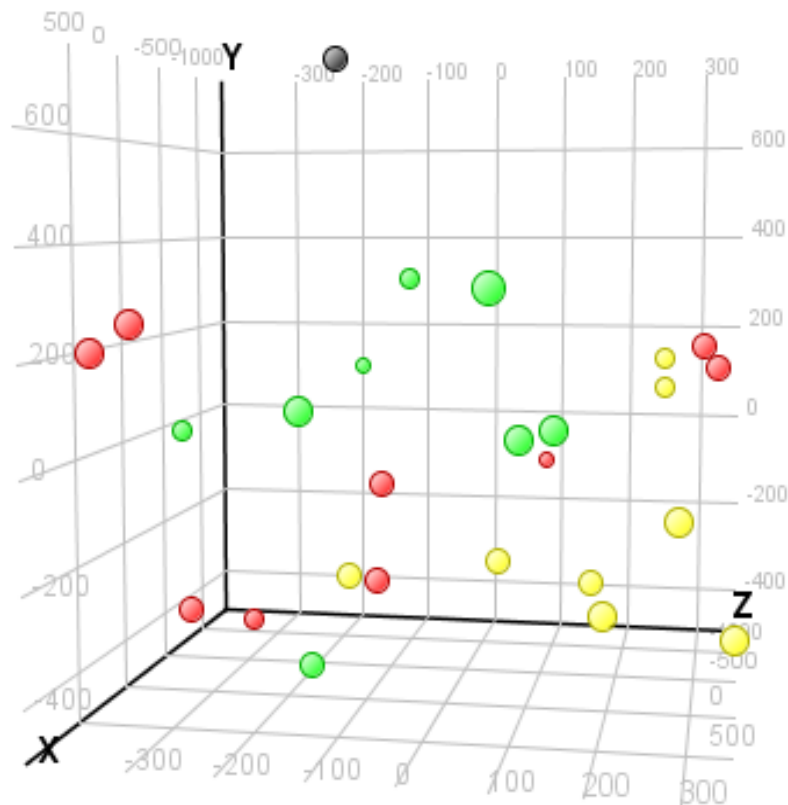
Figure 3.3.3 Principal Component Analysis of all quantile normalised samples (less 10r1h3) coloured by 4-nitrophenol concentration 0mg/l (green), 10mg/l (yellow), 39mg/l (red) the outlying sample (c2h3 i.e. control 2, hour 3) is highlighted in black. The data on axis X, Y and Z are principal components 1, 2 and 3 respectively.

The Principal Component Analysis in *Figure* **3.3.3** shows that the expression profile of the highlighted sample (c2h3, black) is affected by principal component two; the sample is an outlier that does not group with the rest of the samples. The 3'5' ratios of the internal spike in controls (YER022W (34), YER148W (4), & YFL039C (7)) were above the acceptable quality cut off (3) and this identified that this sample had degraded cRNA so it was removed from the experiment for the reasons discussed in **3.4.1** and PCA repeated (*Figure* **3.3.4**)

3.3.4 Principal Component Analysis shows expression profiles of *S. cerevisiae* exposed to 4-nitrophenol are clustered by exposure time and concentration

Principal Component Analysis of all samples (less 10r1h3 & c2h3) was used to identify experimental parameters influencing normalised signal values.
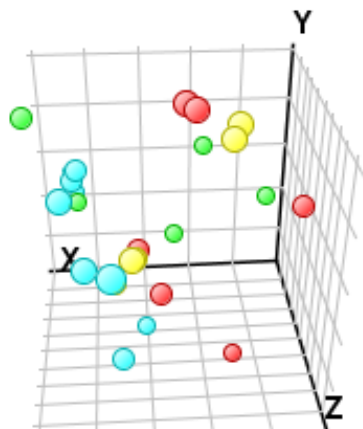
Figure 3.3.4a light blue highlighted samples taken after one hour exposure
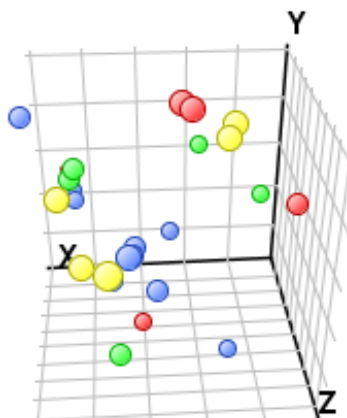
Figure 3.3.4b medium blue highlighted samples taken after two hours exposure
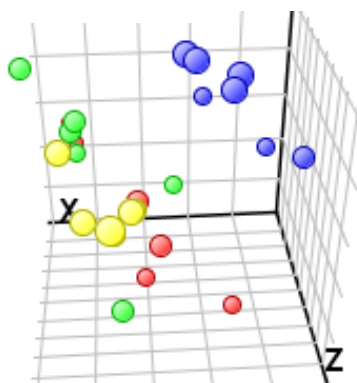
Figure 3.3.4c dark blue highlighted samples taken after three hours exposure

Figure 3.3.4 a, b & c Principal Component Analysis of all samples (less 10r1h3 & c2h3) coloured by exposure concentration (green 0 mg/l, yellow 10 mg/l, red 39 mg/l) and taken after 1 (*3.3.3a* light blue) 2 (*3.3.3b* medium blue) & 3 (*3.3.3c* dark blue) hours exposure. The data on axis X, Y and Z are principal components 1, 2, & 3 respectively.

The Principal Component Analysis in *Figure* 3.3.4 shows that samples exposed to 4-nitrophenol group by concentration and time, samples taken after three hours exposure to 4-nitrophenol have different expression profiles to those taken after one & two hours. Averaged normalised intensity values for the remaining samples were visualised using a Box Whisker plot (*Figure* 3.3.5).

3.3.5 Box Whisker plot of data identifies biological differences between samples taken at different exposure times

Box Whisker plots were used to assess the quality of the data by visually inspecting the summary statistics within and between treatments (*Figure 3.3.5*)
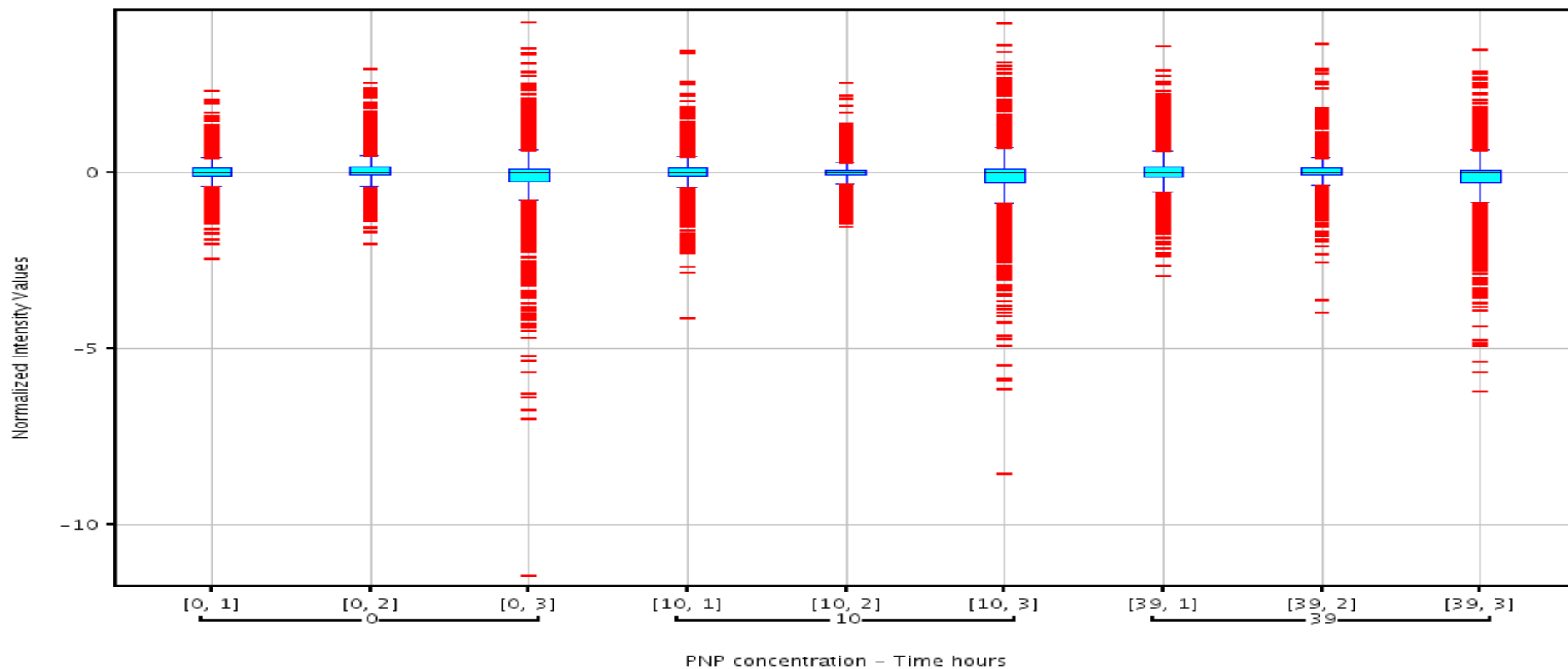
Figure 3.3.5 Box Whisker plot of mean Quantile normalised intensity values for each treatment (less 2 samples, 10r1h3 & c2h3) grouped by 4-nitrophenol (PNP) concentration and time.

The Box Whisker plot in *Figure* **3.3.5** shows the medians for all treatment groups are centrally aligned. The spread of the data (in particular the Lower Quartile) shows that there is a difference between samples taken after three hours ([0, 3], [10, 3], [39, 3], and those taken after one and two hours exposure to 4-nitrophenol. In addition, samples taken after three hours 4-nitrophenol exposure contain more outliers than those taken after one or two hour's exposure.

3.3.6 Comparing two Multiple Testing Correction (MTC) methods to identify significantly differentially expressed genes

To identify parameters and parameter interactions significantly affecting gene expression Two Way ANOVA with Multiple Testing Correction was applied to the data. Benjamini –Hochberg and Bonferroni – Holm corrected p-values were used to determine differentially expressed genes (**3.2.3**) and find the best MTC method for identification of differentially expressed genes. The results (**Table 3.3.6**) show that Bonferroni-Holm identifies fewer differentially expressed genes but more of the differentially expressed genes are expected by chance.

Table 3.3.6 The number of significantly differentially expressed genes from a Two Way ANOVA with Benjamini –Hochberg (Ben) and Bonferroni-Holm (Bon) Multiple Testing Correction for p-values of increasing stringency (p<0.01 – p<0.001), and the number of genes expected to be found as significantly differentially expressed by chance (Expected by chance) for each p-value.

Two Way ANOVA P-values	P<0.01		P<0.005		P<0.001	
MTC method used	Ben	Bon	Ben	Bon	Ben	Bon
Time	2882	850	2520	740	1930	539
Time – PNP concentration	564	31	326	23	63	12
PNP concentration	1126	124	862	100	400	58
Expected by chance	28	70	12	35	1	7

Table 3.3.6 shows that most of the genes significantly differentially expressed are affected by time, and Benjamini –Hochberg identifies the most significantly differentially expressed genes. An interaction between time and 4-nitrophenol (PNP) concentration is present, at p<0.01 using Benjamini-Hochberg more genes in this category are identified as significantly differentially expressed than when Bonferroni-Holm MTC is used. However at p<0.01 using Benjamini-Hochberg 28 genes are suspected to be found as differentially expressed by chance, using Bonferroni-Holm 70 genes are suspected to be found as differentially expressed by chance at p<0.01.

Two Way ANOVA with Benjamini-Hochberg Multiple Testing Correction with a p-value of 0.01 identified 2,882 differentially expressed genes that were significantly affected by time and

1,690 genes affected by 4-nitrophenol (PNP) concentration. Pathway analysis was conducted (**3.3.6**) on the resulting 4,572 genes to identify significant pathways affected by 4-nitrophenol.

3.3.7   Pathways affected by significantly (p<0.01) differentially expressed genes

To provide an overview of the effects of 4-nitrophenol on *S. cerevisiae* pathway analysis was carried out on differentially expressed genes identified using Two Way ANOVA with Benjamini-Hochberg Multiple Testing Correction (p<0.01). Only pathways significantly (p<0.05) affected by 4-nitrophenol are reported in **Table 3.3.7.** Three separate genes lists were created from Two Way ANOVA. Pathway analysis was run on individual gene lists to ascertain the impact of individual experimental parameters. The pathways significantly affected by each parameter (time and 4-nitrophenol concentration) and an interaction between the two can be found in **Table 3.3.7**.

Table 3.3.7 Pathways significantly (p<0.05) affected by 4-nitrophenol treatment

Two Way ANOVA gene lists	Pathway name
Time	Actin organisation
	Filamentous growth
	Glucose sensing
	Hyperosmotic stress response
	Main glucose repression pathway
	Meiotic initiation
	Pheromone response
	Polarised cell growth, budding & cytokinesis
	Ub and cell cycle inter-regulation
Time – 4-nitrophenol concentration	Glucose sensing
	Hyperosmotic stress response
4-nitrophenol concentration	Cell wall integrity
	Glucose sensing
	Hyperosmotic stress response
	Pheromone response
	Polarised cell growth, budding & cytokenesis

The results in **Table 3.3.7** show that nine pathways were significantly affected by time. Pathway analysis conducted with genes affected by 4-nitrophenol (PNP) concentration and time only identified two pathways. However, 4-nitrophenol concentration alone identifies four pathways that are significantly altered by treatment. There is significant overlap in the results, with glucose sensing and hyperosmotic stress response being significantly affected in all gene lists. Additionally pheromone response and polarised cell growth, budding & cytokinesis can be found in gene lists generated from genes affected by time as well as 4-nitrophenol (PNP) concentration. A large number of genes (4,572) were represented in the gene lists, and the time parameter of the experiment clearly affects a large number of pathways. To reduce the number of genes considered in subsequent analysis genes affected

by time only were separated from genes affected by 4-nitrophenol concentration using a Venn diagram (*Figure* **3.3.8**).

3.3.8   Genes affected by 4-nitrophenol treatment selected for subsequent analysis

To separate 4-nitrophenol affected genes from others (genes only affected by time) the differentially expressed genes that passed Two Way ANOVA (p<0.01) with Benjamini-Hochberg Multiple Testing Correction (**3.4.3**) were overlapped in a Venn diagram using the methods in section **3.2.5**. The results in *Figure* **3.3.8** show that 509 genes are affected by 4-nitrophenol concentration and an interaction between 4-nitrophenol concentration and time (covered by pink circle). In addition, 848 of the 2,882 genes significantly affected by time were also significantly affected by 4-nitrophenol concentration (covered by pink and purple circles). In total 3,391 differentially expressed genes are represented in the Venn diagram, of those genes 1,357 are affected by 4-nitrophenol (covered by pink and purple circles).
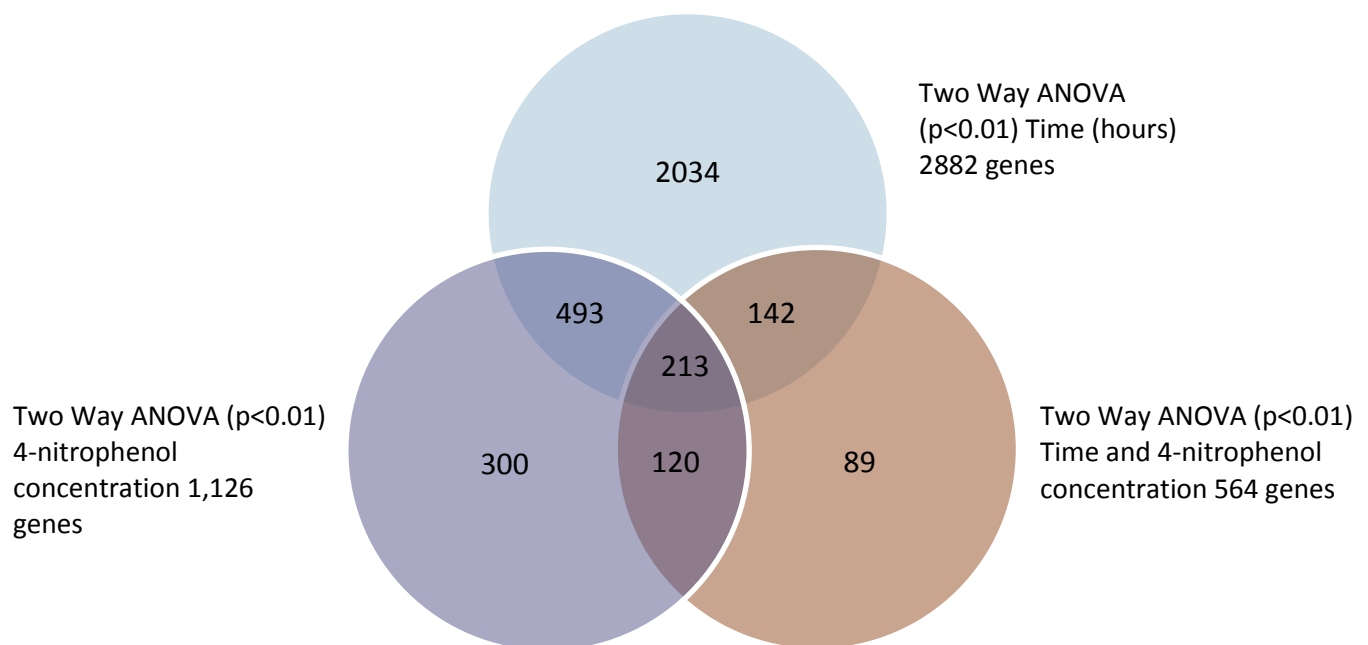
Figure 3.3.8 venn diagram of genes found to be significantly (p<0.01) affected by 4-nitrophenol treatment using Two Way ANOVA with Benjamini-Hochberg Multiple Testing Correction.

The results in the Venn diagram (*Figure 3.3.8*) show that 1,357 genes are significantly affected by 4-nitrophenol concentration (all genes covered by pink and purple cirles). All genes affected by 4-nitrophenol treatment (1,357) were separated from genes affected by time alone (2034 green) and saved.

In excess of 1,300 genes were identified as significantly (p<0.01) differentially expressed because of 4-nitrophenol treatment; such a large number of genes makes analysis difficult. Gene ontology was therefore used to classify differentially expressed genes into functional groups to provide an overview of the effects of 4-nitrophenol on *S. cerevisiae*.

3.3.9 Gene Ontology categories significantly affected by 4-nitrophenol treatment identified

To provide an overview of 4-nitrophenol effects Gene Ontology categories significantly (p<0.05) affected by 4-nitrophenol treatment were assessed using the methods in section **3.2.7**. The Gene Ontology categories affected by 4-nitrophenol treatment; and the number of significantly (p<0.01) differentially expressed genes in each category are summarised in **Table 3.3.9**. The results show that four cellular components, one biological process and one molecular function are significantly affected by 4-nitrophenol treatment.

Table 3.3.9 Gene Ontology categories of genes significantly (p<0.05) affected by 4-nitrophenol treatment.

Gene Ontology category	Process / component / function affected	Number of genes	p-value
Biological process	Ribonucleoprotein complex biogenesis and assembly	145	0.00
Cellular component	Cytoplasm	795	0.01
	Mitochondria	320	0.00
	Nucleolus	85	0.01
	Cytosolic ribosome	59	0.01
Molecular function	Oxidoreductase activity	120	0.00

Genes significantly differentially expressed in response to 4-nitrophenol treatment are associated with four cellular components; cytoplasm, mitochondria, nucleolus, and the cytosolic ribosome (**Table 3.3.9**). Most of the genes significantly affected by 4-nitrophenol treatment are associated with the cytoplasm, followed by the mitochondria. A large number of genes involved in ribonucleoprotein complex biogenesis (145) and oxidoreductase activity (120) were also significantly affected by 4-nitrophenol treatment.

3.4 Discussion

3.4.1 Significant correlation of microarray and qRT-PCR results confirms microarray data

In many microarray studies, genes, which are chosen for examining effects on expression generally, have a four fold or greater change to ensure that the changes in expression level are significant (Rockett & Hellmann 2004). If doing this other genes that are significant and may have lower fold changes in expression will be missed. However, microarrays and qRT-PCR are extremely sensitive techniques so in this work to avoid only confirming genes with large changes in expression, genes between one fold and thirteen fold were selected for confirmation. In addition, two reference genes PGU1 and PDA1 were selected, as their expression levels remained constant across treatment conditions. The results in **Table 3.3.1** confirmed that the expression results from microarrays were accurate; the selected genes had similar fold changes in expression regardless of which method was used to measure them. FIT2 had measured expression levels slightly lower than those measured using microarrays and this may have resulted from in-efficiencies in the amplification of the FIT2 gene (Yuan *et al* 2006). At 10 mg/l 4-nitrophenol there was a discrepancy, using qRT-PCR the expression of FIT2 was shown to increase approximately one fold at all exposure times, whereas the microarray data FIT2 gave expression levels below controls after one and three hours exposure to 4-nitrophenol. All other results were accurate with not only the direction of change but also the fold change in expression being almost the same regardless of the method chosen for measurement. The qRT-PCR results for FIT2 may therefore have resulted due to variation in amplification efficiencies during qRT-PCR. The results of qRT-PCR show that overall the microarray data is accurate and reproducible.

Pearsons correlation was performed on fold change data from microarrays and qRT-PCR and identified significant ($r^2 > 0.95$) correlations between microarray and qRT-PCR fold changes for FIT2, HXT5, PDR15, PDR16 at both concentrations (10 & 39 mg/l 4-nitrophenol) and significant correlation between data for HAC1 and GRE2 at 39 mg/l 4-nitrophenol. The correlation for HAC1 & GRE2 were not significant at 10 mg/l 4-nitrophenol, however HAC1 was increased in expression using both technologies, and HXT5 was decreased in expression using both technologies. The results of qRT-PCR show that overall the microarray data is accurate and reproducible.

3.4.2 Implications of outliers identified using Principal Component Analysis

The purpose of Principal Component Analysis is to explore the structure of the relationships among experimental variables, and determine whether unknown underlying factors can account for the observed patterns in the data (Han and Wang 2009). PCA is a robust

multivariate analysis method that identifies and minimises the influence of individual outliers, i.e. expression of individual genes on the overall patterns in the sample (Moller *et al* 2005; Hilsenbeck *et al* 1999). The distance between any pair of points in a PCA plot is related to the similarity between the samples in high dimensional space (Dillman & Phillips 2005). This experiment measured the expression levels reported by 5,744 *S. cerevisiae* specific probe sets, and each probe set contained eleven probe pairs. The PCA plot represents the average expression values reported by 5,744 probe sets and thus 5,744 response variables. Samples that are near each other in a PCA plot have similar expression levels for probe pairs within probe sets. Samples that are far apart in the plot (outliers) generated and identified by PCA are samples which have different expression values in a large number of probe pairs within probe sets (Dillman & Phillips 2005; Moller *et al* 2005). The Principal Component Analysis in *Figure 3.3.2* and *Figure 3.3.3* identified two outlying samples (10r1h3 & c2h3), further investigation using the 3'5' ratios for the samples showed the cRNA hybridised with the microarrays was degraded. Degradation of cRNA shortens oligonucleotide sequences and results in non-specific hybridisation of samples to probe sets (Copois *et al* 2007). This explains why the expression profiles of these samples did not follow the same pattern as the rest of the samples. Outliers in multivariate data can distort measures of location and scale, due to the fact that multivariate analysis is based on least square procedures (Li *et al* 2004; Hoo *et al* 2002). Consequently including indentified outliers in data analysis can skew the means of the data and have a large effect on the quality of results produced resulting in misleading conclusions (Lalor and Zhang 2001). In addition outliers can distort measures of covariance (how variables change together), a method commonly used to determine relationships between genes whose expression is altered by treatment conditions (Raychaudhuri *et al* 2000). Due to the potential detrimental effects of accommodating outlying samples, the outliers identified in this study were removed from the dataset used in subsequent analysis.

3.4.3   Implications of data patterns identified during quality checking procedures

Once samples with degraded cRNA were removed from the dataset Principal Component Analysis (PCA) identified that samples clustered by 4-nitrophenol exposure time and concentration. The results (*Figure 3.3.3)* show that the expression profiles of samples taken after three hours exposure to 4-nitrophenol cluster separately from those taken after one or two hours exposure to 4-nitrophenol. Separate clustering of these samples is indicative of variation, variation in gene expression profiles can arise due to biological differences or technical errors (Baldi & Hatfield 2002).

Biological differences in gene expression studies are interesting because they reflect true variation among individual samples (Zakharkin *et al* 2005). Time series analysis considers gene

expression profiles of samples taken at different time points, and biological differences can be observed due to changes in gene expression over time (Lloyd and Murray 2006; Rustici *et al* 2004). Box Whisker plots (*Figure 3.3.5*) showed that the median of samples was centrally aligned and thus did not identify biological differences in samples taken after three hours, it is unlikely biological differences account for the variation.

Normalisation procedures were used to scale data to compensate for errors arising from sample handling procedures or technical variation. Box Whisker plots (*Figure 3.3.5*) did not identify specific problems with hybridisation or normalisation procedures so these factors did not influence the results. However, both Box Whisker plots and PCA identified the data from three hours exposure is slightly different from data from one and two hour's exposure. Two samples were removed from the three hour data due to cRNA degradation. Additionally the microarrays for three hours exposure were processed in a different laboratory at Manchester University and this could account for the variation. Genes affected by time alone were filtered and genes differentially expressed at individual exposure times were analysed (**3.3.7**) to minimise the impact of these differences.

3.4.4 Choosing statistical analysis, Multiple Testing Correction methods and p-values for identification of significantly differentially expressed genes

Data quality checking identified that data from the three hour time point differed and to compensate for this Two Way ANOVA was chosen as the statistical analysis method because it identifies genes significantly affected by each of the experimental parameters, time and 4-nitrophenol concentration. Genes differentially expressed as a function of time can be filtered from the genes affected by 4-nitrophenol using Two Way ANOVA. Two Way ANOVA with a low p-value (0.01) and Multiple Testing Correction were used to reduce false positives. Two Multiple Testing Correction methods with different stringencies (Benjamini-Hochberg, medium stringency, and Bonferroni-Holm, highest stringency) were assessed to identify the method which reduces type I errors (false positives) whilst avoiding type II errors (false negatives). The results in **Table 3.3.6** show that at p< 0.01 Bonferroni-Holm identifies fewer significantly different genes than Benjamini-Hochberg. However Benjamini-Hochberg identifies fewer false positives. Benjamini-Hochberg Multiple Testing Correction therefore effectively reduces type I errors. In addition, it identifies more significantly differentially expressed genes than Bonferroni-Holm, showing it is not over stringent in producing type II errors (false negatives). In the analysis of gene expression data Benjamini-Hochberg has been found to be extremely robust in comparison with other Multiple Testing Correction methods (Kim & Wiel 2008) and was thus selected for the identification of differentially expressed genes in this study.

3.4.5   Pathways affected by 4-nitrophenol treatment

Two Way ANOVA generated three separate lists of genes that were significantly affected by experimental parameters. Some of the genes were significantly affected by time and pathway analysis of this gene list identified that nine pathways were significantly affected by time. They were: actin organization, filamentous growth, glucose sensing, hyperosmotic stress response, main glucose repression pathway, meiotic initiation, pheromone response, polarized cell growth, budding & cytokinesis, Ubiquitone and cell cycle inter-regulation. Only two of these pathways (glucose sensing, & hyperosmotic stress response) were affected by time and 4-nitrophenol concentration. Genes involved in sensing glucose have previously been reported to be increased in expression in response to amiodarone, reminiscent of a starvation response (Zhang & Rao 2007), identifying that chemical treatment can affect glucose sensing. The hyperosmotic stress response increases the concentration of glycerol in the cell to adapt the cellular osmotic pressure and prevent the loss of water (Santos *et al* 2005). Chemical exposure frequently results in increased expression of genes involved in response to osmotic stress, such as seen in the yeast response to *Pichia membranifaciens*. It has been reported that osmotic stress results in actin re-organisation, cell cycle arrest, morphological changes and decreased expression of genes (Kim & Shah 2007). 4-nitrophenol was found to affect glucose sensing, hyperosmotic stress, pheromone response and polarized cell growth budding and cytokinesis. Pheromones of *S. cerevisiae* are equivalent to hormones in mammals, and differential expression of pheromones is interesting because studies with rats have found that 4-nitrophenol affects reproductive glands (**1.7.7**). Pheromones are used to signal to mates and result in greater genetic diversity of the population; they are thus generally produced when cells are in good condition to mate (Malleshaiah *et al* 2010). The overall analysis of data did not reveal whether these genes were increased or decreased in expression. Another pathway significantly affected by 4-nitrophenol treatment was polarized cell growth budding & cytokinesis which is the process of bipolar cell growth and division site selection. A significant alteration of this pathway suggests changes in cell morphology have occurred in response to 4-nitrophenol exposure (Sheu *et al* 2000). Changes in morphology are also linked to the cell cycle, as well as osmotic stress, and changes in morphology may reflect changes in cell cycle (Nehil *et al* 2007). Interestingly the osmotic stress pathway also controls pheromone response, filamentous growth, cell size and budding.

Some of the pathways were affected only by time; these included actin organization, filamentous growth, main glucose repression pathway, meiotic initiation, Ub, and cell cycle inter-regulation. All of these pathways are altered by changes in cell cycle and because they are only affected by time they are related to differences in growth rates and not 4-nitrophenol presence. For the purposes of this study these genes are not of interest and were filtered from

genes affected by 4-nitrophenol concentration and an interaction between 4-nitrophenol concentration and time (**3.3.8**)

The overview of pathways affected shows that four pathways were significantly affected by 4-nitrophenol treatment, but the assessment used does not identify whether the genes in these pathways were increased or decreased in expression and at which 4-nitrophenol concentration.

3.4.6   Genes in functional classes provide direction for subsequent analysis

The large number of genes identified as differentially expressed in response to 4-nitrophenol makes studying individual genes difficult. Gene Ontology was used to identify functional groups significantly affected by treatment so that they could be analysed in detail in subsequent chapters (**Table 3.3.9**). Analysing gene expression changes in terms of whole processes rather than individual genes has the advantage of being less laborious and informative (Schwartz *et al* 2007). Extensive knowledge of metabolic networks (Schwartz *et al* 2007) and standardized language for gene function description (Dillman and Phillips 2005) currently available permits the use of metabolic pathways in identifying effects of chemical stress on cells (Schwartz *et al* 2007). Zhang *et al* (2003) are not alone in classifying genes into functional categories, this method has been used by many authors; consequently the applications of this method have been reviewed and refined by Schwartz *et al* (2007). The study by Schwartz *et al* (2007) investigated three sets of gene expression data (Causton *et al* 2001; Gasch *et al* 2000; Iwahashi *et al* 2006) produced by exposing *S. cerevisiae* to various environmental stressors. Schwartz *et al* (2007) used elementary modes (describe the transcriptomic activity of parts of pathways involved in metabolic functions) rather than whole pathways to identify metabolic pathways affected by environmental stress.

3.4.7   Cellular components affected by 4-nitrophenol

Four cellular components were significantly affected by 4-nitrophenol treatment; the cytoplasm, mitochondrion, nucleolus, and the cytosolic ribosome. Previous toxicology studies have also identified specific cellular components that are affected by exposure to toxins. In a study by Lam *et al* (2006) gene expression responses of zebrafish liver found that genes associated with the cytoplasm were significantly affected by arsenic exposure. The cytoplasm is involved in a wide variety of cellular processes subsequently differential expression of genes associated with the cytoplasm are not indicative of any specific response to 4-nitrophenol. However, processes in the cytoplasm alter how other cellular components function. Effects of 4-nitrophenol on mitochondria have previously been reported by TenBrook *et al* (2005), Williamson *et al* (1995), Cyboron & Dryer (1976) and Bastos (1975). In addition structurally related chemicals (2-azido-4-nitrophenol, 2, 4-dinitrophenol, and 3-trifluoromethyl-4-

nitrophenol) have also been reported to affect mitochondrial processes (Niblett and Ballantyne 1976). However, all of the studies looking at the effects of 4-nitrophenol and related compounds on mitochondria so far have been based on physical and biochemical results and this work thus provides extra confirmation that 4-nitrophenol does affect mitochondria (or at least genes associated with mitochondria).

The nucleolus has traditionally been thought to have roles in transcription of ribosomal RNA and ribosome biogenesis, however it also has roles in stress sensing, control of cell ageing, nuclear export, assembling ribonucleoprotein, and control of telomerase activity (Radis-Baptista *et al* 2008; Lin *et al* 2000). It is therefore thought that many of the proteins reported to have been localized to the nucleolus (and thus associated with it by functional class) may have been stored there in anticipation of release (Lin *et al* 2000). No previous gene expression studies have published results that show that genes associated with the nucleolus are significantly affected by toxin exposure. Additionally genes associated with cytosolic ribosomes were significantly affected by 4-nitrophenol treatment. Similar results were reported in a gene expression study by Chung *et al* (2005) who found that exposing rat livers to carbon tetrachloride significantly altered the expression levels of genes involved in ribosome biogenesis. In addition to their main functions (transcription and translation) (Nakamoto and Tokumasa 2009) cytosolic ribosomes can be tethered to, associated with proteins and sometimes act as chaperones, assisting in protein folding (Spooner *et al* 2008). Ribosomes are implicated in response to toxins (Spooner *et al* 2008; Vago *et al* 2005), and are the targets of some toxins (Watson and Spooner 2006).

3.4.8 Molecular functions and biological processes significantly affected by 4-nitrophenol treatment

Molecular functions associated with genes significantly differentially expressed in response to 4-nitrophenol treatment were involved in oxidoreductase activity. Quinone oxidoreductase is one member of this group that localizes to the cytoplasm of *S. cerevisiae*, and is involved in the detoxification of quinone (Porte *et al* 2009). The oxidoreductase enzymes include the cytochromes located in the mitochondria, they respond to a wide variety of chemicals including hydrogen peroxide, and are involved in cellular detoxification and the electron transport chain (Cortes-Rojo *et al* 2007). The redox potentials of chemicals are correlated with oxidoreductase activity, chemicals with high redox potentials such as 4-nitrophenol (redox potential 1.23) are oxidized more slowly, resulting in greater electron deficiencies, and intermediates with greater oxidative capacity (Ayala *et al* 2007). The redox potential of oxidoreductase is determined by the cellular ratio of Fe^{2+} to Fe^{3+}, and oxidative stress causes the conversion of soluble Fe^{2+} to less soluble Fe^{3+} (Longo *et al* 1999), which decreases the

availability of iron required in the cell for the production of oxidoreductases Alterations in the expression of genes involved in producing oxidoreductases indicate cells are under oxidative stress.

Genes involved in ribonucleoprotein complex biogenesis and assembly was significantly affected by 4-nitrophenol treatment. Ribonucleoproteins have been found in abundance in cancer cells resistant to drug treatment, they have been implicated in response to oxidative stress (Suprenant *et al* 2007; Pollard *et al* 1997). Ribosome biogenesis is reduced in the presence of toxic compounds in *S. cerevisiae* (Slepak *et al* 2005) these genes have been named repressed Environmental Stress Response (ESR) genes because they are decreased in expression in response to environmental stress and decreased growth rates (Gasch *et al* 2000).

3.5 Summary & Conclusions

To confirm the microarrays had been successful the results were normalized with eight genes by qRT-PCR, the gene expression changes measured using microarrays and qRT-PCR was highly correlated showing that microarray results were reliable. Quality checks carried out on gene expression data revealed two samples that had degraded cRNA, degradation of the cRNA was not detected using the bioanalyser and therefore contamination of the sample may have occurred after the cRNA quality was assessed. Samples with degraded cRNA were removed to avoid the results skewing the remaining data. Two way ANOVA with Benjamini and Hochberg multiple testing correction was chosen as the statistical method because it is reported to be the most reliable, and produced fewer false positives than Bonferroni Holm produced. A large number of genes were significantly differentially expressed in this study and ways of reducing the complexity of the results were needed. To extract meaningful information from the data pathways significantly affected by treatment were identified. Whilst these identified affected pathways they did not identify whether genes in these pathways were increased or decreased in expression. The second step in reducing complexity removed genes significantly affected by time from genes significantly affected by 4-nitrophenol treatment; this reduced the number of genes considered to 1,357. Gene Ontology was then used in an attempt to reduce data complexity. This approach identified that oxidoreductase activity was affected by 4-nitrophenol treatment, and oxidoreductases are associated with the cytoplasm and mitochondria. Oxidoreductases are known to respond to oxidative stress and the presence of chemicals. They perform vital functions including cellular detoxification and are components of the electron transport chain. Oxidoreductases have a requirement for iron and use complexed iron to metabolise chemicals.

Ribonucleoprotein complex biogenesis was also significantly affected by 4-nitrophenol treatment, and the genes involved have previously been identified as environmental stress response genes because they are always decreased in expression under stress conditions. The overview of results identifies that *S. cerevisiae* treated with 4-nitrophenol are under stress, but do not identify any specific responses to treatment. To identify specific effects of 4-nitrophenol on *S. cerevisiae* significantly differentially expressed genes are examined in more detail on an hour by hour basis in subsequent chapters.

Chapter 4

4. *S. cerevisiae* genes significantly differentially expressed after one hour exposure to 10 & 39 mg/l 4-nitrophenol

4.1 Introduction

4.1.1   Adapting to the presence of 4-nitrophenol

In toxicogenomic studies short lived dramatic changes in gene expression occur during initial exposure to xenobiotics, and subtle changes in gene expression required for adaptation are maintained for a longer period of time (Gasch *et al* 2001). Toxicogenomic studies with microbes measure gene expression changes over a few hours are used to identify genes involved in adapting to the presence of chemicals i.e. resistance. Micro-organisms such as *S. cerevisiae* cells have a short life cycle and genes expressed in the first few hours can identify processes are occurring in the first stages of resistance to xenobiotics (Hirsawa *et al* 2007; Pandey *et al* 2007) and this can be applied to 4-nitrophenol.   In addition to identifying genes involved in resistance, toxicogenomics studies can also be used to identify mechanisms of toxicity, classify chemicals according to toxic mechanisms and identify toxicity markers (Suter *et al* 2004). Toxicogenomic experiments with *S. cerevisiae* usually measure the expression changes occurring in the first three hours of toxin exposure (**1.6.4**).   In this work gene expression changes over one, two and three hours exposure to 4-nitrophenol at the EC_{10} & EC_{50} were measured (**2.4.9** & **2.4.10**) to identify genes that could be used as indicators of 4-nitrophenol exposure, as well as identifying genes and pathways that lead to resistance to 4-nitrophenol. This chapter focuses on genes expression changes that occur after one hour exposure to 4-nitrophenol, to ensure clarity of data and provide a detailed account of changes occurring.

4.1.2   Methods used to identify differentially expressed genes for detailed analysis

In Chapter 3 significantly (p<0.01) differentially expressed genes (1,357) were classified using gene ontology providing a useful overview of 4-nitrophenol effects. To examine gene expression changes over time smaller lists of genes differentially expressed at each time point are required so that genes can be examined in detail. In previous toxicogenomic studies with *S. cerevisiae* genes two fold changes in expression have been published (**1.6.4**). Fold change in expression identifies genes with large changes in expression and is a useful method for reducing the number of genes considered in toxicogenomic studies. Using statistical analysis alongside fold change increases confidence in the results obtained and has been used in

previous toxicogenomics studies (Yin *et al* 2007; Chen *et al* 2007 and Zhang *et al* 2003). In this study to generate smaller lists of specific differentially expressed genes for detailed examination the stringency of statistical analysis was increased and where appropriate used alongside fold change in expression.

4.1.3   Statistical analysis used in identifying differentially expressed genes

Previously (Chapter 3) two way ANOVA (p<0.01) was used to identify differentially expressed genes.   The analysis identified 1,357 genes that were differentially expressed in response to 4-nitrophenol overall. Further analysis revealed that ~650 genes were increased and decreased in expression at each 4-nitrophenol concentration and exposure time (**Table 4.1 Appendix III**). These results showed that gene expression was changing over time.   The aim of this chapter and the next two chapters is to reduce the number of genes considered and identify *S. cerevisiae* genes highly significantly differentially expressed after each hours exposure to 10 & 39 mg/l 4-nitrophenol compared to an un-treated (0 mg/l) control. Data cannot be considered as dependent because cells undergoing each treatment were cultured independently, and other variables such as glucose concentrations (*Figure* **2.3.6**) may affect the results. Since the aim is to identify differentially expressed genes in independent samples (treated (10 & 39 mg/l) and un-treated (0 mg/l)) at individual time points an un-paired t-test was used. The un-paired t-test has the added advantages that the number of replicates in different treatments don't need to be the same (in Chapter 3 two data sets were removed because cRNA was degraded) and data with unequal variance; such as the data from three hours 4-nitrophenol exposure (*Figure 3.3.4*) can be analysed (Hindle *et al* 2010; Jiang *et al* 2010).

4.1.4   Clustering of differentially expressed genes

Clustering methods based on the correlation of gene expression profiles have been used to identify relationships between gene expression profiles (**1.6.8**). Clustering can, and has been used to identify genes with related functions, because genes with correlated expression profiles are often involved in related processes. Several types of clustering analysis are available in GeneSpring GX 10® and clustering is an important tool for identifying patterns in multivariate data. The most commonly used clustering methods are self organizing maps followed by hierarchical clustering. In the studies by Hirasawa *et al* (2007) and Pandey *et al* (2007) self organizing maps and hierarchical clustering were used to identify genes whose altered expression conferred resistance to ethanol and salt stress respectively. In this study the aims are to identify genes whose expression is changed in response to 4-nitrophenol exposure and potentially use them to identify the presence of 4-nitrophenol in water samples. For this purpose statistical analysis and fold change in gene expression were used to identify differentially expressed genes. Hierarchical clustering was also used to aid analysis of results

by clustering genes with similar expression profiles to identify genes with related functions and identify cellular processes affected by 4-nitrophenol exposure.

Aims of chapter 4

Aim 1 Increase the stringency of filtering methods to reduce the number of 4-nitrophenol affected genes (after 1 hour exposure) considered in detail

Objectives to meet Aim 1
1. Use unpaired t-tests to identify genes significantly ($p<0.001$) differentially expressed after one hours exposure to 10 and 39 mg/l 4-nitrophenol
2. Filter significantly ($p<0.001$) differentially expressed genes to identify genes with two fold or more changes in expression after one hours exposure to 10 & 39 mg/l 4-nitrophenol

Aim 2 Identify genes with correlated expression levels that may have related functions

Objectives to meet Aim 2
1. Use Hierarchical clustering to identify differentially expressed genes with correlated expression levels
2. Use literature searches to gather information on factors leading to differential expression of identified correlated differentially expressed genes and identify cellular processes affected by 4-nitrophenol treatment

4.2 Methods

Previously (Chapter 3) two way ANOVA identified 1,357 genes that were affected by 4-nitrophenol treatment over the time course of exposure. Genes were classified using gene ontology and genes associated with cellular components and processes were identified. Most differentially expressed genes were associated with the cytoplasm and mitochondria, and were involved in ribosome biogenesis and oxidoreductase activity therefore providing an overview of 4-nitrophenol effects on the cell. In this chapter specific processes affected by one hours exposure to 4-nitrophenol at 10 and 39 mg/l were identified.

4.2.1   Identifying genes with significant (p<0.001) changes in expression

A large number of genes were significantly (p<0.01) differentially expressed making detailed analysis difficult. To reduce the number of genes going into subsequent analysis, genes affected by 4-nitrophenol treatment (**3.2.6**) were filtered using un-paired t-tests to identify genes significantly (p<0.001) increased and decreased in expression after one hour exposure to 10 and 39 mg/l 4-nitrophenol and were saved as separate lists. This analysis method identified twelve genes that were differentially expressed at 10 mg/l, however more than 100 genes were found to be differentially expressed at 39 mg/l (**Table 4.3.1**). To further reduce the number of genes selected for further analysis fold change in expression was used alongside significance (**4.2.2**)

4.2.2   Identifying genes with significant (p<0.001) and two fold changes in expression

To decrease the number of genes considered in detail genes significantly (p<0.001) differentially expressed at 10 & 39 mg/l were further filtered so that only genes with a two fold or greater change in expression were considered . The number of genes significantly (p<0.001) and two fold differentially expressed at 10 & 39 mg/l 4-nitrophenol are recorded in **Table 4.3.2**. Only one gene was differentially expressed at 10 mg/l 4-nitrophenol using this method. To ensure genes with significant changes in expression at the lower 4-nitrophenol concentration were not disregarded all genes significantly (p<0.001) differentially expressed at 10 mg/l 4-nitrophenol are discussed (12 genes).

4.2.3   Using hierarchical clustering to identify patterns in differentially expressed genes

Clustering methods are widely used in the analysis of gene expression data to identify genes involved in related processes (**4.1.3**). Differentially expressed genes identified using the methods in **4.2.1** & **4.2.2** were clustered using hierarchical clustering with Pearson's absolute

correlation (**1.6.8**) and Wards linkage rule (Van Delft *et al* 2005; Ryan *et al* 2007; He & Zeng 2006; Little *et al* 2005). Relationships between genes that cluster together are discussed in detail in section **4.4**.

4.3 Results

To provide an overview of the effects of 4-nitrophenol on *S. cerevisiae* over the time course of the experiment a p-value of 0.01 was used to maximize the amount of information provided by the data. The overview provided in Chapter 3 identified the overall effects of 4-nitrophenol on *S. cerevisiae,* however more than 1,000 genes were identified as differentially expressed. One purpose of this study is to identify a small sample of differentially expressed genes that could be used to identify potentially toxic concentrations of 4-nitrophenol in water samples. To meet this aim and increase confidence in the results obtained gene expression data was further filtered with increasing stringency to identify a subset of genes that can be analysed in detail.

4.3.1   Identifying genes with significant (p<0.001) gene expression changes

The number of genes identified as significantly (p<0.01) increased in expression (670) after one hours exposure to 4-nitrophenol (**Table 4.1 Appendix III**) is too great to examine in detail. To reduce the number of genes considered unpaired t-tests were used to identify genes with even greater significance (p<0.001). The number of genes significantly (p<0.001) increased and decreased in expression after one hours treatment with 10 and 39 mg/l 4-nitrophenol are shown in **Table 4.3.1.**

Table 4.3.1 Number of genes significantly (p<0.001) differentially expressed after one hours exposure to 10 and 39 mg/l 4-nitrophenol

4-nitrophenol (mg/l)	Number of genes increased	Number of genes decreased
10	7	5
39	39	67

Table 4.3.1 shows that increasing the stringency of statistical analysis reduces the number of genes differentially expressed at 10 mg/l 4-nitrophenol to twelve genes that can be analysed in detail. However, the number of genes identified as differentially expressed at 39 mg/l 4-nitrophenol using statistical analysis alone is too great to analyse in detail. To further reduce the number of genes considered in subsequent analysis fold change filters were used to identify genes with a two fold or more change in expression.

4.3.2 Genes with significant (p<0.001) and two fold changes in expression

At 39 mg/l 4-nitrophenol too many genes to analyse in detail were identified using significance alone so genes were further filtered using fold change in expression, the number of genes significantly (p<0.001) and two fold increased and decreased in expression at 10 & 39 mg/l 4-nitrophenol are shown in **Table 4.3.2**

Table 4.3.2 Number of genes significantly (p<0.001) and two fold differentially expressed after one hours exposure to 10 and 39 mg/l 4-nitrophenol

4-nitrophenol concentration mg/l	Number of genes significantly (p<0.001) and two fold increased	Number of genes significantly (p<0.001) and two fold decreased
10	0	1
39	16	27

Table 4.3.2 shows that applying a two fold change filter and increasing the stringency of statistical tests dramatically reduces the number of genes identified as differentially expressed at 10 & 39mg/l 4-nitrophenol.

Only one gene (YJR115W) was significantly (p<0.001) and two fold decreased in expression in response to one hours treatment with 10mg/l 4-nitrophenol, and no genes were increased in expression at 10mg/l 4-nitrophenol. This analysis method is too stringent for the gene expression data from *S. cerevisiae* exposed to 10 mg/l 4-nitrophenol. For this reason, different analysis methods were used to identify genes differentially expressed at 10 & 39 mg/l 4-nitrophenol, genes significantly (p<0.001) differentially expressed at 10 mg/l 4-nitrophenol are clustered using hierarchical clustering and analysed in detail, whereas genes significantly (p<0.001) and two fold differentially expressed at 39 mg/l 4-nitrophenol are clustered using hierarchical clustering and analysed in detail.

4.3.3 Genes significantly (p<0.001) increased in expression after one hour exposure to 10 mg/l 4-nitrophenol

Seven genes (**Table 4.3.3**) were increased in expression after one hour exposure to 10 mg/l 4-nitrophenol.

Table 4.3.3 The functions of genes increased in expression after one hours exposure to 10 mg/l 4-nitrophenol

Gene function	Gene name
Response to osmotic stress	SFA1, SLN1
Exit from mitosis	SW15, POP3
Cell wall maintenance	DCW1
Chaperone protein	HLJ1
Sulphur uptake	BDS1

The results (**Table 4.3.3**) show that genes are involved in a range of processes and two of the genes (SFA1 & SLN1) increased in expression after one hour's exposure to 4-nitrophenol respond to osmotic stress. Hierarchical clustering was used to identify similarities in gene expression profiles and this demonstrated that the genes separated into three clusters (*Figure 4.3.3*)
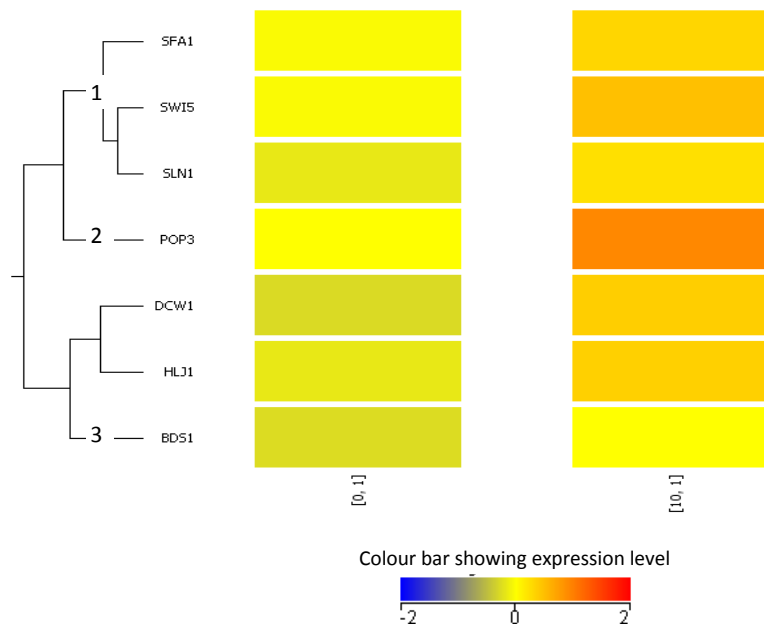
Colour bar showing expression level

Figure **4.3.3** Dendogram produced by hierarchical cluster analysis (r^2<0.95) of genes significantly (p<0.001) increased in expression after one hours exposure to 10 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after one hour's exposure to 0 mg/l ([0, 1]) and 10 mg/l ([10, 1]) 4-nitrophenol is shown by the leaves on the right.

The results in *Figure 4.3.3* show that the expression levels of each of the *S. cerevisiae* genes increased in expression relative to the untreated control *S. cerevisiae*. Hierarchical clustering groups genes with the most similar expression values within treatment conditions, thereby identifying genes that may be involved in related processes. Three clusters are formed by genes increased in expression after one hour exposure to 10 mg/l 4-nitrophenol. SWI5, SLN1 & SFA1 cluster together as do DCW1, HLJ1 & BDS1. POP3 shows a high increase in expression after one hour treatment with 10 mg/l 4-nitrophenol and does not belong to other clusters.

4.3.4   Genes significantly (p<0.001) decreased in expression after one hour exposure to 10 mg/l 4-nitrophenol

Five genes (**Table 4.3.4**) were significantly (p<0.001) decreased in expression after one hours treatment with 10 mg/l 4-nitrophenol.

Table 4.3.4 the functions of genes decreased in expression after one hours exposure to 10 mg/l 4-nitrophenol

Gene name	Gene function

RPL32	RNA splicing
TSA2	Antioxidant enzyme
YJR115W	Unknown function
MDY2	Shmoo formation during mating
PAN3	Cell division

Genes decreased in expression after one hours exposure to 10 mg/l 4-nitrophenol have a range of functions, two of the genes decreased in expression (MDY2 & PAN3) are involved in cell replication. To gain further information about the relationships between genes hierarchical clustering (*Figure 4.3.4*) was used to separate the genes into clusters according to their expression profiles.
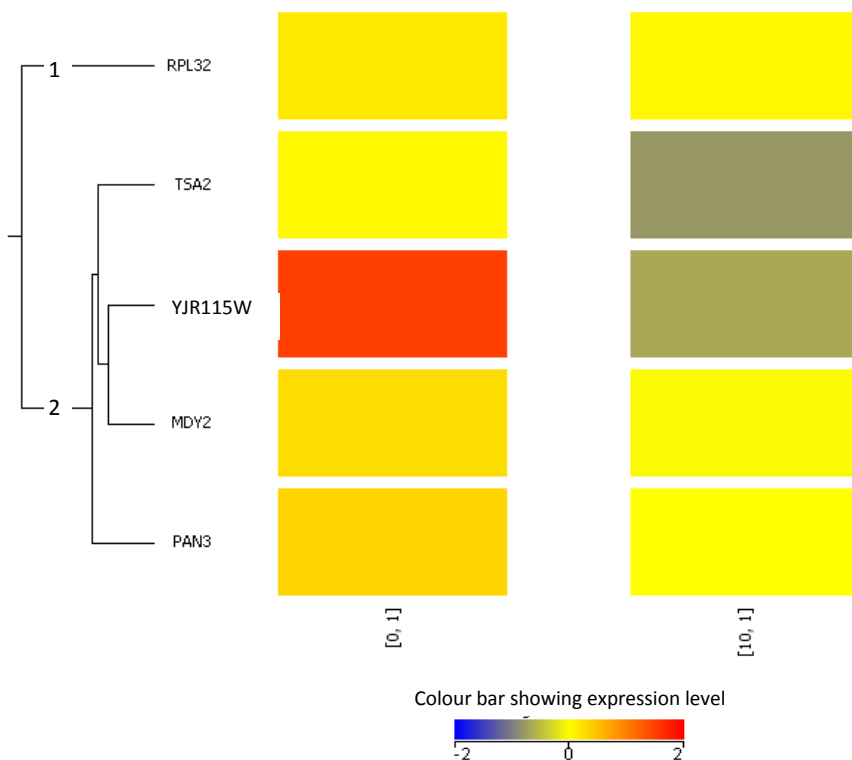
Figure **4.3.4** Dendogram produced by hierarchical cluster analysis ($r^2 < 0.95$) of genes significantly ($p < 0.001$) decreased in expression after one hours exposure to 10 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after one hour's exposure to 0 mg/l ([0, 1]) and 10 mg/l ([10, 1]) 4-nitrophenol is shown by the leaves on the right.

The expression levels of genes significantly ($p < 0.001$) decreased in expression after one hours exposure to 10 mg/l 4-nitrophenol. Two clusters are formed by genes decreased in expression after one hour exposure to 10 mg/l 4-nitrophenol. RPL32 is significantly ($p < 0.001$) decreased in expression, however *Figure 4.3.4* shows that a subtle change in expression occurs, and RPL32 does not cluster with the remaining genes decreased in expression after one hour exposure to 10 mg/l 4-nitrophenol. MDY2 and YJR115W have gene expression profiles with the highest similarity and cluster with PAN3 and TSA2.

4.3.5 Genes increased in expression after one hours exposure to 39mg/l 4-nitrophenol

Only 16 genes (**Table 4.3.5**) were significantly (p<0.001) and two fold increased in expression after one hours exposure to 39mg/l 4-nitrophenol.

Table 4.3.5 the function of genes increased in expression after one hours exposure to 39 mg/l 4-nitrophenol

Function	Genes increased in expression
Drug resistance	PDR3, PDR15, YLR346C, PDR16, YPL088W
Iron homeostasis	ARN2, FIT3
Cell division regulation during stress	GPG1, NQM1, HXT5
Cell membrane and wall maintenance	GRE2, YGP1, GSC2, ICT1, RSB1
Unknown	YMR102C

The results in **Table 4.3.5** show that five of the genes increased in expression (PDR3, PDR15, YLR346C, PDR16, & YPL088W) are involved in drug resistance, three (GPG1, NQM1, & HXT5) are involved in regulating cell division, four are involved in cell wall and membrane maintenance (YGP1, GSC2, GRE2 & ICT1) and two (ARN2 & FIT3) are involved in iron homeostasis. Hierarchical clustering was used to identify genes with related expression profiles (*Figure 4.3.5*).
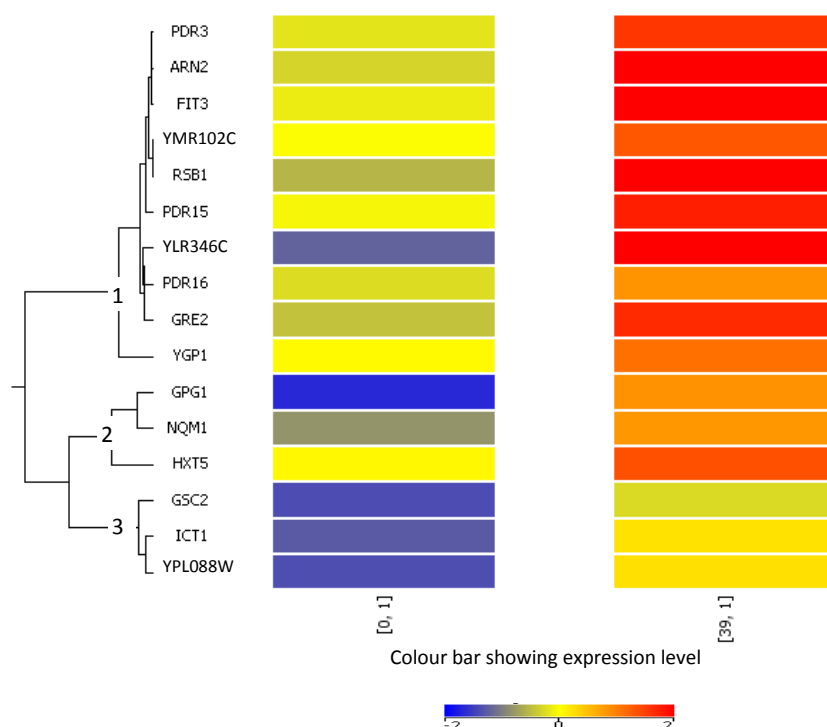
Colour bar showing expression level

Figure **4.3.5** Dendogram produced by hierarchical cluster analysis (r^2<0.95) of genes that were significantly (p<0.001) and two fold increased in expression after one hour exposure to 39 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the leaves on the right show the expression level of each gene after one hour's exposure to 0 mg/l ([0, 1]) and 39 mg/l ([39, 1]) 4-nitrophenol.

Three clusters of genes are produced using hierarchical clustering, the first cluster contains ten genes that are increased in expression after one hours exposure to 4-nitrophenol (*Figure 4.3.5*). Two unnamed genes (YMR102C & YLR346C) demonstrate greater than two fold changes in expression and have expression levels that are highly correlated with genes in the first cluster that are involved in drug resistance and iron uptake. The genes in the second cluster have all been characterised and are involved in cell division regulation. The third cluster contains three genes whose expression levels are highly correlated, interestingly another uncharacterized gene (YPL088W) clusters with the characterized genes GSC2 and ICT1, which are both, involved in cell wall and membrane maintenance.

4.3.6   Genes decreased in expression after one hours exposure to 39mg/l 4-nitrophenol

Only 16 genes (**Table 4.3.6**) were significantly (p<0.001) and two fold increased in expression after one hours exposure to 39mg/l 4-nitrophenol. The results in **Table 4.3.6** show that most of the genes decreased in expression after one hours exposure to 39 mg/l 4-nitrophenol are involved in RNA processing, (ENP1, DBP2, DBP3, ROK1, UTP21, UTP4, UTP8, LIA1 and DUS3) as well as ribosome assembly and maturation (YTM1, ARX1, MAK21, UTP10, URB1, URB2 NOC2, and NOP56).

Table 4.3.6 the functions of genes decreased in expression after one hours exposure to 39 mg/l 4-nitrophenol

Function	Genes decreased in expression
RNA processing	ENP1, DBP3, ROK1, UTP21, LIA1, DBP2, UTP4, UTP8, DUS3
Ribosome assembly and maturation	YTM1, ARX1, MAK21, UTP10, URB2, NOP56, URB1, NOC2
Unknown	YBL029W, YKR075C,
Thiamine uptake	PHO3
Resistance to fluconazole	YGR283C, FCY2
Entry into stationary phase	AAH1, ACO2
Amino acid synthesis	LEU9, ARO4
Resistance to flurocytosine	FCY21

Other genes include genes involved in entry into stationary phase, amino acid synthesis, thiamine uptake and resistance to chemicals. To identify genes with correlated expression levels Hierarchical clustering was used (*Figure 4.3.6*)
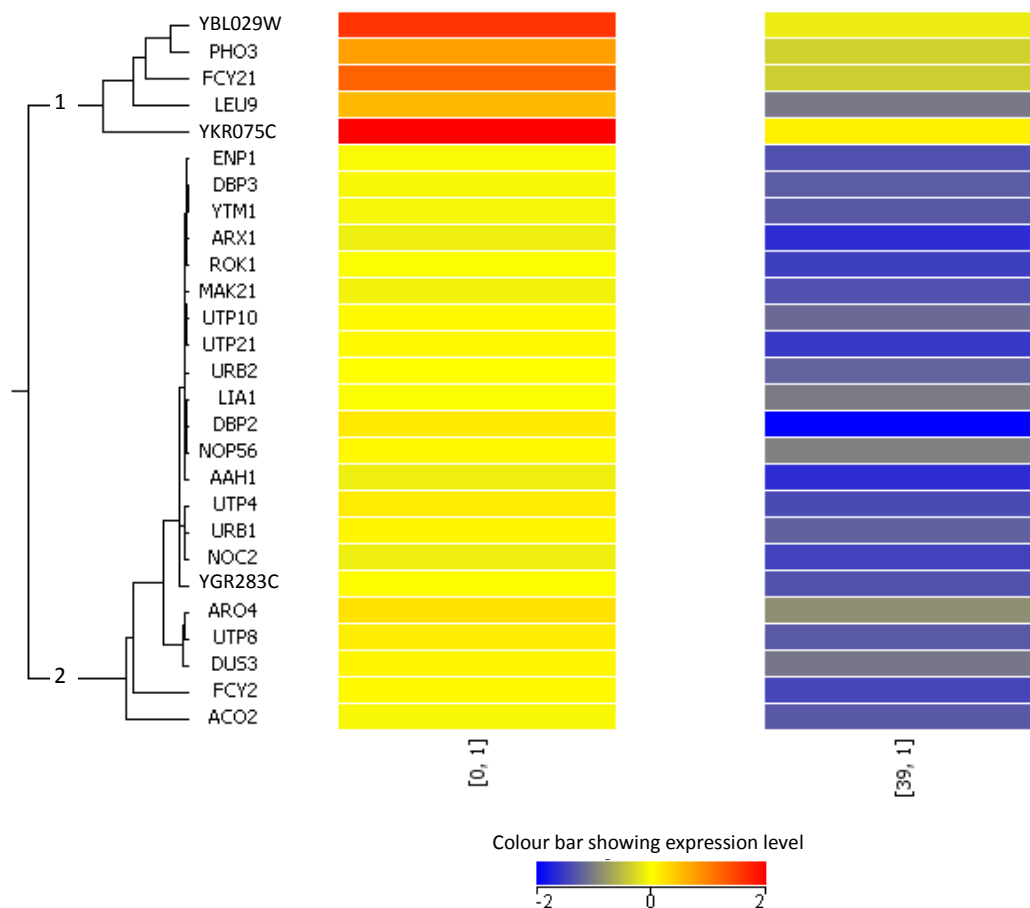
Figure 4.3.6 Dendogram produced by hierarchical cluster analysis (r²<0.95) of genes significantly and two fold decreased in expression after one hours exposure to 39 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the leaves on the right show the expression level of each gene after one hour's exposure to 0 mg/l ([0, 1]) and 39 mg/l ([39, 1]) 4-nitrophenol.

The results of hierarchical clustering in *Figure 4.3.6* show that genes decreased in expression after one hour's exposure to 39 mg/l 4-nitrophenol form two clusters. The first cluster contains two uncharacterized genes (YBL029W & YKR075C) whose expression is correlated with three genes of known function (PHO3, FCY21 & LEU9). The genes in the second cluster are highly correlated and contain one uncharacterized gene (YGR283C) and twenty one genes that have known functions.

4.4 Discussion

4.4.1 significantly (p<0.001) differentially expressed genes

The overall aim was to reduce the number of genes identified as differentially expressed to a manageable level so that specific chemical effects could be identified. Increasingly stringent statistical analysis was used to increase confidence in the resulting data and ensure the data analysed was relevant. The 4-nitrophenol concentrations chosen (10 & 39 mg/l) caused 10

and 50% reduction in cell division respectively. The lower concentration (10 mg/l) has less of an effect on cells as fewer genes show highly significant changes in expression. However the twelve genes that do have significant (p<0.001) differential expression are important indicators of the effects of low concentrations of 4-nitrophenol on cells, consequently they are examined in more detail in sections **4.4.2** and **4.4.3**. At 39 mg/l more genes (106) are significantly (p<0.001) differentially expressed identifying that the higher concentration has more of an effect on *S. cerevisiae* but making detailed analysis difficult. Therefore genes affected by 39 mg/l 4-nitrophenol were further filtered using a two fold change in expression to reduce the number of genes analysed in detail and the results are discussed in sections **4.4.4** and **4.4.5.**

4.4.2   Genes significantly (p<0.001) increased in expression after one hours treatment with 10 mg/l 4-nitrophenol

Seven genes (SFA1, SWI5, SLN1, POP3, DCW1, HLJ1, & BDS1) were significantly (p<0.001) increased in expression after one hours treatment with 10 mg/l 4-nitrophenol. Hierarchical clustering identified that SFA1, SWI5 and SLN1 had similar expression profiles and clustered together. SLN1 is associated with response to osmotic stress (*Figure 4.4.2*)
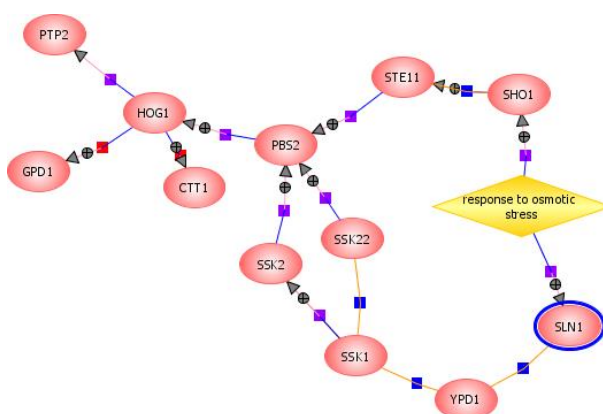
Figure **4.4.2** The Hyperosmotic stress response pathway contained a gene (SLN1) affected by p-nitrophenol exposure. SLN1 is highlighted (blue circle). This gene is positively regulated by osmotic stress and SLN1 was significantly (p<0.001) increased in expression after one hours exposure to 4-nitrophenol.

Increased expression of SLN1 activates and deactivates the High Osmolarity Glycerol pathway by activating Hog1 (product of HOG1 *Figure* **4.4.2**) (He *et al* 2009). HOG1 was not significantly increased in expression in this study, however the pathway is dependent on activation and deactivation of Hog1 protein not its level of production (related to gene expression level). Pathway analysis identified that the hyperosmolarity stress response pathway was significantly (p<0.01) affected by one hours treatment with 10 mg/l 4-nitrophenol (**3.3.7**). Further investigation identified that SLN1 was significantly increased in expression at both 10 & 39 mg/l 4-nitrophenol. SLN1 is a negative regulator of HOG1, deletion of the gene results in lethal HOG1 activation (Avery *et al* 2008), so increased expression of

SLN1 indicates HOG1 is deactivated. HOG pathway deactivation occurs under hypo osmotic conditions, it leads to the increased expression of AQY2 (increased two fold in expression in this study) and decreased glycerol production and uptake (Avery *et al* 2008). In addition osmotic stress leads to temporary cell cycle arrest (Reiser *et al* 2006), and exit from mitosis via action of SWI5 (Kishi *et al* 2008; Toyn *et al* 1997; Asakawa & Toh-e 2002) and other genes such as POP3 (Reiser *et al* 2006; Orchotorena *et al* 2001). These results indicate that *S. cerevisiae* treated with 10mg/l 4-nitrophenol have reduced cell division in response to osmotic stress. Osmotic stress is caused by damage to cell walls and membranes and DCW1 (Defective Cell Wall) codes for a protein required for cell wall development (Kitigaki *et al* 2002). The increased expression of DCW1 found in this work suggests 4-nitrophenol treated *S. cerevisiae* have damaged cell walls. The importance of the cell wall in protecting cells from osmotic and oxidative stress was illustrated by (Sun *et al* 2007) who encapsulated *S. cerevisiae* with alginate-chitosan-alginate, and found that encapsulation of *S. cerevisiae* lead to increased tolerance to osmotic and oxidative stress. Damage to cell walls caused by exposure to 4-nitrophenol initiates the transcription of genes involved in responding to osmotic stress i.e. the HOG pathway, which has also been found to regulate response to oxidative stress (Bisland *et al* 2004). In *Figure* **4.3.3** SFA1 clusters closely with SLN1 and SWI5, which are both involved in response to osmotic stress but SFA1 is involved in response to oxidative stress (Sahoo *et al* 2009; Hickman *et al* 2004) and both osmotic and oxidative stress frequently occur simultaneously. Osmotic and oxidative stress affect a large number of cellular processes including protein folding a process in which HLJ1 is involved, and the increased expression of HLJ1 may be related to oxidative and osmotic stress (Youker *et al* 2004). BDS1 is thought to code for a sulphatase required for the use of sulphur and its increased expression is indicative of a sulphur deficiency or increased requirement for sulphur in treated *S. cerevisiae* (Hall *et al* 2005; Rollini & Manzoni 2006).

In summary genes expression changes in *S. cerevisiae* cells exposed to 10 mg/l, 4-nitrophenol suggests cells are responding to osmotic and oxidative stress by exiting from mitosis. Increased expression of genes involved in cell wall maintenance indicates a damaged cell wall may contribute to osmotic stress. Additionally genes involved in protein folding and acquiring sulphur are increased in expression.

4.4.3   Genes decreased in expression after one hours exposure to 10 mg/l 4-nitrophenol

Only five genes were significantly (p<0.001) decreased in expression after one hours exposure to 10 mg/l 4-nitrophenol. RPL32 does not cluster with any of the other genes and regulates RNA splicing (Vilardell & Warner 1997), decreased expression of RPL32 indicates that RNA

splicing events have been reduced and this may be the result of decreased growth rates or the fact that different genes are required, or more important for survival in the presence of 4-nitrophenol. The expression profiles of TSA2, YJR115W and MDY2 were correlated; however, the functions of these do not appear to be related. TSA2 codes for an antioxidant enzyme that is increased in expression during the unfolded protein response (Kimata *et al* 2006), YJR115W is a gene of unknown function, and MDY2 (mating deficient yeast) (**4.3.3**) is involved in mating (Hu *et al* 2005). These gene expression results indicate that mating has been reduced by one hour's treatment with 10mg/l and indicate that YJR115W and TSA2 may play a role in this process. PAN3 regulates cell survival in response to replication stress (Hammet *et al* 2002) decreased expression of PAN3 suggests that cell division is reduced by treatment with 10mg/l 4-nitrophenol. As observed cell growth is decreased (Chapter 2) this is a good indication that cell division is also decreased.

4.4.4   Genes increased in expression after one hour exposure to 39 mg/l 4-nitrophenol

Increasing the stringency of statistical analysis and applying a two fold change in expression filter identified 16 genes that were increased in expression after one hour exposure to 39 mg/l 4-nitrophenol. Hierarchical clustering was used to identify genes with correlated expression levels, the results (*Figure 4.3.5*) show that three clusters of genes were formed. The first cluster contains PDR3, ARN2, FIT3, YMR102C, RSB1, PDR15, YLR346C, PDR16, GRE2, and YGP1 whose expression levels are highly correlated. Increased expression of PDR3 causes the transcription of drug resistance genes to be increased (Jungwirth & Kuchler 2005; Gulshan *et al* 2008). PDR3 clusters most closely with ARN2 and FIT3, which are both involved in iron siderophore uptake (Emmerson *et al* 2002; Rutherford *et al* 2003). Both ARN2 and FIT3 are increased in expression during iron deprivation and iron deprivation has previously been linked to increased susceptibility to xenobiotics in *S. cerevisiae* (Emerson *et al* 2002; Prasad *et al* 2006; Rojas *et al* 2008). The results from this experiment lend support to previous studies that show iron plays a role in drug resistance in *S. cerevisiae* (Tuttle *et al* 2003; Rojas *et al* 2008; Kimura *et al* 2007; Fairn *et al* 2007; Almeida *et al* 2008). It was previously found that iron depletion results in increased membrane fluidity leading to increased xenobiotic sensitivity in the yeast *Candida albicans* (Prasad *et al* 2006). This is consistent with other studies that have suggested increased iron concentrations result in increased membrane rigidity (Garcia *et al* 2005) so iron may play a role in maintaining membrane fluidity in *S. cerevisiae*. Changes in membrane fluidity inhibit electron transport by complex III of the electron transport chain, resulting in increased generation of oxygen radicals by the Rieske iron sulphur complex of complex III, and this process has been shown to result in increased sensitivity of *S. cerevisiae* to Farnesol (Fairn *et al* 2007). It would appear that 4-nitrophenol causes membrane damage and

the cell thus starts to require more Fe to maintain the correct membrane fluidity; hence the increase in expression of ARN2 and FIT3. Increased membrane fluidity and inhibition of electron transport are processes that previous biochemical studies have shown to be affected by 4-nitrophenol exposure (**1.7.4** & **2.1.3**) and alterations in membrane composition and structure are further supported by the increased expression of PDR16. PDR16 is a drug resistance gene that increases the production of phospholipids in response to membrane damage (Le Crom *et al* 2002); its deletion leads to increased sensitivity to azoles, a characteristic that has been attributed to impaired sterol synthesis, and alterations in plasma membrane composition (Mattal *et al* 2001; Van Den Hazel *et al* 1999; Hazel *et al* 1999). In this experiment, the expression of PDR16 is most closely correlated with GRE2 that increases ergosterol synthesis during membrane damage and osmotic stress (Warringer *et al* 2006; Warringer & Blomberg 2006; Chen *et al* 2003; Kalapos 1999). Although not closely correlated in this experiment increased expression of YGP1 and ARN2 has previously occurred upon exposure to weak organic acids (Kawahata *et al* 2006; Fernandes *et al* 2005) a group of chemicals to which 4-nitrophenol belongs (**1.7.2**). YGP1 clusters with genes involved in drug resistance ; it encodes a poorly characterized cell wall glycoprotein whose increased expression suggests 4-nitrophenol may cause cell wall damage, or that alterations in cell wall composition are required for adapting to the presence of 4-nitrophenol. Genes in cluster three that are also increased in expression are involved in the maintenance of cell walls and membranes. The three genes in cluster three are GSC2, ICT1 and YPL088W. GSC2 is a gene that codes for a glucan synthase involved in cell wall formation (Iwamoto *et al* 2005) that has previously been reported to be increased in expression as a consequence of cell wall damage caused by exposure of *S. cerevisiae* to anti-fungal agents (Agarwal *et al* 2002). The increased expression of GSC2 and YGP1 indicate cell wall damage occurs during exposure to 39 mg/l 4-nitrophenol. Frequently cell wall damage occurs alongside cell membrane damage and increased expression of ICT1 has previously been linked to membrane damage caused by organic acid stress. ICT1 increases the production of phospholipids during organic acid stress (Anderson *et al* 2009) alongside the increased expression of PDR16 and GRE2 these results increase the evidence that cell wall and membrane damage are caused by one hours exposure to 39 mg/l 4-nitrophenol. YPL088W clusters with GSC2 and ICT1 however it is a poorly characterized gene that has previously been implicated in drug resistance (Le Crom *et al* 2002); its actual function remains unknown. However, these results indicate its involvement in maintenance of cell membranes and or walls. One of the consequences of increased membrane fluidity (membrane damage) is increased passive transport of substances into cells. To counter this activation of pleiotropic drug resistance pumps that can actively remove unwanted and harmful substances from cells occurs. PDR15 is a pleiotropic drug resistance

gene that codes for a membrane localized yeast ATP Binding Cassette (ABC) that uses an ATP dependent pump that eliminates a multitude of toxic chemicals from cells (Frelet & Klein 2006; Annilo *et al* 2006; Wolfger *et al* 1997; Junwirth & Kuchler 2005). Increased expression of PDR15 suggests that *S. cerevisiae* are attempting to actively transport 4-nitrophenol out of the cell. PDR15 is in the same cluster as and has similar expression profiles to RSB1, YMR102C and YLR346C all of which are under the control of pleiotropic drug resistance transcription factors. While RSB1 is known to be involved in transporting lipids in the cell the functions of YMR102C and YL346W are unknown (Gulshan *et al* 2008; Anderson *et al* 2003; Manente & Ghislain 2009; Le Crom *et al* 2002; Valle Matta *et al* 2001).

Other genes increased in expression include the three genes in the second cluster (***Figure 4.3.5***) NQM1, GPG1 and HXT5 which are all involved in regulating cell division under stress conditions (Chang *et al* 2000; Van Suyllekom *et al* 2007; Ishiwata *et al* 2008). Increased expression of genes involved in reducing growth rates shows that cells are responding to 4-nitrophenol exposure by inhibiting their growth and is consistent with the results in **2.3.2** that show 39 mg/l 4-nitrophenol causes significant reduction in cell division.

Overall, the results show that after one hour's exposure to 39mg/l 4-nitrophenol drug resistance mechanisms that include removal of 4-nitrophenol and modification/repair of cell walls and membranes have been activated. These typical responses to xenobiotic exposure have previously been characterized using gene expression profiling. Additionally the results show that iron uptake is increased after one hours exposure to 39 mg/l 4-nitrophenol. Increased uptake of iron has previously been reported to increase resistance to xenobiotics, and the cell appears to be attempting to increase Fe uptake to maintain the appropriate level of membrane fluidity.

4.4.5 Genes decreased in expression after one hour exposure to 39 mg/l 4-nitrophenol

More genes were decreased in expression than increased in expression after one hour exposure to 39 mg/l 4-nitrophenol. Genes decreased in expression separate into two clusters the first of which contains the fewest genes. The first cluster contains two unknown genes, YBL029W and YKR075C, as well as PHO3 which codes for a phosphatase involved in the uptake of thiamin (Nosaka *et al* 2005), FCY21 is a gene whose disruption and decreased expression leads to increased resistance to 5-flurocytosine (Paluszynski *et al* 2006), and LEU9, which codes for a protein involved in leucine biosynthesis required for maintaining growth on non-fermentable carbon sources (Casalone *et al* 2000). The genes in cluster one that are decreased in expression do not appear to have related functions, however both PHO3 and PCY21 are

involved in the uptake of chemicals and their decreased expression may be related to the cell trying to reduce 4-nitrophenol uptake from the culture media.

The second cluster contains more genes that are decreased in expression after one hour exposure to 39 mg/l 4-nitrophenol. Examination reveals that most of the genes are involved in gene transcription and translation processes such as RNA processing, ENP1, DBP2, DBP3, ROK1, UTP21, UTP4, UTP8, LIA1 and DUS3 (Chen *et al* 2003; Weaver *et al* 1997; Champion *et al* 2008; Bond *et al* 2001; Vos *et al* 2004; Strub *et al* 2007; Thompson *et al* 2003; Gregio *et al* 2009; Wang *et al* 1997), as well as ribosome assembly and maturation YTM1, ARX1, MAK21, UTP10, URB1, URB2 NOC2, and NOP56 (Miles *et al* 2005; Bradatsch *et al* 2007; Edskes *et al* 1998; Dez *et al* 2007; Milkereit *et al* 2003; Mnaimneh *et al* 2004; Milkereit e*t al* 2001; Gautier *et al* 1997). Previous studies have shown that ribosome biogenesis is reduced in the presence of toxic compounds in *S. cerevisiae* (Slepak *et al* 2005). Genes involved in ribosome biogenesis and RNA processing have been grouped together and named repressed Environmental Stress Response (ESR) genes because they are decreased in expression in response to environmental stress and decreased growth rates (Gasch *et al* 2000). A diverse array of environmental changes result in the decreased expression of approximately 600 genes involved in gene transcription, RNA processing, and protein production known as the Environmental Stress Response (ESR) or Common Environmental Response (CER) (Berry & Gasch 2008). Investigation of this response has identified that it is the inhibition of oxidative phosphorylation that initiates the ESR and it functions to rebalance energy supply and demand as well as co-coordinating cell growth rates (Lai *et al* 2008). Previous work suggesting that the decreased expression of these genes was related to decreased growth rates appears to be wrong as the study by Lai *et al* (2008) showed varying ESR gene expression levels under different growth conditions that reduced cell growth rate.

Iron plays an important role in translation initiation, termination and thus RNA processing and ribosome biogenesis. Iron Responsive Proteins (IRPs) control translation initiation of specific genes containing iron responsive elements (IREs) (Goforth *et al* 2010). IREs are contained in the 5' or 3' regions of mRNA coding for proteins of iron and oxidative metabolism and iron depletion results in the binding of Iron Responsive Proteins to IREs and prevent their translation (Ke *et al* 1998). These observations are interesting because they provide a potential link between increased iron uptake and decreased expression of genes involved in RNA processing and protein production (ESR genes).

Metal ions play an important role in the structural organisation and activation of enzymes involved in the transfer of genetic material from DNA, resulting in the synthesis of specific proteins (Regupathy & Nair 2010). The iron chelators Deferiprone and ciclopirox have been

found to inhibit the formation of the eukaryotic translation initiation factor eIF5A by preventing the modification of a lysine residue by deoxyhypusine hydroxylase. Reduction of eIF5A slows proliferation and leads to cell cycle arrest in *S. cerevisiae* (Hoque *et al* 2009). Likewise, iron displacement has been found to cause conformational changes in the structure of Lia1 (the *S. cerevisiae* deoxyhypsuine hydroxylase enzyme), identifying that iron plays an important role in translation initiation (Cano *et al* 2010). Additionally iron-sulphur cluster containing RNase L inhibitor is involved in ribosome subunit maturation, and translation through interaction with the eukaryotic initiation factor 3 (EIF3) complex, and TPA1 which terminates translation mRNA are both responsive to iron (Khoshnevis *et al* 2010; Regupathy & Nair 2010).

Some of the genes decreased in expression in this experiment (AAH1 & ACO2) are involved in responding to glucose concentrations and are decreased in expression upon entry into stationary phase (Escusa *et al* 2006; Tatusov *et al* 2000). ARO4 codes for a protein involved in the biosynthesis of amino acids a process which is reduced as a direct consequence of decreased growth rates (Valerius *et al* 2003), this leads to the conclusion that most of the genes decreased in expression are involved in cell replication and thus growth, and their decreased expression is thus linked to decreases in growth, replication and inhibition of oxidative phosphorylation. Exceptions include both YGR283C and FCY2 whose decreased expression results in resistance to fluconazole (Anderson *et al* 2003; Florent *et al* 2009) and their decreased expression may also play a role in resistance to 4-nitrophenol.

4.5 Summary & Conclusions

The changes in gene expression resulting from exposure to 39 mg/l were more pronounced than those observed after exposure to the lower 4-nitrophenol concentration (10 mg/l); however, both concentrations appear to cause cell wall and membrane damage. At 10 mg/l, 4-nitrophenol differentially expressed genes were involved in decreasing growth rates, decreasing mating frequency and activation of the osmotic stress response pathway. At the higher 4-nitrophenol concentration (39 mg/l) genes involved in drug resistance and the maintenance of cell walls and membranes were increased in expression, identifying that exposure of *S. cerevisiae* to 4-nitrophenol elicits a similar response as exposure to other xenobiotics. Additionally the results lend support to previous suggestions that iron uptake plays a role in drug resistance as genes involved in iron uptake were correlated with drug resistance genes. Genes decreased in expression in response to 4-nitrophenol treatment were involved in RNA processing and ribosome biogenesis and are genes that have previously been grouped together and named repressed Environmental Stress Response (ESR) genes, on account of the fact they are always decreased in expression in response to environmental

stress. RNA processing, ribosome biogenesis and the transcription and translation of genes has been found to be affected by iron concentrations and the presence of iron chelators inhibits the formation and maturation of translation initiation factors. The genes repressed by iron deficiency contain Iron Responsive Elements and are genes coding for enzymes involved in iron and oxidative metabolism. These results and observations indicate a link between iron concentrations and the expression of ESR genes and increased expression of iron siderphore transporters. Iron was not limited in the growth media so these results suggest available iron is used to maintain membrane activity reducing the availability of iron for other processes such as oxidative phosphorylation.

Chapter 5

5*. S. cerevisiae* genes significantly differentially expressed after two hours exposure to 10 & 39 mg/l 4-nitrophenol

5.1 Introduction

5.1.1   Overview of data filtering methods used Chapter 4

In the previous chapter (Chapter 4), the effects of 10 & 39 mg/l 4-nitrophenol on gene expression in *S. cerevisiae* after one hour exposure were identified. A large number of genes were differentially expressed after two hours exposure to 4-nitrophenol, to reduce the number of genes considered in detail data was further mined using un-paired T-tests with a more stringent p-value (p<0.001). This method identified over 100 genes that were differentially expressed at 39 mg/l 4-nitrophenol. Further filtering was needed to reduce these genes, so at 39 mg/l unpaired t-tests were coupled with a two fold or greater change in expression. Clustering was then used to elucidate relationships between identified genes. (**4.1.1 – 4.1.4**). The results were used to identify time related changes in gene expression resulting from 4-nitrophenol exposure.

Aims of chapter 5

The overall aim is to see gene expression changes occurring after two hours exposure to 4-nitrophenol and below are individual objectives to meet the overall aim.

Aim 1 Increase the stringency of filtering methods to reduce the number of genes considered in detail

Objectives to meet Aim 1

1. Use unpaired T-tests to identify genes significantly (p<0.001) differentially expressed after two hours exposure to 10 and 39 mg/l 4-nitrophenol

2. Filter significantly (p<0.001) differentially expressed genes to identify genes with two fold or more changes in expression after two hours exposure to 10 & 39 mg/l 4-nitrophenol

Aim 2 Identify genes with correlated expression levels that may have related functions

Objectives to meet Aim 2

1. Use Hierarchical clustering to identify differentially expressed genes with correlated expression levels

2. Use literature searches to gather information on factors leading to differential expression of identified correlated differentially expressed genes and identify cellular processes affected by 4-nitrophenol treatment

5.2 Methods

Previously (Chapter 4) genes differentially expressed after one hour's treatment with 10 & 39 mg/l 4-nitrophenol were filtered from the 1,357 genes that were affected by 4-nitrophenol treatment over the time course of exposure (Chapter 3). To obtain a small subset of genes that could be analysed in detail statistical tests with increased stringency were used alongside fold change in expression (where appropriate). The resulting gene lists indicated exposure to 10 & 39 mg/l 4-nitrophenol caused cell membrane and wall damage leading to osmotic stress. At the higher 4-nitrophenol concentration, (39 mg/l) genes involved in drug resistance, iron acquisition, as well as cell wall and membrane repair were increased in expression.

In this chapter the methods described in **4.2** were used to identify specific processes affected by two hours exposure to 4-nitrophenol at 10 and 39 mg/l. Briefly unpaired t-tests ($p<0.001$) were used to reduce the genes considered in detail. This process identified a large number of genes (121) that were differentially expressed at 39 mg/l 4-nitrophenol, so these genes were further filtered using a two fold change in expression. This process reduced the number of genes differentially expressed at 39 mg/l 4-nitrophenol to 43 which was more manageable. Two fold change in expression filters were not used on genes differentially expressed at 10 mg/l because it did not identify and differentially expressed genes so to avoid disregarding subtle affects of low concentrations. All of the genes that passed the filters were clustered using Hierarchical clustering with Pearsons correlation ($r^2<0.95$) to identify highly correlated genes with potentially related functions.

5.3 Results

The primary purpose of this study is to identify a small sample of differentially expressed genes that could be used to identify potentially toxic concentrations of 4-nitrophenol in water samples. To meet this aim and increase confidence in the results obtained gene expression data was further filtered with increasing stringency to identify a subset of genes that can be analysed in detail.

5.3.1   Identifying genes with significant (p<0.001) gene expression changes

The number of genes identified as significantly (p<0.01) increased in expression after two hours exposure to 4-nitrophenol (**Table 4.1 Appendix III**) was too great to examine in detail. To reduce the number of genes considered un-paired t-tests were used to identify genes with even greater statistically significant change in expression (p<0.001). The number of genes significantly (p<0.001) increased and decreased in expression after three hours treatment with 10 and 39 mg/l 4-nitrophenol are shown in **Table 5.3.1.**

Table 5.3.1 Number of genes significantly (p<0.001) differentially expressed after two hours exposure to 10 and 39 mg/l 4-nitrophenol

4-nitrophenol (mg/)l	Number of genes increased	Number of genes decreased
10	4	17
39	68	53

Table 5.3.1 shows that increasing the stringency of statistical analysis reduces the number of genes differentially expressed at 10 mg/l 4-nitrophenol to twenty one genes that can be analysed in detail. However, the number of genes identified as differentially expressed at 39 mg/l 4-nitrophenol using statistical analysis alone was too large for detailed analysis. To further reduce the number of genes considered in subsequent analysis fold change filters were used to identify genes with a two fold or more change in expression.

5.3.2   Genes with significant (p<0.001) and two fold changes in expression

The number of genes significantly (p<0.001) and two fold increased and decreased in expression at 10 & 39 mg/l 4-nitrophenol are shown in **Table 5.3.2**

Table 5.3.2 Number of genes significantly (p<0.001) and two fold differentially expressed after two hours exposure to 10 and 39 mg/l 4-nitrophenol

4-nitrophenol concentration mg/l	Number of genes significantly (p<0.001) and two fold increased	Number of genes significantly (p<0.001) and two fold decreased
10	0	2
39	36	34

Table 5.3.2 shows that applying a two fold change filter and increasing the stringency of statistical tests dramatically reduces the number of genes differentially expressed at 10 & 39mg/l 4-nitrophenol.

Only two genes (YDR034W-B & SPG4) were significantly (p<0.001) and two fold decreased in expression in response to two hours treatment with 10mg/l 4-nitrophenol, and no genes were increased in expression at 10mg/l 4-nitrophenol. This analysis method is too stringent for the gene expression data from *S. cerevisiae* exposed to 10 mg/l 4-nitrophenol. For this reason, different analysis methods were used to identify genes differentially expressed at 10 & 39 mg/l 4-nitrophenol. Genes significantly (p<0.001) differentially expressed at 10 mg/l 4-nitrophenol are clustered using hierarchical clustering and analysed in detail, whereas genes significantly (p<0.001) and two fold differentially expressed at 39 mg/l 4-nitrophenol are clustered using hierarchical clustering and analysed in detail.

5.3.3   Genes significantly (p<0.001) increased in expression after two hours exposure to 10 mg/l 4-nitrophenol

Four genes (**Table 5.3.3**) were significantly (p<0.001) increased in expression after two hours exposure to 10 mg/l 4-nitrophenol.

Table 5.3.3 The functions of genes increased in expression after two hours exposure to 10 mg/l 4-nitrophenol

Gene function	Gene name
Mitochondrial carrier protein	RIM2
Ergosterol synthesis	ERG1
Unknown	YML018C
Silencing of genes involved in mating	SAS5

The results in **Table 5.3.3** show that genes increased in expression after two hours exposure to 10 mg/l 4-nitrophenol are involved in a range of cellular processes including energy generation (RIM2) ergosterol synthesis (ERG1) and reducing mating (SAS5). Hierarchical clustering was used to identify genes with correlated expression levels, differentially expressed genes separated into two clusters (*Figure 5.3.3*).
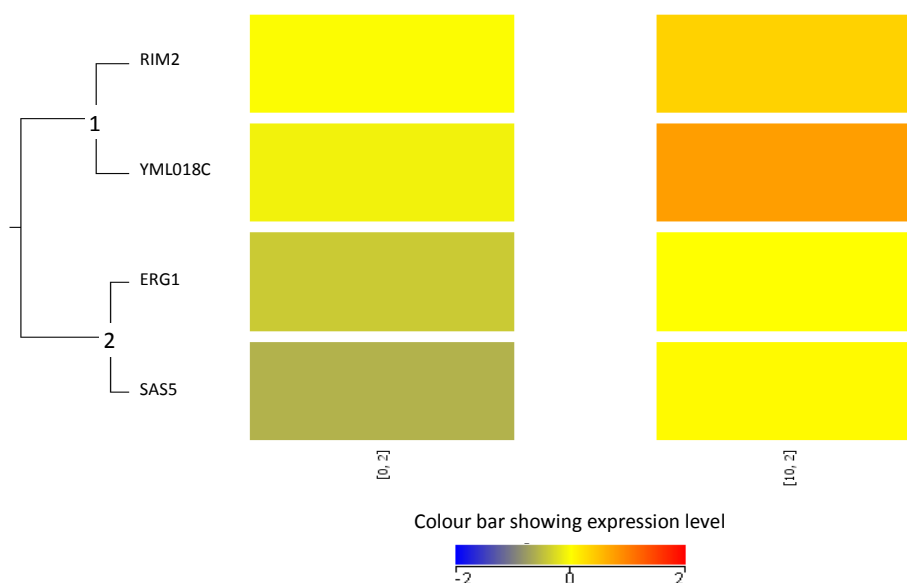
Figure **5.3.3** Dendogram produced by hierarchical cluster analysis (r^2<0.95) of genes significantly (p<0.001) increased in expression after two hours exposure to 10 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after two hours exposure to 0 mg/l ([0, 2]) and 10 mg/l ([10, 2]) 4-nitrophenol is shown by the leaves on the right.

The results in *Figure 5.3.3* show the expression levels of each of the *S. cerevisiae* genes increased in expression relative to the untreated control *S. cerevisiae*. Hierarchical clustering groups genes with the most similar expression values within treatment conditions, thereby identifying genes that may be involved in related processes. The results in *Figure 5.3.3* show that RIM2 clusters with YML018C and ERG1 clusters with SAS5.

5.3.4 Genes significantly (p<0.001) decreased in expression after two hours exposure to 10 mg/l 4-nitrophenol

Seventeen genes (**Table 5.3.4**) were significantly (p<0.001) decreased in expression after two hours treatment with 10 mg/l 4-nitrophenol.

Table 5.3.4 Functions of genes decreased in expression after two hours exposure to 10 mg/l 4-nitrophenol

Function	Genes decreased in expression	Total
Energy generation	ATP15, ATP18, QCR10, VPS1, THI11, SPG4	6
Mitochondrial biogenesis	AIM2, RPM2, MRP10	3
Unknown	ISR1, TMA108, YDR034W-B, YER158C	4
Cell wall maintenance	AGA2, ACE2	2
Mitosis	SWR1, RPN2	2

The results in **Table 5.3.4** show that three of the genes decreased in expression are involved in energy generation (ATP15, ATP18 & QCR10) and two of the genes are involved in mitochondrial biogenesis (AIM2 & RPM2). Hierarchical clustering was used to identify genes

with correlated expression levels and this technique separated the genes into three clusters (*Figure 5.3.4*).
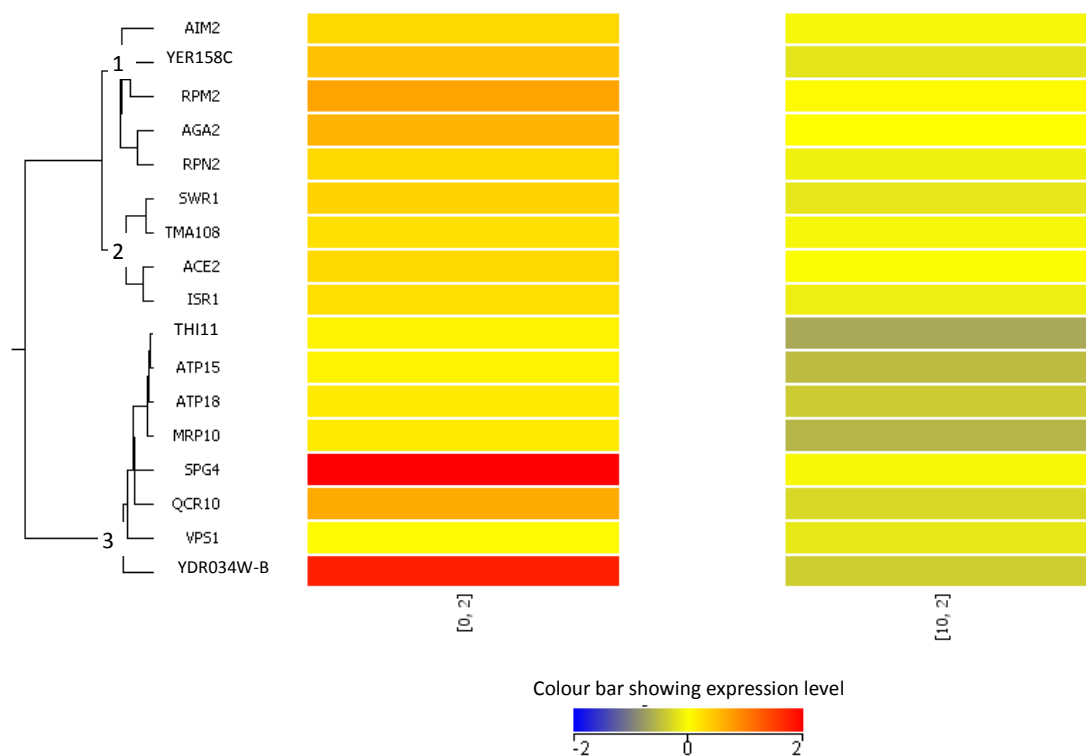
Figure 5.3.4 Hierarchical cluster analysis (r^2<0.95) of genes significantly (p<0.001) decreased in expression after two hours exposure to 10 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after two hours exposure to 0 mg/l ([0, 2]) and the leaves on the right show 10 mg/l ([10, 2]) 4-nitrophenol.

The results in *Figure* 5.3.4 show the expression levels of genes significantly (p<0.001) decreased in expression after two hours exposure to 10 mg/l 4-nitrophenol. Three clusters are formed by genes decreased in expression; cluster 3contains the most genes. Genes in this cluster show the greatest change in expression compared to untreated controls and are involved in mitochondrial biogenesis and energy generation.

5.3.5   Genes increased in expression after two hours exposure to 39mg/l 4-nitrophenol

Thirty six genes (**Table 5.3.5**) were significantly (p<0.001) and two fold increased in expression after two hours exposure to 39mg/l 4-nitrophenol. The results in **Table 5.3.5** show that most of the genes increased in expression are involved in iron homeostasis and drug resistance, followed by lipid and membrane synthesis.

Table 5.3.5 The functions of genes increased in expression after two hours exposure to 39 mg/l 4-nitrophenol

Function	Genes increased in expression
Drug resistance	PDR3, PDR15, SNQ2, PDR5, YOR1, YLR346C
Iron homeostasis	ARN2, FIT2, FIT3, ARN1, FRE1, AKR1, SIT1, CCC2, FIT1, YAP5, ENB1, YTP1, NFS1, TIS11, YLR126C
Energy generation	YGL157W, YKL071W
Lipid synthesis	RSB1, GRE2, RTA1, ICT1, VHR1
Unknown	YOR152C, YML131W, YMR102C, YLR460C, YGR035C, PHM6, BCP1
RNA processing	CPA1

Hierarchical clustering was used to identify genes with correlated expression levels (*Figure 5.3.5*)
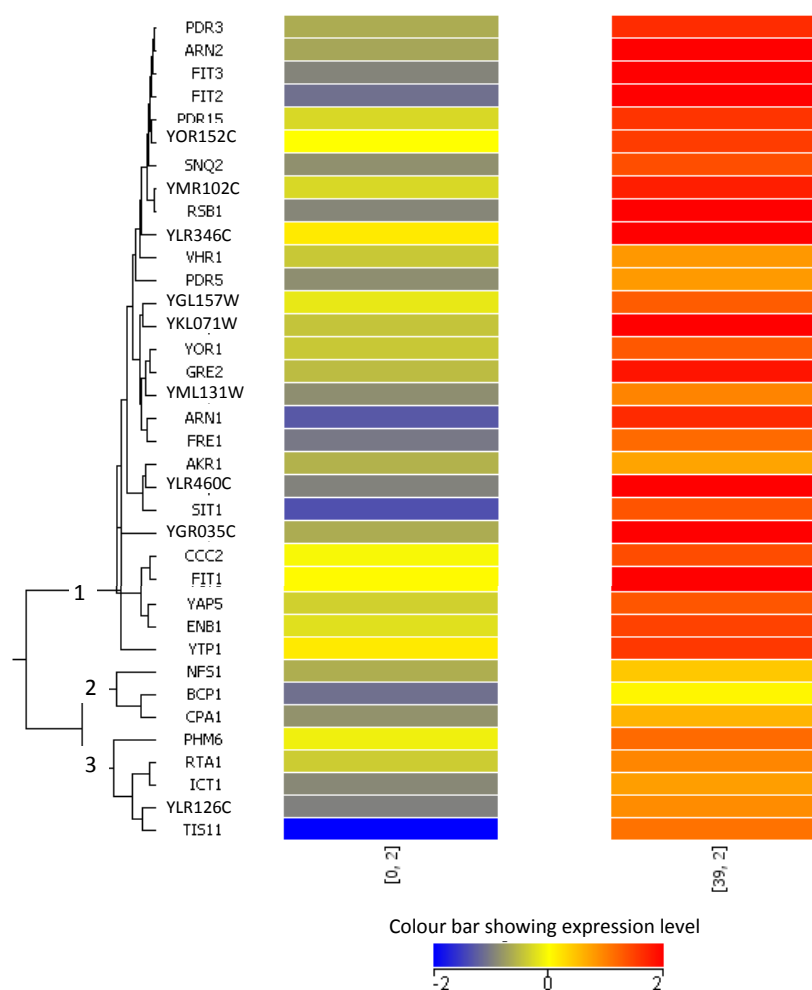
Figure 5.3.5 Hierarchical cluster analysis (r^2<0.95) of genes that were significantly (p<0.001) and two fold increased in expression after two hours exposure to 39 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after two hours exposure to 0 mg/l ([0, 2]) and 39 mg/l ([39, 2]) 4-nitrophenol is shown by the leaves on the right.

Most of the genes in cluster one is involved in iron homeostasis and drug resistance. The

second cluster contains only three genes and all are of known function, but their functions

vary. The third cluster contains three genes of known function including ICT1, which was discussed in Chapter 4, and one previously uncharacterized gene YLR126C.

5.3.6 Genes decreased in expression after two hours exposure to 39mg/l 4-nitrophenol

Only 34 genes (**Table 5.3.6**) were significantly ($p < 0.001$) and two fold increased in expression after two hours exposure to 39mg/l 4-nitrophenol.

Table 5.3.6 Functions of genes decreased in expression after two hours exposure to 39 mg/l 4-nitrophenol

Function	Genes decreased in expression
Energy generation	CYC1, STF2, COX5B, PHM7, OM45, OM14, ALD4, NCE103
Nutrient absorption	HXT2, HXT4, HXT6/7, MTH1, HXT5, RTN2
Oxidative stress	BTN2, CTT1
Unknown	ICS2, YMR196W, YBR285W, YDR034W-B, FMP16, UIP4, DDR2, YNR034W-A, YER067W, ISF1, SPG4, RPL22B, YKR075C, MSC1
Osmolyte metabolism	GUT2, GDB1, TMA10, GPH1,

The results in **Table 5.3.6** show that most of the genes decreased in expression are unknown, additionally a large proportion of the genes are involved in energy generation and nutrient absorption. Additionally some genes decreased in expression are involved in stress resistance and one is involved in meiosis.

The results of hierarchical clustering in *Figure 5.3.6* show that genes decreased in expression after two hours exposure to 39 mg/l 4-nitrophenol form four clusters. The first cluster contains a total of 13 genes, most of which are involved in energy generation, and one of which (YMR196W) is uncharacterized. The second cluster contains the most genes (17) four of which are uncharacterized, but most of the genes in this cluster are involved in nutrient absorption, followed by energy generation. The third and fourth clusters both contain only two genes each which are neither highly correlated nor perform similar functions.
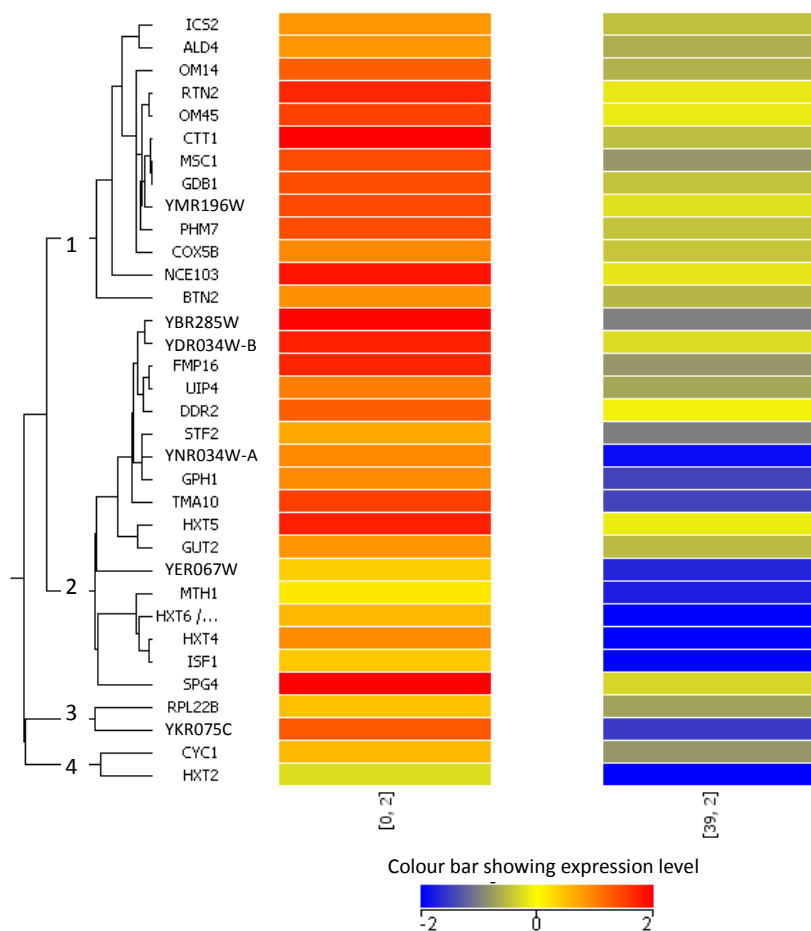
Figure **5.3.6** Hierarchical cluster analysis ($r^2 < 0.95$) of genes significantly and two fold decreased in expression after two hours exposure to 39 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after two hours exposure to 0 mg/l ([0, 2]) and the leaves on the right show 39 mg/l ([39, 2]) 4-nitrophenol.

5.4 Discussion

5.4.1 Significantly (p<0.001) differentially expressed genes

The overall aim was to reduce the number of genes identified as differentially expressed to a manageable level so that specific chemical effects could be identified. Increasingly stringent statistical analysis was used to increase confidence in the resulting data and ensure the data analysed was relevant. The 4-nitrophenol concentrations chosen (10 & 39 mg/l) caused 10 and 50% reduction in cell division respectively. The lower concentration (10 mg/l) has less of an effect on cells as fewer genes show highly significant changes in expression. However the twenty three genes that do have significant (p<0.001) differential expression are important indicators of the effects of low concentrations of 4-nitrophenol on cells, consequently they are examined in more detail in sections **5.4.2** and **5.4.3**. At 39 mg/l more genes (121) are significantly (p<0.001) differentially expressed identifying that the higher concentration has more of an effect on *S. cerevisiae* and making analysis difficult. Genes affected by 39 mg/l 4-

nitrophenol were further filtered using a two fold change in expression to reduce the number of genes analysed in detail to a manageable level and the results are discussed in sections **5.4.4** and **5.4.5.**

5.4.2   Genes significantly (p<0.001) increased in expression after two hours treatment with 10 mg/l 4-nitrophenol

Four genes (RIM2, ERG1, YML018C, SAS5) were significantly (p<0.001) increased in expression after two hours exposure to 10 mg/l 4-nitrophenol. None of these genes were increased in expression after one hours exposure to 10 mg/l 4-nitrophenol. These four genes formed two clusters each containing two genes (*Figure 5.3.3*). ERG1 and SAS5 clustered together, but these genes do not have related functions. SAS5 belongs to a group of genes that regulate modification of histones and the assembly and disassembly of chromatin, thereby regulating the silencing of genes involved in mating (Xu *et al* 1999), indicating mating frequency is reduced and decreased mating frequency accompanies decreased cell division rates (**4.4.2** & **4.4.3**). ERG1 codes for squalene epoxidase an important precursor for ergosterol biosynthesis (Pedroso *et al* 2009). Increased expression of ERG1 occurs when ergosterol concentrations are limited (Leber *et al* 2000). Ergosterol deficiencies could result from increased requirement for ergosterol for drug export as sterols serve as substrates for the transport of xenobiotics across membranes by pleiotropic drug resistance genes (Kontoyiannis 2000). However pleiotropic drug resistance genes were not significantly increased in expression upon exposure to 10 mg/l 4-nitrophenol. Alternatively, mutations in ergosterol synthesis genes have been found to lead to increased sensitivity to terbinafine, and nystatin (Klobucnikova *et al* 2003; Beezer *et al* 1986). This suggests that increased expression of ERG1 may be a drug resistance response. A study by Klobucnikova *et al* (2003) found that *S. cerevisiae* with mutations in the ERG1 gene also had respiration deficiencies. The cytochrome P450 (a iron requiring oxidoreductase) is important for the biosynthesis of ergosterol and mutations in the gene coding for cytochrome P450, have been found to result in decreased resistance to azoles in *C. albicans* and *S. cerevisiae* (Sanglard *et al* 1997; Kontoyiannis 2000; Hanioka *et al* 2007). Cytochrome P450 is also an enzyme of toxicological importance and is known for its ability to metabolise a wide range of xenobiotics including 4-nitrophenol (Hanioka *et al* 2007). It could be hypothesized that the use of cytochrome P450 in detoxification of 4-nitrophenol by the cell results in this enzyme being less utilized for ergosterol biosynthesis. Interestingly, cluster two (*Figure 5.3.3*) contains RIM2 which codes for a mitochondrial carrier protein whose increased expression has been attributed to rescue of respiration deficient cells (Vandyck *et al* 1995; Marobbio *et al* 2006), indicating that respiration is affected by 4-nitrophenol exposure. YML018C, which clusters, with RIM2 are a gene of unknown function that may also be involved in the rescue of respiration deficient cells.

These results indicate that cells have respiration deficiencies, ergosterol deficiencies, as well as reduced mating frequency and cell growth after two hours exposure to 10 mg/l 4-nitrophenol.

5.4.3   Genes decreased in expression after two hours exposure to 10 mg/l 4-nitrophenol

Seventeen genes (AIM2, YER158C, RPM2, AGA2, RPN2, SWR1, TMA108, ACE2, ISR1, THI11, ATP15, ATP18, MRP10, SPG4, QCR10, VPS1, YDR034W-B) were significantly (p<0.001) decreased in expression after two hours exposure to 10 mg/l 4-nitrophenol. None of these genes were significantly decreased in expression after one hours exposure to 10 mg/l 4-nitrophenol. The results in *Figure 5.3.4* show that the genes significantly (p<0.001) decreased in expression formed three clusters. Analysis of genes in clusters did not reveal that genes clustered according to function with the exception of cluster three which contained eight genes, six of which (ATP15, ATP18, QCR10, VPS1, THI11, & SPG4) are involved in energy generation. Briefly, ATP15, ATP18, & QCR10 are essential components of the electron transport chain, which code for iron requiring oxidative phosphorylators, ATP synthases and oxidoreductases respectively (Marsy *et al* 2008; Arnold *et al* 1998; Brandt *et al* 1994; Rogers *et al* 2002). THI11 is involved in the synthesis of thiamine, which is used to generate energy (Wightman & Meacock 2003), and VPS1 is involved in the production of peroxisomes, which metabolise fatty acids (Kuravi *et al* 2006). SPG4 is a gene that responds to nutritional stress and is thought to be involved in energy generation (Pir *et al* 2006). Additionally MRP10, AIM2 and RPM2 (cluster one, *Figure 5.3.4*) are involved in mitochondrial biogenesis (Hess *et al* 2009; Stribinskis *et al* 2001; Jin *et al* 1996) implying that the production of proteins required for mitochondrial biogenesis and energy production have been reduced by two hours exposure to 10 mg/l 4-nitrophenol. Interestingly many of these genes are affected by iron concentrations as many of the gene products have a requirement for iron, and some of these genes are under the control of Iron Responsive Elements as they code for oxidoreductases and genes involved in iron metabolism.

Decreased energy production is also a direct consequence of reduced growth/division rates which occurs as a consequence of exposure to 10 mg/l 4-nitrophenol (reduced cell growth was observed in Chapter 2 in response to 10 mg/l 4-nitrophenol exposure). Cell division is a process with a high-energy requirement and reduction in cell division rates would directly affect energy requirements, and thus energy production. Genes involved in cell division include : ACE2 which activates enzymes responsible for locally specific cell wall degradation, and is required for the separation of mother and daughter cells (Voth *et al* 2005). RPN2 whose decreased expression results in growth inhibition (Beuudenon *et al* 1999); AGA2 that codes for a cell adhesion protein, the α-agglutinin binding subunit (de Nobel *et al* 2001) that mediates

the cell-cell adhesion of α haploid cells during mating (Shen *et al* 2001). As well as SWR1, an ATP dependent chromatin remodeling complex that plays a role in mediating the repair of double strand breaks in DNA (Attikum *et al* 2007). These results show that meiosis and cell division appear to be reduced by 4-nitrophenol treatment and are consistent with previous results (**4.4.2, 4.4.3** & **5.4.2**). Decreased expression of SWR1 indicates lower frequency of double strand breaks in DNA occur in *S. cerevisiae* treated with 10 mg/l 4-nitrophenol for two hours. However this does not necessarily mean that 4-nitrophenol is a chemical that reduces DNA damage at 10 mg/l because DNA damage occurs during cell replication particularly meiosis (Johnson *et al* 2007). Therefore, reduced cell division would lead to decreased double strand DNA breaks and a reduced requirement for SWR1. Decreased cell division rates, as well as decreased respiration rates are both processes that have been attributed to decreased expression of ESR genes (**4.4.4**). Although these genes were not identified as significantly decreased in expression at 10 mg/l 4-nitrophenol (at a high stringency level) these results imply that they could have been affected. To investigate whether this was the case, data was further analysed by identifying genes two fold and significantly (p<0.01 rather than p<0.001) decreased in expression after one and two hours exposure to 10 mg/l 4-nitrophenol. The results identified that ESR genes were two fold and significantly (p<0.01) decreased in expression after two hours exposure to 10 mg/l 4-nitrophenol. This additional analysis appears to explain the probable cause of the observed results, and shows that the stringency of statistical analysis used did not allow identification of all genes of interest.

TMA108, YER158C, YDR034W-B, and ISR1 code for a proteins of unknown function. However, it is known that decreased expression of ISR1 protects *S. cerevisiae* from the anti-fungal drug staurosporine (Miyahara *et al* 1998). ISR1 may act to protect *S. cerevisiae* from 4-nitrophenol, in the same way it does with staurosporine.

5.4.4   Genes increased in expression after two hours exposure to 39 mg/l 4-nitrophenol

Thirty six genes (PDR3, ARN2, FIT3, FIT2, PDR15, YOR152C, SNQ2, YMR102C, RSB1, YLR346C, VHR1, PDR5, YGL157W, YKL071W, YOR1, GRE2, YML131W, ARN1, FRE1, AKR1, YLR460C, SIT1, YGR035C, CCC2, FIT1, YAP5, ENB1, YTP1, NFS1, BCP1, CPA1, PHM6, RTA1, ICT1, YLR126C, & TIS11) were significantly (p<0.001) and two fold increased in expression after two hours exposure to 39 mg/l 4-nitrophenol. Eight genes with unknown functions were increased in expression (YLR346C (**4.4.4**), YOR152C, YML131W (implicated in response to osmotic stress), YMR102C & YGR035C (implicated in drug resistance), YLR460C, PHM6 (implicated in phosphate

acquisition), BCP1 (implicated in heat shock), & YLR126C). Other genes increased in expression include CPA1, which modulates destabilisation of mRNA (Zhang & Dietrich 2005), and this implies that mRNA is being degraded by the cells. Two of the unknown genes increased in expression are implicated in drug resistance and this result is consistent with increased expression of drug resistance genes in Chapter 4.

 Eight of the genes (PDR3, ARN2, FIT3, YMR102C, RSB1, PDR15, YLR346C, & GRE2,) increased in expression after two hours exposure to 39 mg/l 4-nitrophenol were also increased in expression after one hours exposure to 39 mg/l 4-nitrophenol and were discussed in detail (**4.4.4**). Genes increased in expression after two hours exposure to 39 mg/l 4-nitrophenol formed three clusters, the first of which contained the most (28) genes. Comparing **Table 5.3.5** and *Figure 5.3.5* identifies that all the genes involved in drug resistance (PDR3, PDR15, SNQ2, PDR5, YOR1) can be found in the first cluster. PDR3 and PDR15 were increased in expression after one hour exposure to 39 mg/l 4-nitrophenol, briefly PDR3 is the transcription activator for the pleiotropic drug resistance response and PDR15 is a cell membrane located pump that actively removes xenobiotics (**4.4.4**). In addition, YOR1 and PDR5 are ATP-dependent efflux pumps that stimulate drug export from cells (Leppert *et al* 1990; Kolaczkowska *et al* 2008; Le Crom *et al* 2002). SNQ2 is involved in drug resistance (Mahe *et al* 1996) and primarily responds to 4-nitroquinoline-N-oxide mediating resistance to free radicals (oxidants) (Cui *et al* 1998). Oxidative stress caused by 4-nitrophenol exposure is further supported by the increased expression of YGL157W which codes for an NADPH dependent reductase (Liu & Moon 2009; Katz *et al* 2003) and YKL071W which codes for an NADPH dependent oxidoreductase (Heer *et al* 2009); both are involved in generating energy via the pentose phosphate pathway. Generation of energy via the pentose phosphate pathway is thought to occur during oxidative stress and provides the necessary reducing power for antioxidant enzymes (Shanmuganathan *et al* 2004). These results show that *S. cerevisiae* exposed to 39 mg/l 4-nitrophenol are probably experiencing oxidative stress as well as actively removing 4-nitrophenol from cells via the pleiotropic drug resistance pathway.

Also in cluster one were fourteen genes involved in iron homeostasis again suggesting that drug resistance and iron homeostasis are highly correlated and thus may be linked. These results clearly highlight the importance of iron in responding to 4-nitrophenol treatment. Other gene expression studies have identified that exposure to chemicals causes increased expression of genes involved in iron homeostasis. Yasokawa *et al* (2010), Emerson *et al* (2001), and Cusick *et al* (2009), Kim *et al* (2006) conducted toxicogenomic studies with zinc pyrithione, chloroquine, and saxitoxin, Textile Mix Effluents respectively. All authors identified that genes involved in iron homeostasis were increased in expression. Emerson *et al* (2001) extended

their investigation by conducting further studies in the presence of iron chelators and with *S. cerevisiae* strains with deleted iron uptake genes. Both treatments increased sensitivity to chloroquine and they concluded chloroquine was a competitive inhibitor of iron uptake. An additional study by Rojas *et al* (2008) found that increased sensitivity of *S. cerevisiae* to cryptolene occurred in strains unable to increase iron uptake. These investigations identify that iron plays an important role in resistance to chemicals.

To elucidate why so many genes involved in iron homeostasis were increased in expression in this study the function of each gene was investigated. As previously discussed (**4.4.4**) ARN2 and FIT3 uptake iron siderophores, (iron chelating compounds) involved in iron uptake. FRE1, ENB1, & SIT1 are genes that also fall into this category and all are increased in expression during iron deprivation (Emerson *et al* 2002; Prasad *et al* 2006; Rojas *et al* 2008; Aronova *et al* 2007; Lamb e*t al* 2001; Emerson *et al* 2001). Overall, it appears that *S. cerevisiae* cells are increasing their uptake of iron chelating compounds (siderophores) which scavenge Fe^{3+}. *S. cerevisiae* treated with 39 mg/l 4-nitrophenol for two hours have an increased requirement for iron as well as increasing production of siderophores that chelate iron (specifically Fe^{3+}). A logical explanation is that the siderophores are increased to scavenge Fe^{3+} and iron transporters increase in expression to provide *S. cerevisiae* with iron that can be used.

Iron has numerous functions in cells and increased uptake of iron could occur because of

- Iron limitation (unlikely as sufficient iron available in culture media)
- Reduced iron availability (4-nitrophenol has the potential to react with iron)
- Iron is being used maintain membrane stability, therefore extra iron is required to carry out other functions such as respiration
- Iron is required by enzymes (i.e. cytochromes P450) used in metabolizing 4-nitrophenol

A study by Longo *et al* (1999) found superoxide's caused the disassembly of 4Fe – 4S clusters, increasing concentrations of free iron. Increased free iron increases Fenton-type reactions (Fe^{3+} converts one Oxygen free radical and water into two hydroxyl free radicals) producing insoluble Fe^{2+}. This process reduces the availability of soluble iron (Fe^{2+}) and this could also explain the increased expression of ARN1, FIT1 & YTP1, all of which are involved in the uptake of soluble iron Fe^{2+} (van Bakel *et al* 2005; Protchenko *et al* 2001; De Freitas *et al* 2004). These genes are all regulated by the transcription factor TIS11 (Lamb *et al* 2001; Haurie *et al* 2003) which is also increased in expression. Additionally genes involved in iron homeostasis (AKR1, CCC2, & YLR126C), storage (YAP5), and the synthesis of mitochondrial and cytosolic iron-

sulphur proteins (NFS1), were increased in expression and iron is required for their formation (De Freitas *et al* 2004; Serrano *et al* 2004; Nett *et al* 2001).

Interestingly iron is contained in cytochromes, and oxidoreductases known to have roles in chemical transformation of 4-nitrophenol and other xenobiotics. A recent paper by Liu *et al* (2010) used mixtures of iron and EDTA (a chelating compound) and found they degrade 4-nitrophenol. They proposed that iron acts as an oxidant initiating the reaction, while the chelating agent increases the generation of a free radical and then competes for the free radical causing the 4-nitrophenol to be degraded. This mechanism is similar to the way in which oxidoreductase degrade chemicals to protect cells from damage (Ayala *et al* 2007). This highlights the possibility that iron coupled with iron chelants could be being used by *S. cerevisiae* to detoxify 4-nitrophenol. A similar result was reported by Zhou & Lei (2005) who used Fe^{3+} and UV light to degrade 4-nitrophenol, showing that iron is plays a role in 4-nitrophenol degradation. Additionally previous discussion (**5.4.2**) implicated the iron containing oxidoreductase cytochrome P450 as an enzyme that metabolises 4-nitrophenol and other xenobiotics (Hanioka *et al* 2007). Suggesting 4-nitrophenol exposure would result in an increased requirement for iron, so that cytochrome P450 concentrations could be increased. Either of these explanations could help explain;

1. Why iron limitation causes increased sensitivity to xenobiotics
2. Why the addition of iron to culture medium decreases sensitivity to xenobiotics
3. Why chemicals (a variety of organic pollutants) cause increases in the expression of genes involved in iron uptake and chelation.
4. Why the addition of iron chelators to culture medium increases sensitivity to xenobiotics
5. Why deleting genes involved in iron transport render cells more susceptible to xenobiotic effects.

As with after one hour exposure to 39 mg/l 4-nitrophenol the expression of genes involved in lipid and membrane synthesis were also increased in expression after two hours. Three of the genes increased after one hour exposure were also increased after two hours exposure (RSB1, GRE2, & ICT1) and will not be discussed in this chapter. The other two genes RTA1 and VHR1 have not previously been discussed, RTA1 is involved in lipid, fatty acid and sterol metabolism, and is thought to play a role in drug resistance (Vermitsky *et al* 2006), whereas VHR1 is a transcriptional regulator of the vitamin H (biotin) transporter gene (VHT1). Biotin is required by *S. cerevisiae* as a coenzyme in the metabolism of fatty acids (Weider *et al* 2005). These results show that *S. cerevisiae* cells are producing and metabolising fats, and suggests these processes play a role in protecting cells from the toxic effects of 4-nitrophenol. As mentioned

in Chapter 4 (**4.4.3**) it has previously been established that reduced iron availability results in increased membrane fluidity and in this study both changes in iron uptake and membrane composition were affected by 4-nitrophenol. Changes in membrane fluidity can inhibit electron transport resulting in increased generation of oxygen free radicals, increased Fenton reactions and consequently decreased respiration (resulting from decreased iron availability).

5.4.5   Genes decreased in expression after two hours exposure to 39 mg/l 4-nitrophenol

Thirty four genes (ICS2, ALD4, OM14, RTN2, OM45, CTT1, MSC1, GDB1, YMR196W, PHM7, COX58, NCE103, BTN2, YBR285W, YDR034W-B, FMP16, UIP4, DDR2, STF2, YNR034W-A, GPH1, TMA10, HXT5, GUT2, YER067W, MTH1, HXT6/HXT7, HXT4, ISF1, SPG4, RPL228, YKR075C, CYC1, HXT2) were significantly (p<0.001) and two fold decreased in expression after two hours exposure to 39 mg/l 4-nitrophenol. Thirty eight percent of the genes decreased after two hours exposure were genes of unknown function and were spread evenly across the clusters (*Figure 5.3.6*).

Interestingly the second largest group of genes decreased in expression after two hours exposure to 39 mg/l 4-nitrophenol were genes involved in energy generation and have a requirement for iron (**Table 5.3.6**). In total eight genes decreased in expression were involved in energy generation, seven of those (CYC1, ALD4, OM14, OM45, STF2, COX5B, PHM7, NCE103) genes code for constituents of the mitochondria (Burri *et al* 2006; Waizenegger *et al* 2003; Boubekeur *et al* 2001). At least two of these genes are linked; CYC1 codes for an ion binding electron carrier that transfers electrons to the product of COX5B in the electron transport chain (Burke *et al* 1997; Trueblood & Poyton 1987). Both require heme and are regulated by the availability of iron and oxygen (Lodi *et al* 1999; Hon *et al* 2003; Hickman & Winston 2007). Decreased expression of all of these genes could result from decreased iron and/or oxygen availability, and the result is consistent with increased expression of iron transporters, reductases and siderophores, which suggest limited or an increased requirement for Fe^{2+}. The other genes involved in energy generation included PHM7, which participates in phosphate metabolism and NCE103 a carbonic anhydrase that responds to low carbon conditions, and is increased in expression during the unfolded protein response (Ogawa *et al* 2000; Amoroso *et al* 2005; Kimata *et al* 2006).

Decreased expression of genes involved in energy generation could result from decreased growth rates, or decreased availability of substrates. The suggestion that substrates for energy generation are limited is supported by the decreased expression of four genes involved in glucose transport. HXT2, HXT4, HXT6/7 and HXT5 code for high affinity glucose transporters that are increased in expression when glucose is limited (Kashara *et al* 2003; Aronova *et al*

2007; Lai *et al* 2007; Verwaal *et al* 2002). The expression of these genes is under the control of glucose sensors including MTH1 whose expression is repressed by high glucose concentrations (lafuente *et al* 2000). Alongside these results, one of the genes (RTN2) has been linked to nitrogen limitation (Mendes-Ferreira *et al* 2007) and this suggests that nitrogen is not limited in treated cultures, but may be limited in the un-inhibited control cultures. In line with these results is the decreased expression of CTT1, which may be increased in expression during carbon and nitrogen limitation in un-inhibited control culture (Demasi *et al* 2006; Parrou *et al* 1999). As well as BTN2, whose increased expression is seen in cultures undergoing ethanol stress. These results are consistent with those in Chapter 2 (*Figure 2.3.6*) which showed that cultures treated with 39 mg/l 4-nitrophenol for two hours had significantly higher glucose concentrations than un-inhibited control cultures. Differences in glucose concentrations resulting from reduced growth rates in treated *S. cerevisiae* cultures (*Figure 2.3.2*) are a likely explanation for these results. Because decreased glucose concentrations occur alongside nitrogen limitation and lead to the increased concentration of ethanol in the culture medium. Decreased growth / division rates in *S. cerevisiae* cultures treated with 39 mg/l 4-nitrophenol may also account for the decreased expression of MSC1 which is thought to be involved in meiosis (Thompson & Stahl 1999).

However, previous reports have indicated that hexose transporter genes are also targets of the pleiotropic drug resistance transcription factors (i.e. PDR3). Deletion of hexose transporters results in resistance to cycloheximide, sulformethuron methyl, and 4-nitroquinoline-N-oxide, and over expression of hexose transporters results in increased sensitivity to these chemicals (Nourami *et al* 1997). This suggests hexose transporters play a role in transporting these chemicals into *S. cerevisiae*. It has also been proposed that Pleiotropic Drug Resistance (PDR) genes negatively regulate hexose transporters (Boles *et al* 1997). Likewise in a study by Santos *et al* (2009) 15 minutes exposure to quinine (1g/l) was found result in decreased expression of genes coding for glucose transporters in *S. cerevisiae* namely HXT2, HXT4, HXT6/7, MAL11, MAL31, and the glucose sensing MTH1. Interestingly HXT2, HXT4, HXT6/7 and MTH1 were also decreased in expression in this study. The quinine concentrations inside cells with deleted glucose transporter genes were found to be lower than those with functional glucose transporter genes and the decreased expression of glucose transporters was attributed to decreased accumulation of quinine. The concentrations of quinine tested by Santos *et al* (2009), exerted a very slight effect on cell growth, suggesting their results indicate glucose transporters facilitate drug uptake. Similarly Liu *et al* (2004) reported that glucose transporters facilitate the transport of Arsenic trioxide into cells, and deletion mutants exhibit reduced uptake. An interesting paper by Mangat *et al* (2009) reported that glycogen binds to glucose sensors inhibiting activation of nutrient stress pathways. This therefore suggests that

decreased glycogen metabolism (increased glycogen concentrations) may be inhibiting the transcription of starvation induced genes i.e. glucose and nitrogen transporters. Indicating a link between decreased glycogen metabolism and reduced glucose uptake. Genes involved osmolyte homeostasis were decreased in expression. GUT2 codes for a glycerol-3-phosphate shuttle whose decreased expression has been linked to decreased respiration (Overkamp *et al* 2002), GUT2 is also required for glycerol utilization (Lu et al 2005). Glycerol is an important compound for survival under osmotic stress conditions, (Hounsa *et al* 1998) however it is exported from cells during hypo-osmotic stress (Kayingo *et al* 2001) hypo-osmotic stress was implicated in **4.4.1**, and reduced glycerol availability may be linked to decreased expression of genes involved in its transport.   Other genes involved in energy generation that were decreased in expression include three genes involved in glycogen metabolism (GPH1, GDB1, & TMA10) (Favre *et al* 2008; Teste *et al* 2000; Dienhart 2002; Pir *et al* 2006; Caba 2005) showing that glycogen metabolism is lower in treated *S. cerevisiae*. Glycogen accumulation has been shown to play a role in stress tolerance in *S. cerevisiae* decreased glycogen metabolism may serve to protect cells from stress, by reducing osmotic stress (Zhang *et al* 2010). It is possible that osmotic stress (**5.4.4**) caused expression of genes involved in glucose uptake and glycogen and glycerol metabolism to decrease. Alterations in glycogen and glycerol concentrations as well as decreased glucose uptake aid cells in adapting to osmotic stress (Parrou *et al* 1997; Davenport *et al* 1999; Ando *et al* 2006; Greatrix *et al* 2006).

5.5 Summary & Conclusions

Genes increased in expression after two hours exposure to 10 mg/l 4-nitrophenol indicate that cells have respiration deficiencies, ergosterol deficiencies, as well as reduced mating frequency and cell growth/division after two hours exposure to 10 mg/l 4-nitrophenol. Genes decreased in expression identified that in general the production of proteins required for mitochondrial biogenesis and energy production were reduced by 4-nitrophenol exposure.

Twenty two percent of the genes increased in expression after two hours exposure to 39 mg/l 4-nitrophenol had unknown functions. However, two of them had previously been implicated in drug resistance. Consistent with this four of the genes with known functions were involved in removal of drugs and one (SNQ2) responds to oxidative stress. Increased expression of this gene suggested cells were under oxidative stress. This observation was further supported by increased expression of two genes involved in generating energy via the pentose phosphate pathway. The pentose phosphate pathway is used to generate energy during oxidative stress to provide additional reducing power for antioxidants. However, the most significant result was that 39% of the genes increased expression were involved in iron homeostasis. Increased requirement for iron occurs during oxidative stress, iron concentrations affect membrane

fluidity and genes involved in maintaining and altering the composition of membranes were increased in expression. Genes decreased in expression were involved in mitochondrial processes, some were members of the electron transport chain that had a requirement for, and thus were regulated by iron and oxygen. Genes involved in glycogen metabolism were also decreased in expression, evidence of a decreased requirement for energy, inability to generate energy using carbon storage compounds, or increased requirement for glycerol. Other genes decreased in expression included glucose transporters. Decreased expression of glucose transporters could result from decreased respiration or reduced cell division in treated *S. cerevisiae*. However, it could also be an artifact; untreated *S. cerevisiae* cultures had significantly lower glucose concentrations (**2.3.6**) than treated cultures. The expression of glucose transporters may have been increased in control cultures rather than decreased in treated cultures. Additionally some studies have reported that glucose transporters are under the control of PDR3, and glucose transporters are decreased in expression during osmotic stress and with changes in membrane fluidity.

Chapter 6

6. S. cerevisiae **genes significantly differentially expressed after three hours exposure to 10 & 39 mg/l 4-nitrophenol**

6.1 Introduction

6.1.1 Overview of data filtering methods used

In the previous chapters (Chapter 4 & 5), the effects of 10 & 39 mg/l 4-nitrophenol on gene expression in *S. cerevisiae* after one and two hour's exposure were identified. In this chapter to reduce the number of genes considered in detail data was further mined using un-paired T-tests with a more stringent p-value ($p<0.001$). Further filtering used T-tests were coupled with a two fold change in expression. Clustering was then used to elucidate relationships between identified genes. (**4.1.1 – 4.1.4**)

6.1.2 Overview of effects of one & two hours exposure to 10 mg/l 4-nitrophenol on gene expression

One hours exposure to 10 mg/l 4-nitrophenol caused the increased expression of genes, significantly ($p<0.01$) associated with the hyperosmotic stress response pathway. Reduced cell division was implied as well as, increased production of cell wall proteins, increased production of chaperone proteins, and an increased requirements for sulphur. After two hours exposure to 10 mg/l 4-nitrophenol genes increased in expression were once again involved in reducing cell division rates. However, some of the genes increased in expression were indicative of respiration deficiencies and ergosterol deficiencies, suggesting cell metabolism was affected by treatment.

Five genes were decreased in expression after one hours exposure to 10 mg/l 4-nitrophenol. Genes decreased in expression were involved in mating events, cell division and RNA splicing, showing that treatment with low concentrations of 4-nitrophenol caused reduced growth rates. The genes decreased in expression after two hours exposure to 10 mg/l 4-nitrophenol were involved in production of mitochondria and energy production.   These results confirmed that treated cells had respiration deficiencies.

6.1.3 Overview of effects of one & two hours exposure to 39 mg/l 4-nitrophenol on gene expression

After one hour of exposure to 39 mg/l 4-nitrophenol five genes (PDR15, PDR16, PDR3, YL346C, YPL088W) increased in expression were involved in drug resistance. After two hours, exposure to 39 mg/l 4-nitrophenol two additional drug export genes (YOR1 & PDR5) and one oxidative

stress responsive gene (SNQ2) were increased in expression. Additionally gene expression indicated increased use of the pentose phosphate pathway (a known oxidative stress response). Consistent with an osmotic stress response five genes involved in the maintenance and modification of cell membranes and walls were also increased in expression after one and two hours 4-nitrophenol exposure. Two genes involved in the uptake of iron were increased in expression after one hour suggesting an increased requirement for iron. After two hours the number of genes involved in iron homeostasis increased sevenfold, suggesting iron deficiency or an increased requirement for iron.

More genes were decreased in expression after one hour exposure to 39 mg/l 4-nitrophenol (27) and the majority (22) of these genes were environmental stress response genes involved in RNA processing, ribosome assembly and maturation, and nutrient uptake. After two hours exposure to 39 mg/l 4-nitrophenol 34 genes were decreased in expression, 38% were genes of unknown function, 35% of them were involved in energy generation, and 18% were involved in glucose sensing and transport. Decreased glucose transport was either the cause, or a consequence of decreased respiration rates. It was concluded that decreased respiration might have resulted from decreased iron availability, as iron was used to maintain membranes and was required by enzymes that detoxify 4-nitrophenol.

Aims of chapter 6

The overall aim of Chapter 6 was to assess the effects of 4-nitrophenol on gene expression in *S. cerevisiae* after three hours exposure

Aim 1 Increase the stringency of filtering methods to reduce the number of genes considered in detail

Objectives to meet Aim 1
1. Use unpaired T-tests to identify genes significantly ($p<0.001$) differentially expressed after three hours exposure to 10 and 39 mg/l 4-nitrophenol
2. Filter significantly ($p<0.001$) differentially expressed genes to identify genes with two fold or more changes in expression after three hours exposure to 10 & 39 mg/l 4-nitrophenol

Aim 2 Identify genes with correlated expression levels that may have related functions

Objectives to meet Aim 2

1. Use Hierarchical clustering to identify differentially expressed genes with correlated expression levels

2. Use literature searches to gather information on factors leading to differential expression of identified correlated differentially expressed genes and identify cellular processes affected by 4-nitrophenol treatment

6.2 Methods

Previously genes differentially expressed after one (Chapter 4) and two (Chapter 5) hours treatment with 10 & 39 mg/l 4-nitrophenol were filtered from the 1,357 genes that were affected by 4-nitrophenol treatment over the time course of exposure (Chapter 3). To obtain a small subset of genes that could be analysed in detail statistical tests with increased stringency were used alongside fold change in expression (where appropriate). The resulting gene lists indicated exposure to 10 & 39 mg/l 4-nitrophenol caused cell membrane and wall damage leading to osmotic stress. At the higher 4-nitrophenol concentration, (39 mg/l) genes involved in drug resistance, iron acquisition, as well as cell wall and membrane repair were increased in expression. In previous chapters unpaired t-tests (p<0.001) that assumed equal variance were used to identify differentially expressed genes. This test was chosen because the Box Whisker plot in *Figure 3.3.4* showed that the samples from one and two hour's exposure had few outliers and the number of microarrays for which data was available was equal for all treatment conditions. However in this chapter Welch t-test was used to identify differentially expressed genes at 39 mg/l 4-nitrophenol. This method was chosen because it accounts for the removal of data from two microarrays (one control and one 10 mg/l 4-nitrophenol after three hours exposure to 4-nitrophenol) as it compensates for unequal variances and group sizes. All other methods used to identify specific processes affected by three hours exposure to 4-nitrophenol at 10 and 39 mg/l remained the same.

6.3 Results

The primary purpose of this study is to identify a small sample of differentially expressed genes that could be used to identify potentially toxic concentrations of 4-nitrophenol in water samples. To meet this aim and increase confidence in the results obtained gene expression data was filtered with increasing stringency to identify a subset of genes that can be analysed in detail.

6.3.1 Identifying genes with significant (p<0.001) gene expression changes

The number of genes identified as significantly (p<0.01) increased in expression after three hours exposure to 4-nitrophenol (**Table 4.1 Appendix III**) is too great to examine in detail. To reduce the number of genes considered un-paired t-tests were used to identify genes with even greater significance (p<0.001). The number of genes significantly (p<0.001) increased and decreased in expression after three hours treatment with 10 and 39 mg/l 4-nitrophenol are shown in **Table 6.3.1.**

Table 6.3.1 Number of genes significantly (p<0.001) differentially expressed after three hours exposure to 10 and 39 mg/l 4-nitrophenol

4-nitrophenol concentration mg/l	Number of genes increased	Number of genes decreased
10	10	9
39	40	33

Table 6.3.1 shows that increasing the stringency of statistical analysis reduces the number of genes differentially expressed at 10 mg/l 4-nitrophenol to nineteen genes that can be analysed in detail. However, the number of genes identified as differentially expressed at 39 mg/l 4-nitrophenol using statistical analysis alone is too great to analyse in detail. To further reduce the number of genes considered in subsequent analysis fold change filters were used to identify genes with a two fold or more change in expression.

6.3.2 Genes with significant (p<0.001) and two fold changes in expression

The number of genes significantly (p<0.001) and two fold increased and decreased in expression at 10 & 39 mg/l 4-nitrophenol are shown in **Table 6.3.2**. Only two genes were significantly (p<0.001) and two fold decreased in expression in response to three hours treatment with 10mg/l 4-nitrophenol, and eight genes were increased in expression at 10mg/l 4-nitrophenol. In previous chapters, (Chapter 4 & Chapter 5) different analysis methods were used to identify genes differentially expressed at 10 & 39 mg/l 4-nitrophenol. To ensure the data from each time-point is comparable genes significantly (p<0.001) differentially expressed at 10 mg/l 4-nitrophenol were clustered using hierarchical clustering and analysed in detail, whereas genes significantly (p<0.001) and two fold differentially expressed at 39 mg/l 4-nitrophenol were clustered using hierarchical clustering and analysed in detail.

Table 6.3.2 Number of genes significantly (p<0.001) and two fold differentially expressed after three hours exposure to 10 and 39 mg/l 4-nitrophenol

4-nitrophenol concentration mg/l	Number of genes significantly (p<0.001) and two fold increased	Number of genes significantly (p<0.001) and two fold decreased
10	8	2
39	35	22

Table 6.3.2 shows that applying a two fold change filter and increasing the stringency of statistical tests reduces the number of genes differentially expressed at 10 & 39mg/l 4-nitrophenol.

6.3.3   Genes significantly (p<0.001) increased in expression after three hours exposure to 10 mg/l 4-nitrophenol

Ten genes were significantly increased in expression after three hours exposure to 10 mg/l 4-nitrophenol. To identify similarities in differentially expressed genes they were grouped by function (**Table 6.3.3**)

Table 6.3.3 The functions of genes increased in expression after three hours exposure to 10 mg/l 4-nitrophenol

Gene function	Gene name
RNA processing	DUS3, ENP1, NOP13, BUD23, REX4, SER3, DAS2
Energy generation	MIS1, PPT1,
Response to Oxidative stress	YHB1

The results in **Table 6.3.3** show that genes increased in expression after three hours exposure to 10 mg/l fell into three categories, 70% of genes were involved in RNA processing, 20% energy generation and the remaining gene (YHB1) responds to oxidative stress. Hierarchical clustering was used to identify genes with similar expression profiles.
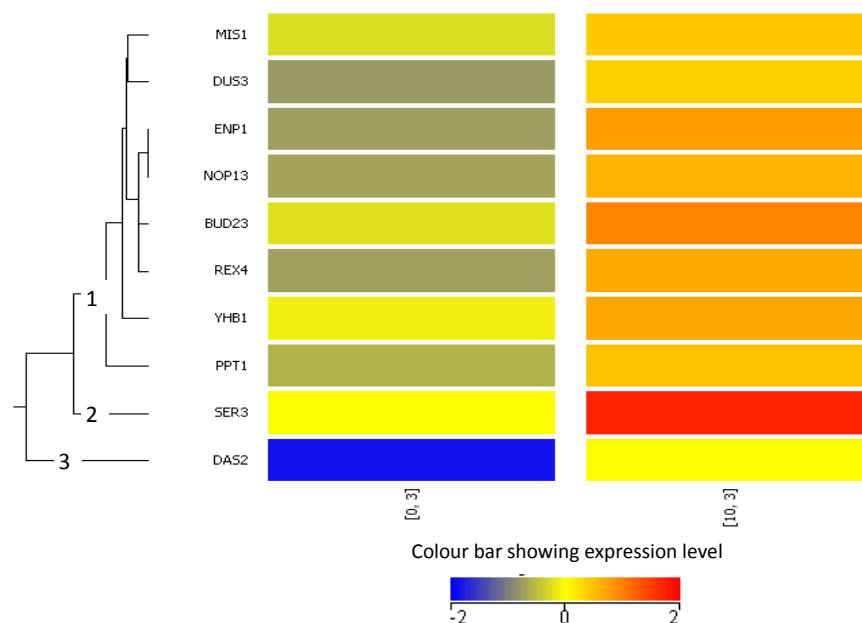
Colour bar showing expression level

Figure **6.3.3** Dendogram produced by hierarchical cluster analysis (r^2<0.95) of genes significantly (p<0.001) increased in expression after three hours exposure to 10 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after three hours exposure to 0 mg/l ([0, 3]) and the leaves on the right show 10 mg/l ([10, 3]) 4-nitrophenol.

The results in *Figure 6.3.3* show that ENP1, NOP13, BUD23 and REX4 have the most similar expression profiles and Table 6.3.3 shows that all these genes are involved in RNA processing. In total three clusters are formed, DAS2 and SER3 have more pronounced changes in expression and cluster separately from other genes.

6.3.4   Genes significantly (p<0.001) decreased in expression after three hours exposure to 10 mg/l 4-nitrophenol

Nine genes were significantly decreased in expression after three hours exposure to 10 mg/l 4-nitrophenol. Genes were grouped by function to identify overall patterns in the data and identify cellular processes decreased by treatment with 4-nitrophenol.

Table 6.3.4 the functions of genes decreased in expression after three hours exposure to 10 mg/l 4-nitrophenol

Gene function	Gene name
Energy generation	OM14, NDE2, GRE3
Endocytosis	SNC1
DNA repair	YNL133W
Response to stress	UBC8, GTT3
Mitosis	NUM1
Unknown	YPR159C-A

The results in **Table 6.3.4** show that genes decreased in expression after three hours exposure to 10 mg/l 4-nitrophenol are involved in a range of cellular functions. The function categories containing the highest number of genes are energy generation and response to stress.

The results in *Figure 6.3.4* show that genes decreased in expression form three clusters, genes involved in energy generation can all be found in cluster one alongside genes involved in endocytosis, response to stress, and DNA repair. The gene of unknown function (YPR159C-A) clusters separately from other genes and NUM1 has an identical expression profile to GTT3 and they cluster together.
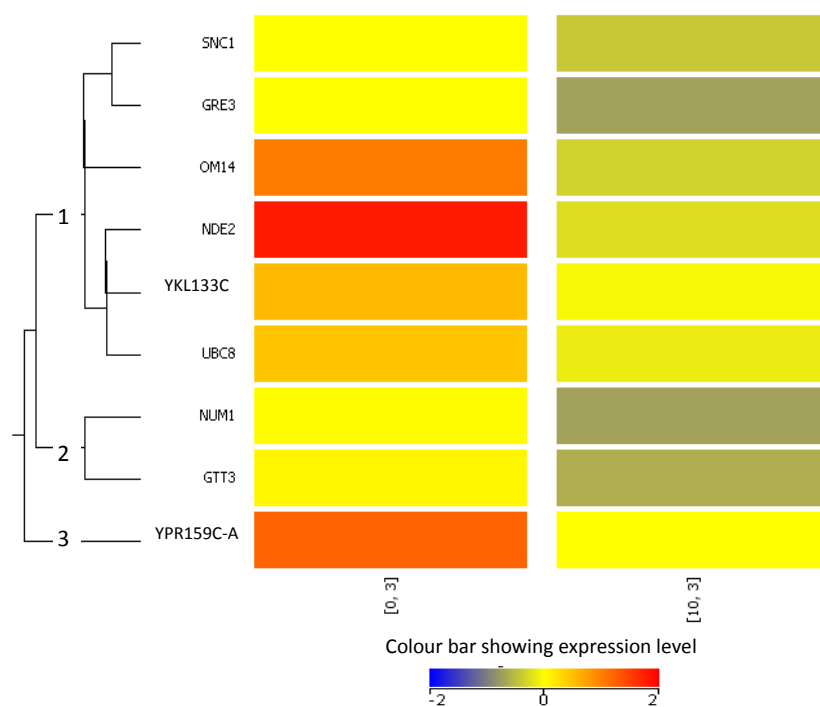
***Figure* 6.3.4** Dendogram produced by hierarchical cluster analysis ($r^2 < 0.95$) of genes significantly (p<0.001) decreased in expression after three hours exposure to 10 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after three hours exposure to 0 mg/l ([0, 3]) and the leaves on the right show 10 mg/l ([10, 3]) 4-nitrophenol.

6.3.5 Genes significantly (p<0.001) and two fold increased in expression after three hours exposure to 39 mg/l 4-nitrophenol

Thirty five genes were significantly and two fold increased in expression after three hours exposure to 39 mg/l 4-nitrophenol. Genes were classified by function (**Table 6.3.5**) to identify functional categories affected by three hours treatment with 39 mg/l 4-nitrophenol

Table 6.3.5 the functions of genes increased in expression after three hours exposure to 39 mg/l 4-nitrophenol

Gene function	Gene name
Energy generation	MAE1, PCS60, YHF1, MRPS18
Cell signalling/ sulphate metabolism/	RBD2/ SAM1/ YNL095C, LOT5, YGR035C
RNA processing	RPA14, ECM1, NMD3, PWP1, NOG1, YTM1, ENP2, NOP2, YNL096C, RPC40, DBP2, EMG1, RPA135, PWP1
Iron homeostasis / Drug resistance	FRE6, PGU1 ARN2, FIT2 FIT3 / PDR5, YLR346C
Phospholipid synthesis	SCS3, ICT1, PHO4, LAC1

The results in **Table 6.3.5** show that the functional category that contains the most genes increased in expression is RNA processing. Hierarchical clustering was used to identify genes with related functions that clustered together
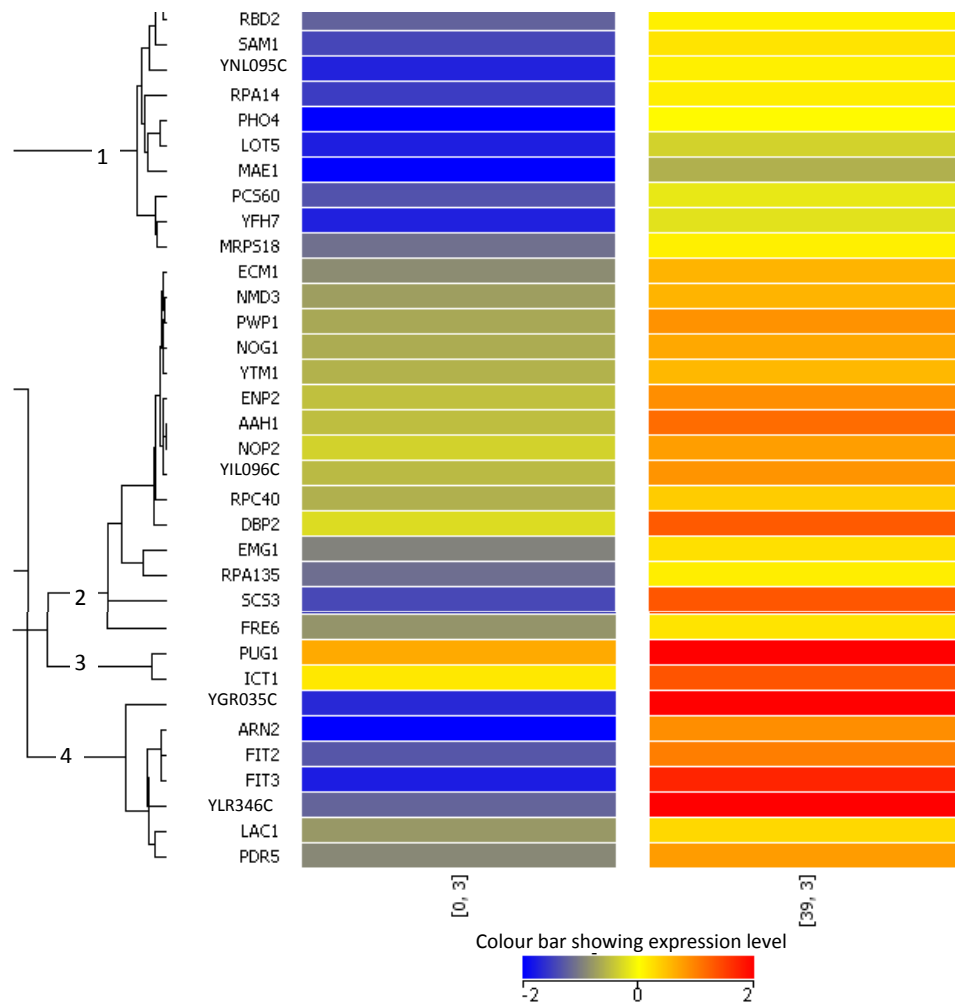
Figure **6.3.5** Dendogram produced by hierarchical cluster analysis ($r^2<0.95$) of genes that were significantly (p<0.001) and two fold increased in expression after three hours exposure to 39 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after three hours exposure to 0 mg/l ([0, 3]) and the leaves on the right show 39 mg/l ([39, 3]) 4-nitrophenol.

6.3.6 Genes significantly (p<0.001) and two fold decreased in expression after three hours exposure to 39 mg/l 4-nitrophenol

Twenty two genes were significantly and two fold decreased in expression after three hours exposure to 39 mg/l 4-nitrophenol. Genes were grouped by function (**Table 6.3.6**) to identify functional processes reduced by three hours treatment with 39 mg/l 4-nitrophenol.

Table 6.3.6 the functions of genes decreased in expression after three hours exposure to 39 mg/l 4-nitrophenol

Gene function	Gene name
Chaperone protein	HSP26, SSA4, HSP78, SYM1,
Unknown	YDR034W-B, PHM8, EOS1, YHC3, YLR326W, YPR157W, PET18, YPR159C-
Fatty acid/peptide synthesis	ELO1, RMI2, OPT2
Energy generation	GOR1, PNC1, GDB1, ALD4, ALD2, TFS1, DIP5

The results in **Table 6.3.6** show that most of the genes decreased in expression have unknown functions. However a large number of the genes are involved in energy generation, some code for chaperone proteins and some are involved in fatty acid synthesis Hierarchical clustering was used to identify genes with correlated expression levels.
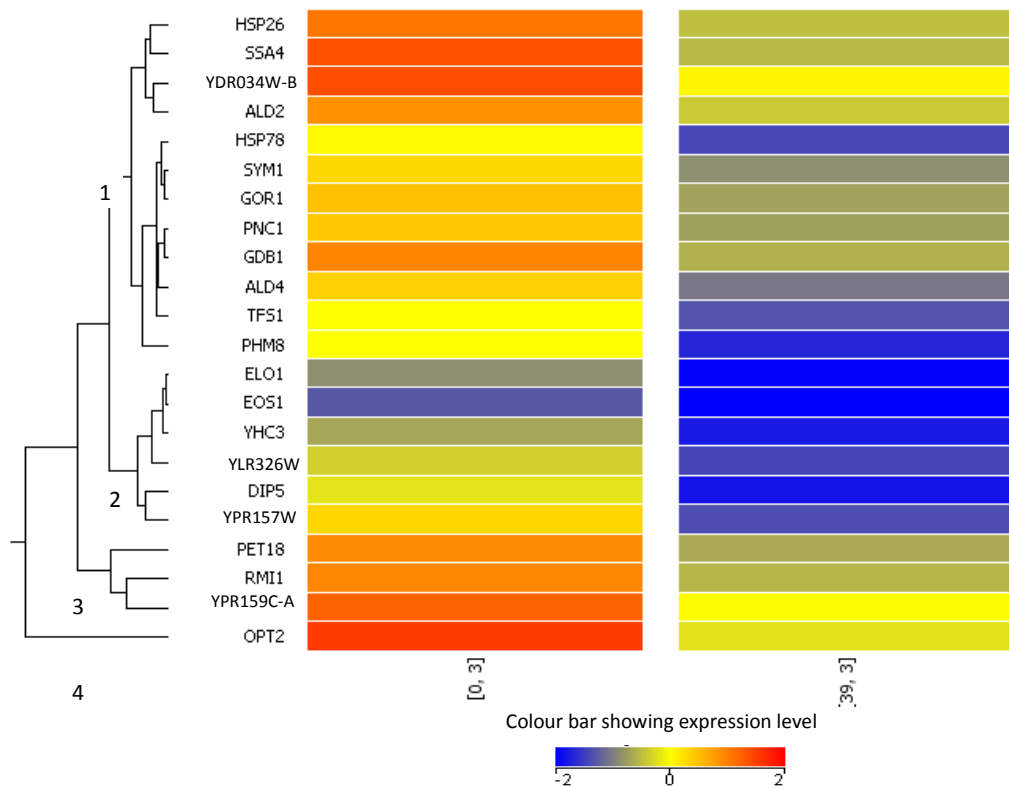
***Figure* 6.3.6** Dendogram produced by hierarchical cluster analysis (r^2<0.95) of genes significantly and two fold decreased in expression after three hours exposure to 39 mg/l 4-nitrophenol. Gene identifiers are shown to the right of nodes and the expression level of each gene after three hours exposure to 0 mg/l ([0, 3]) and the leaves on the right show 39 mg/l ([39, 3]) 4-nitrophenol.

6.4 Discussion

6.4.1   Significantly (p<0.001) differentially expressed genes

The overall aim was to reduce the number of genes identified as differentially expressed to a manageable level so that specific chemical effects could be identified. Increasingly stringent statistical analysis was used to increase confidence in the resulting data and ensure the data analysed was relevant.   The 4-nitrophenol concentrations chosen (10 & 39 mg/l) caused 10 and 50% reduction in cell division respectively. The lower concentration (10 mg/l) has less of an effect on cells as fewer genes show highly significant changes in expression. However the nineteen genes that do have significant (p<0.001) differential expression are important indicators of the effects of low concentrations of 4-nitrophenol on cells, consequently they are examined in more detail in sections **6.4.2** and **6.4.3**. At 39 mg/l more genes (73) are significantly (p<0.001) differentially expressed identifying that the higher concentration has more of an effect on *S. cerevisiae*. At 39 mg/l, 4-nitrophenol a large number of genes were differentially expressed making analysis difficult. Genes affected by 39 mg/l 4-nitrophenol were further filtered using a two fold change in expression to reduce the number of genes analysed in detail and the results are discussed in sections **6.4.4** and **6.4.5.**

6.4.2   Genes significantly increased in expression after three hours exposure to 10 mg/l 4-nitrophenol

Ten genes were significantly increased in expression after three hours exposure to 10 mg/l 4-nitrophenol. The results in **Table 6.3.3** identified that most (70%) of the genes increased in expression were genes involved in processing RNA (Pir *et al* 2006; White *et al* 2008; Faber *et al* 2004; Eppens *et al* 2002; Thompson & Parker 2006; Sinclair *et al* 1993). The following genes; DUS3, ENP1, NOP13, BUD23, REX4, SER3 & DAS2 which are involved in RNA processing have previously been identified as repressed Environmental Stress Response (ESR) genes because they are usually decreased in expression in response to a range of environmental stressors (Gasch *et al* 2000). Interestingly a large number of genes in this category were significantly and two fold decreased in expression after one hours exposure to 39 mg/l 4-nitrophenol. After three hours exposure to 10 mg/l 4-nitrophenol seven ESR genes were increased in expression and two of these genes (ENP1 & DUS3) were decreased in expression after one hours exposure to 39 mg/l 4-nitrophenol. At first sight, the results from the present study appear incongruous to previously published data but overall it would appear that the expression of ESR genes in response to 4-nitrophenol exposure is time dependent. This work shows that cells had previously decreased the expression of ESR genes after one hour exposure to 4-nitrophenol (as they had at 39 mg/l) as identified in **5.4.3.** ESR genes were

subsequently increased in expression once the toxic effects of 4-nitrophenol (i.e. osmotic stress) were addressed by the increased production of cell wall and membrane constituents (as observed after one hour exposure to 10 mg/l 4-nitrophenol (**4.4.2**)). These observations suggest cells are recovering from the initial shock of adding 4-nitrophenol.

Other genes increased in expression included two genes involved in energy production (MIS1 & PPT1). Both MIS1 and PPT1 code for mitochondrial proteins, MIS1 plays a role in initiation of mitochondrial protein synthesis (Holmes *et al* 2000) and PPT1 regulates chaperone protein HSP90. Increased expression of PPT1 results in increased production of ubiquinone, a component of the electron transfer system (Zhang *et al* 2007; Wandinger *et al* 2006). These results indicate *S. cerevisiae* exposed to 10 mg/l 4-nitrophenol for three hours are altering mitochondrial proteins and attempting to synthesise components of the electron transport chain, showing that respiration is affected by treatment. The results are consistent with those seen after two hours exposure to 10 mg/l 4-nitrophenol, as this exposure time also identified genes involved in respiration were increased in expression.

One of the genes YHB1 clustered with genes involved in RNA processing but it has a wide range of functions including; metabolism of nitric oxide, response to oxidative stress, reduction of Fe^{3+}, response to glucose depletion, as well as regulating the expression of some components of the electron transport chain (Castello *et al* 2006; Lewinska *et al* 2006; Zhu *et al* 2006; Castello *et al* 2008).

6.4.3   Genes significantly decreased in expression after three hours exposure to 10 mg/l 4-nitrophenol

Nine genes were significantly (p<0.001) decreased in expression after three hours exposure to 10 mg/l 4-nitrophenol. Genes decreased in expression spanned a range of functions and most functions contained a single gene (**Table 6.3.4**), however three of the genes (OM14, NDE2 & GRE3) were involved in energy generation, and two (UBC8 & GTT3) are known to respond to stress.   OM14 codes for a constituent of the mitochondria was discussed in Chapter 5 because it was also decreased in expression after two hours exposure to 39 mg/l. NDE2 codes for a mitochondrial NADH dehydrogenase (Kleijn *et al* 2006) that feeds electrons from NADH into the electron transport chain (Davidson & Schiestl 2001). Deletion of NDE2 reduces the amount of hydrogen peroxide produced by the mitochondria (Davidson & Schiestl 2001), so its decreased expression may be related to oxidative stress, or reduced respiration. GRE3 codes for an aldose reductase that reduces xylose generate energy (Traff *et al* 2001; Traff *et al* 2002). Decreased expression of these genes in *S. cerevisiae* cultures treated with 4-nitrophenol show that energy generation pathways have been altered by treatment.

In addition to genes involved in energy generation genes that are known to respond to stress were also decreased in expression. UBC8 codes for an ubiquitin conjugating enzyme that increases stress tolerance in *S. cerevisiae* (Hiraishi *et al* 2006). Whereas GTT3 is a gene with an unknown function that has been implicated in glutathione metabolism (Samanta & Liang 2003). Glutathione is used by cells to counter oxidative stress therefore, decreased metabolism of glutathione is indicative of increased oxidative stress in treated cells.

Other genes decreased in expression were SNC1, which codes for a vesicle membrane receptor involved in the fusion of Golgi vesicle bodies that is proposed to have a role in endocytosis (Furuta *et al* 2007). This result shows that cells have reduced their intake of external molecules, and may have occurred to reduce uptake of 4-nitrophenol. YNL133W (FYV6) is a gene of unknown function that has been implicated in the repair of double strand breaks in DNA (Ewa *et al* 2008); decreased expression of this gene indicates cells are not repairing DNA double strand breaks. This result could indicate a reduced frequency of double strand breaks in treated cells, or a higher frequency of doubles strand breaks in untreated cells, or the inability to repair double strand breaks. As previously discussed DNA damage and double strand breaks occur during cell division (**5.4.3**) (Johnson *et al* 2007). Decreased mitosis (cell division) is implied by the decreased expression of NUM1, which codes for protein involved in separation of the bud neck during mitosis (Kormanec *et a*l 1991). One other gene decreased in expression YPR159C-A is a gene with no known function.

6.4.4 Genes significantly and two fold increased in expression after three hours exposure to 39 mg/l 4-nitrophenol

Thirty five genes were increased in expression after three hours exposure to 39 mg/l 4-nitrophenol, genes were grouped by function (**Table 6.3.5**) and this showed that most of the genes increased in expression were involved in RNA metabolism. These genes (RPA14, ECM1, NMD3, PWP1, NOG1, YTM1, ENP2, NOP2, YNL096C, RPC40, DBP2, EMG1, RPA135 & PWP1) were mainly clustered together in cluster two having similar expression values (*Figure 6.3.5*). This result is consistent with the increased expression of genes involved in RNA metabolism three hours after the addition of 10 mg/l 4-nitrophenol. These genes have previously been grouped together and named Environmental Stress Response genes (ESR). They are usually decreased in expression when cells are stressed and have higher expression levels in un-stressed cells. The expression of these genes is thought to be co-regulated and decreased expression occurs alongside changes in the expression of genes involved in carbohydrate metabolism and response to oxidative stress. However, in the previous study by Gasch *et al* (2000) (who named them ESR genes) samples were taken from *S. cerevisiae* exposed to chemical stressors for up to two hours. The effects of longer exposure to chemicals were

therefore not addressed, and these results suggest decreased expression of these genes is a temporary measure aimed at adapting to the presence of chemicals. After the application of cell, adaptation measures (i.e. alterations in cell membrane/wall structures, implementation of drug resistance pathways and metabolic pathways, as well as increased iron uptake and reduction). It would appear that after three hours exposure to 4-nitrophenol the cells have responded to the presence of the chemical via 'coping mechanisms' and are beginning to grow again. Other genes increased in expression after three hours exposure to 39 mg/l 4-nitrophenol included genes involved in iron homeostasis (FRE6, PGU1 ARN2, FIT2 & FIT3). Two of these genes (ARN2 & FIT3) were increased in expression after one hours exposure to 39 mg/l 4-nitrophenol and are involved in iron siderophore uptake (Emmerson $et\ al$ 2002; Rutherford $et\ al$ 2003), in addition FIT2 was increased in expression after two hours exposure to 39 mg/l 4-nitrophenol and is involved in the retention of iron sidrophores in the cell wall (Philpott $et\ al$ 2002). FRE6 codes for a ferric reductase that supplies Fe^{2+} to efflux pathways that transport Fe^{2+} from storage in the vacuole (Singh $et\ al$ 2007). Whereas PGU1 codes for a gene that plays a role in the uptake of protoporphyrin IX and heme efflux. Increased expression of PGU1 reduces the use of exogenous heme, and increases protoporphyrin uptake. Protoporphyrin is a carrier molecule for cations and forms heme containing enzymes such as cytochromes when combined with Fe^{2+} (Protchenko $et\ al$ 2008). These results are consistent with those discussed in **5.4.4**, and increased expression of genes coding for; Fe^{3+} scavenger proteins (iron siderophores ARN2 & FIT3), iron siderophore retention genes (FIT2), ferric reductase (FRE6), and Fe^{2+} utilising proteins (PGU1) suggest an increased requirement for iron. As previously discussed **5.4.4** chelated iron has previously been found to aid 4-nitrophenol metabolism and an increased requirement for iron may constitute a way for $S.\ cerevisiae$ to detoxify 4-nitrophenol.

Once again genes involved in iron homeostasis were closely correlated and clustered with genes involved in drug resistance (PDR5 & YLR346C). The gene YLR346C is of unknown function and has previously been implicated in drug resistance, this observation is supported by the results in this study as it was significantly ($p<0.001$) and two fold increased in expression after one, two and three hours exposure to 4-nitrophenol. It frequently clusters with genes involved in drug resistance and had a consistently high fold change in expression (~16 fold after one hour, 11 fold after two hours, 15 fold after three hours exposure to 39 mg/l 4-nitrophenol). To elucidate the function of YLR346C the genes it is known to interact with were identified and investigated. Briefly YLR346C binds to the protein product of IME4 and promotes its activation: IME4 mediates control of meiosis (Shah & Clancy 1992; Hongay $et\ al$ 2006). HTM1 signals glycoprotein degradation in the endoplasmic reticulum (Clerc $et\ al$ 2009). NSL1 is required for spindle formation during meiosis (Euskirchen 2002; Scharfenberger $et\ al$

2003). SYN8 is involved in vesicle fusion (Lewis & Pelham 2002; Valdez-Taubas & Pelham 2005), and SWI5 codes for a cell cycle regulated transcription factor that activates expression of lag phase specific genes (McBride *et al* 1999). All of the genes with which YLR346C interacts are involved in cell cycle processes (Shah & Clancy 1992; Hongay *et al* 2006; Clerc *et al* 2009; Euskirchen 2002; Scharfenberger *et al* 2003; Lewis & Pelham 2002; Valdez-Taubas & Pelham 2005; McBride *et al* 1999), and act to decrease cell replication via meiosis and mitosis in response to 4-nitrophenol treatment.

PDR5 is an ATP-dependent efflux pump that stimulates drug export from cells, elevated copy numbers of the PDR5 gene confer increased drug resistance and disruption of PDR5 results in drug hypersensitivity (Leppert *et al* 1990; Mache *et al* 1996; Moye-Rowley 2005; Leppert *et al* 1990). The protein produced from transcription of the PDR5 gene Pdr5p is localized to the plasma membrane and transcribed by Pdr3p (product of PDR3) induces transcription of genes involved pleiotropic drug resistance (Moye-Rowley 2005; Katzmann *et al* 1994; Devaux *et al* 2001). DNA mutation also increases the expression levels of PDR5 (Kolaczkowska *et al* 2008; Hallstrom & Moye-Rowley 2000; Devaux *et al* 2001) via PDR3. PDR5 was increased in expression three fold after two hours after exposure to 39 mg/l 4-nitrophenol, and two fold three hours after initial exposure. These results suggest that the export of 4-nitrophenol is occurring and this response could possibly relate to DNA damage. Additionally the location of YGR035C (*Figure 6.3.5*) seems to suggest it is involved in drug resistance and this observation is supported by the study by Anderson *et al* (2009) who found it's increased expression was associated with resistance to amphotericin and fluconazole. Additionally a study by Le Crom *et al* (2002) identified that YGR035C was increased in expression alongside pleiotropic drug resistance genes. As discussed previously (**4.4.4** & **5.4.4**) increased production of membrane constituents is associated with exposure to organic chemicals. Membrane damage is known to occur upon exposure of cells to organic compounds (Xu *et al* 2005) (a group to which 4-nitrophenol belongs). Consistent with this four genes involved in phospholipid synthesis (SCS3, ICT1, PHO4, LAC1) were increased in expression after three hours exposure to 4-nitrophenol. ICT1 has previously been linked to membrane damage caused by organic acid stress and increases the production of phospholipids (Gosch *et al* 2008). Consistent with the increased expression of ICT1 the expression of genes involved in the production of precursors for phospholipid synthesis (SCS3), genes involved in lipid production (LAC1) and phosphate accumulation (PHO4) were also increased in expression (Hosaka *et al* 1994; Guillas *et al* 2001; Ogawa *et al* 2000; Almaguer *et al* 2003) . Increased expression of all of these genes leads to the increased production of phospholipids and indicates remodeling of cell membranes. These results seem to indicate 39 mg/l 4-nitrophenol causes cell membrane damage. The results are also consistent with previous results (Chapters 4 & 5) that have shown genes

involved in response to osmotic stress and the remodeling of cell membranes and walls have been increased in expression.

Four genes involved in energy generation (MAE1, PCS60, YFH7, MRPS18) were also increased in expression after three hours exposure to 39 mg/l 4-nitrophenol. MAE1 codes for a mitochondrial malic enzyme involved in the synthesis of pyruvate (Boles *et al* 1998). MAE1 is needed for NAPH production during galactose metabolism via the pentose phosphate pathway (Velagapudi *et al* 2007). Increased expression of MAE1 shows that after three hours exposure to 4-nitrophenol cells are continuing to generate energy via the pentose phosphate pathway suggesting they are under oxidative stress. Not much is known about MRPS18 other than it codes for a mitochondrial protein subunit that plays a role in energy generation and is essential for viability (Graack & Wittmann-Liebold 1998). PCS60 codes for an AMP binding proteins that localises to the peripheral membrane and the matrix of the mitochondria. Its' expression is induced by oleic acid, and it is involved in formation of ATP (Blobel & Erdman 1996). Additionally YFH7 codes for an ATP dependent kinase involved in ATP binding and phosphotransfer (Gueguen-Chaignon *et al* 2008) implicating its involvement in energy generation. Additionally increased in expression, RBD2 codes for a rhomboid protein that has similarity to RBD1 which plays a role in re-modeling of the mitochondria (van der Bliek & Koehler 2003). Rhomboid proteins are processed by the rhomboid protease Pcp1 located in the inner mitochondrial membrane, and are subsequently thought to be involved in energy generation (van der Bliek & Koehler 2003; Pratje & Elke 2007; McQuibban *et al* 2003). These results indicate that cells have increased ATP production indicating respiration deficiencies have been addressed.

Little is known about the other genes increased in expression; SAM1 codes for gene involved in sulphur metabolism and sulphate assimilation whose increased expression occurs during methionine deficiency (Kim *et al* 2009; Thomas & Surdinkerjan 1991). This result suggests either methionine or sulphur deficiency, most likely is a sulphur deficiency resulting from increased respiration. The two other genes increased in expression (YNL095C, & LOT5) have unknown functions.

6.4.5   Genes significantly and two fold decreased in expression after three hours exposure to 39 mg/l 4-nitrophenol

In total twenty two genes were significantly and two fold decreased in expression after three hours exposure to 39 mg/l 4-nitrophenol. Most of the genes decreased in expression have unknown functions (YDR034W-B, YHC3, YLR326W, YPR157W, PET18, YPR159C-A), these unknown genes are spread across the clusters in *Figure 6.3.6* making it difficult to assign them specific functions. However it is noteworthy that YDR034W-B is located in cluster one

with chaperone proteins, and may share this function. Other chaperone protein encoding genes decreased in expression are the genes HSP26, SSA4, HSP78 & SYM1. HSP26, SSA4 and HSP78, are chaperone protein involved in protein folding and are usually increased in expression in stressed cells (Santos *et al* 2009; Saavedra *et al* 1996). This result is unexpected and to understand why these genes were decreased in expression factors affecting their expression were investigated. HSP26 and HSP78 are expressed during stationary phase (Donalies & Stahl 2001; Dickson & Brown 1998). Interestingly SYM1 is not required for general respiratory growth, but it is required for ethanol metabolism (Trott & Morano 2004). In addition studies by Riou *et al* (1997), and Brosnan *et al* (1999), have shown that during fermentation the expression of genes encoding chaperone proteins is decreased. All of these genes are altered in response to decreased glucose concentrations so these observations are consistent with the results in *Figure 2.3.6* which showed that glucose concentrations in untreated controls were significantly (p=0.001) lower than in cultures treated with 39 mg/l 4-nitrophenol for three hours. However a study by Gibson *et al* (2008) showed that glucose concentrations did not alter gene expression until glucose concentrations were below 20μg/ml, equal to the concentration of cultures exposed to 0mg/l 4-nitrophenol for three hours. Therefore, glucose concentrations in control cultures after three hours may not have altered the expression of glucose and ethanol responsive genes in un-treated controls. Another factor which alters the expression levels of chaperone protein encoding genes is increased translation rates resulting in the increased expression of genes involved in RNA processing (**6.4.4** ESR genes) and (Horton *et al* 2001). Increased accumulation of mRNA (thus increased RNA processing (ESR genes)) (**6.4.4**) and repression of chaperone proteins also occur simultaneously (Izawa 2010). These results suggest that when translation of ESR genes is initiated chaperone proteins are decreased in expression.

Other genes decreased expression were genes involved in energy generation. GOR1 codes for a glyoxylate reductase (an oxidoreductase) that is an NAD+ reductase (Rintala *et al* 2007). PNC1 codes for a nicotinamidase, decreased expression of PNC1 results in decreased intracellular NAD+ concentrations (Ghislain et al 2002). PNC1 is a NAD+ salvage pathway gene that converts nicotinamide back into NAD+ (Sandmeier *et al* 2001). ALD2 codes for a mitochondrial aldehyde dehydrogenase that converts acetaldehyde into acetyl CoA, reducing NAD+ to NADH (Wang *et al* 1998). ALD4 plays a role in the production of NADPH in the absence of NADH kinase reactions (Miyagi *et al* 2009). All of these genes are involved in NADH and NADPH production, which occurs during respiration. Lastly DIP5 codes for an amino acid permase whose expression is dependent on cell cycle, and is decreased in expression when cells use the pentose phosphate pathway (Eckert-Boulet *et al* 2005). The results suggest that

up to two hours after the addition of 4-nitrophenol cells use the pentose phosphate pathway. After three hours they recover and switch to more efficient energy and catabolic pathways.

GDB1 codes for a glycogen debranching enzyme, glycogen is a carbohydrate storage molecule that is metabolized during nutrient starvation (Perez-Torado *et al* 2002), decreased expression of GDB1 suggests that glycogen is not being metabolized by *S. cerevisiae* exposed to 39 mg/l 4-nitrophenol. Additionally OPT2 codes for an oligopeptide transporter that has been implicated in resistance to chemicals (Aouida *et al* 2009; Wiles *et al* 2006).   TFS1 codes for a carboxypeptidase inhibitor that inhibits IRA2, whose increased expression increases production of lipids (Gombault *et al* 2009; Kamisaka *et al* 2005) TFS1 is thus involved in carbohydrate storage and it's decreased expression results in increased production of lipids. ELO1 codes for a protein involved in the elongation of fatty acids (Pedroso *et al* 2009). Decreased expression of this gene implies that elongated fatty acids are not required by cells exposed to 39 mg/l 4-nitrophenol for three hours. Decreased expression of all of these genes increases the concentrations of glycogen and lipids in cells. These represent standard osmotic stress responses and may result from decreased respiration. RMI1 codes for a protein that stabilises the genome and plays a role in DNA replication stress checkpoint preventing DNA mutations from being replicated (Putnam *et al* 2009) it's decreased expression may be related to decreased replication rates and thus reduction in mitosis rates (**6.4.3**).

6.5 Summary & Conclusions

Genes increased in expression after three hours exposure to 10 & 39 mg/l 4-nitrophenol were ESR genes whose expression is generally observed to decrease under stress conditions. The expression of ESR genes is linked to iron and oxygen metabolism and many of them encode oxidoreductase genes involved in energy generation via electron transport. Increased expression of these genes indicates adapatations (cell membrane and wall maintenance, activation of drug resistance pathways, increased iron uptake) have addressed the respiration dieficiencies seen after one and two hours exposure to 4-nitrophenol. This was supported by alterations seen in the expression of genes involved in the pathways used for energy generation. At 39 mg/l genes involved in iron homeostasis were significantly increased in expression and once again clustered with drug resistance genes. This identified that after three hours exposure to 4-nitrophenol cells continued to have and increased requirement for iron / iron deificiencies, and were still exporting 4-nitrophenol from cells, and the two processes appear to be linked. Cells continued to have increased expression of genes involved in maintaining membranes after three hours exposure, indicating that membrane damage was a constant problem for 4-nitrophenol exposed cells. Genes decreased in expression after three hours exposure to 39 mg/l 4-nitrophenol included genes coding for chaperone proteins.

Decreased expression of chaperone proteins occurs when ESR genes are increased in expression as observed in this study, providing an explanation for these findings. Genes involved in metabolism of lipids and glycogen were also decreased in expression, accumulation of these substances is a process that has been associated with osmotic stress, and indicates cells are still facing osmotic stress.

Chapter 7

7. General Discussion

7.1   Sources of 4-nitrophenol

Increased production and use of xenobiotics has lead to increased concentrations in the natural environment. 4-nitrophenol is one such xenobiotic, it is a degradation product of widely used organophosphate pesticides which can result in high concentrations of 4-nitrophenol in soil. Additionally it is used as a tanning agent for the treatment of leather, as well as being a by-product of the production of paracetamol. Together, agricultural and industrial activities have resulted in significant levels of 4-nitrophenol in the environment.

7.2   Modes of 4-nitrophenol toxicity

4-nitrophenol is considered a model organic pollutant, it's toxicity to a wide range of organisms has been established (Bergauer *et al* 2004; Gemini *et al* 2005; Li *et al* 2006; TenBrook *et al* 2005; Mitra and Vaidyanathan 1982). Biochemical studies have found that it is a respiratory uncoupler that damages mitochondrial membranes, interfering with electron transport (Bakkali *et al* 2008; TenBrook *et al* 2005; Williamson *et al* 1995; Braunbeck *et al* 1988). Due to the generality of this mode of toxicity it is toxic to a wide variety of organisms. In mammals low concentrations (0.01mg/l) can affect endocrine and reproductive systems (Li *et al* 2006) but mobility inhibition assays conducted with vertebrates and invertebrates have proved insensitive with concentrations as high as 1mg/l causing no apparent effect (Gemini *et al* 2005). Likewise studies with microorganisms have identified that the EC_{50} concentration of 4-nitrophenol is relatively high (56 mg/l for the prokaryote *E. coli* and 32 mg/l for the eukaryote *S. cerevisiae*) (Brown 2005; Bergauer *et al* 2004). The results clearly demonstrate differences in the sensitivity of different organisms to 4-nitrophenol as measured using traditional methods, highlighting the need for a more effective way of assessing and monitoring toxicity (**1.7**). Traditional methods measure decreased mobility in vertebrates and decreased reproduction in microbes, and are insensitive to subtle effects caused by low concentrations of 4-nitrophenol.

7.3   The need for a high throughput sensitive test for toxic concentrations of 4-nitrophenol and other xenobiotics

The legal limit for 4-nitrophenol in natural waters (0.01mg/l) set by the US EPA has been found to be exceeded in natural waters and this concentration is equal to the concentration that has been found to affect the endocrine and reproductive systems of rats when repeated doses are given (TenBrook *et al* 2003; Karim & Gupta 2003; Li *et al* 2006). This has resulted in the need

for a highly sensitive test that can be used to detect potentially toxic concentrations of 4-nitrophenol and other xenobiotics in water. Transcriptomics is an extremely sensitive technique for monitoring the effects of chemicals on cells, because even low chemical concentrations have been found to cause changes in gene expression (Pollard and Earnshaw 2002; Heijne *et al* 2003; Nuwaysir *et al* 1999; Aardema & MacGregor 2001). It has been shown that toxic chemicals produce specific gene expression patterns that relate to their mechanisms of toxicity and chemicals have been classified into toxicological classes using gene expression patterns (Kier *et al* 2003; Amin *et al* 2002; Bouton *et al* 2001; Burczynski *et al* 2000; Meydan *et al* 2008; Hamadeh *et al* 2002; Thomas *et al* 2001; Suter *et al* 2004; Ellinger-Ziegelbauer *et al* 2008; Falciani *et al* 2008). It is thought that toxicogenomics will eventually allow chemicals acting by specific mechanisms to be recognised based on their toxicogenomic response allowing reassessment of the risks associated with pollutants (Thomas *et al* 2001; Suter *et al* 2004; Ellinger-Ziegelbauer *et al* 2008; Falciani *et al* 2008). It has been proposed that safe concentrations of chemicals can be determined by identifying the No Transcriptional Effect Level (NOTEL) for a chemical (Poynton *et al* 2008; Labenhofer *et al* 2004).

7.4   Using transcriptomics of *S. cerevisiae* to identify 4-nitrophenol toxicity

The sensitivity of *S. cerevisiae* to 4-nitrophenol is relatively low, concentrations as high as 39 mg/l 4-nitrophenol are required to inhibit growth by 50% in Standard Complete (SC) medium (**2.3.2**). However concentrations as high as 10 mg/l causes only 10% inhibition of growth measured by final cell density. The aims of this study were to establish the amount of 4-nitrophenol that effects growth of *S. cerevisiae* using chronic toxicity tests and then extend the study to identify the effects of high and low concentrations of 4-nitrophenol on gene expression. The final aim of the project was to identify and select a subset of genes that can be used as signature genes that indicate exposure to toxic concentrations of 4-nitrophenol or other chemicals acting by similar mechanisms i.e. respiratory uncouplers. The effects of 4-nitrophenol on gene expression were investigated at the EC_{10} and EC_{50} concentrations (Chapter 2), so that comparisons could be made with other toxicogenomic studies. Time series analysis was conducted to identify genes whose expression was significantly altered throughout a period of exposure to 4-nitrophenol, to allow the use of correlation analysis to identify genes with related functions and hypothesise the functions of differentially expressed genes with unknown functions.

7.5   Overview of toxic effects of 4-nitrophenol on gene expression in *S. cerevisiae*

A general analysis of the gene expression data was conducted (Chapter 3), pathway analysis showed that pathways significantly affected by 4-nitrophenol ($p<0.05$) were glucose sensing, osmotic stress response, pheromone production and cell morphology. These results indicated

that osmotic conditions, glucose transport, sexual reproduction and cell shape were altered by 4-nitrophenol treatment.   Most of the genes significantly (p<0.01) affected by 4-nitrophenol exposure were associated with the cytoplasm and most significantly the mitochondria. These results were consistent with previous results from biochemical and physiological studies that have shown 4-nitrophenol affects mitochondrial function. Additionally oxidative stress and respiratory affects were implicated by significant alterations in the number of genes with oxidoreductase activity that were differentially expressed (p<0.01). Another category of genes showing highly significant differential expression were genes involved in ribonucleoprotein complex biogenesis and assembly and formation of the cytosolic ribosome (p<0.01). Showing that translation of mRNA was probably affected by 4-nitrophenol treatment. The results obtained in this work are consistent with previous research that have shown 4-nitrophenol affects mitochondria and provided a useful overview of 4-nitrophenol effects, but did not provide details of which genes were increased and decreased in expression, or how gene expression changes over time. A more detailed analysis of differentially expressed genes was conducted in subsequent chapters to identify specific effects of 4-nitrophenol and specific genes affected by treatment with each concentration.

7.6   Osmotic stress caused by 4-nitrophenol in *S. cerevisiae*

Samples taken after one hour exposure to 10 mg/l 4-nitrophenol showed that genes associated with response to osmotic stress increased in expression were significant (p<0.01). The SLN1 gene which deactivates HOG1 (controls response to hyper-osmotic stress) was significantly increased in expression (p<0.001). Hypo-osmotic stress results in increased production of the water transporter AQY2 (p<0.01) which was two fold over-expressed at 10 & 39 mg/l. Genes which reduce glycerol production and increase glycerol export via FPS1 also showed greater than two fold increases in expression (p<0.01) (Hohmann 2008 p.115).   Hypo-osmotic stress leads to rapid cell swelling (not measured) and at 10 mg/l 4-nitrophenol the growth of *S. cerevisiae* was inhibited. In the first two hours growth is lower than 10% (Appendix II *Figure* 2.7). Increased cell size could account for this, as fewer larger cells would increase the optical density of the sample. Expansion of cells due to hypo-osmotic stress results in decreased cellular ion concentrations, increased import of ions, decreased transcription, and increased glycerol export (Hohmann 2008 p.115). A hypo-osmotic response was supported by the increased expression of genes involved in iron uptake and decreased expression of genes involved in glycogen and glycerol metabolism after two hours exposure to 39 mg/l 4-nitrophenol. In line with this Environmental Stress Response (ESR) genes were decreased in expression, and decreased expression of these genes has previously been linked to decreased iron concentrations and hypo-osmotic stress (Cano *et al* 2010; Khoshnevis *et al* 2010; Regupathy & Nair 2010; Goforth *et al* 2010; Hohmann 2008 p.115). Activation of the

hypo-osmotic pathway also causes cells to decrease division rates and genes associated with decreased cell division were differentially expressed at both 10 & 39 mg/l 4-nitrophenol. At 39 mg/l 4-nitrophenol *S. cerevisiae* had increased expression of pleiotropic drug resistance pumps, known to actively export xenobiotics from cells. Increased concentrations of 4-nitrophenol inside cells could occur as a result of damage to cell walls and membranes, genes involved in cell wall and membrane maintenance, and repair were significantly increased in expression at both 10 & 39 mg/l 4-nitrophenol ($p < 0.001$). Increased expression of genes involved in ergosterol synthesis was also identified and ergosterol is used to maintain cell membranes as well as serving as a substrate for drug export (Klobucnikova *et al* 2003; Beezer *et al* 1986). Ergosterol is synthesised during membrane damage and subsequent osmotic stress. Ergosterol synthesis is aided by cytochrome P450 that is also involved in metabolism and detoxification of 4-nitrophenol. Cytochrome P450 contains iron both of which have been found to have an affinity for 4-nitrophenol (Mordishahla *et al* 2007). This observation is interesting since two of the genes increased in expression coded for genes involved in chelating iron and previous studies have suggested chelated iron can be used to detoxify 4-nitrophenol (Ayala *et al* 2007). Iron acts an oxidant and the chelate acts as an electron receiver in 4-nitrophenol detoxification. 4-nitrophenol and its degradation products are lipophilic so more ergosterol is available to bind to 4-nitrophenol/metabolised 4-nitrophenol and ensure that the pollutant is safely exported from the cell by Pleiotropic Drug Resistance (PDR) genes (Kontoyiannis 2000). In addition in previous studies increased expression of PDR genes have been found to coincide with increased expression of genes involved in ergosterol synthesis in *S. cerevisiae* in response to steroid and chemical treatment (Banerjee *et al* 2008; Cernicka *et al* 2007). The results of this study and the other studies suggest a link between increased expression of PDR genes and ergosterol synthesis, as well as links between membrane damage, ergosterol synthesis, increased expression of PDR genes, and iron accumulation. Increased expression of all of these genes acts to decrease membrane fluidity, suggesting all of these responses might be aimed at countering osmotic stress or entry of 4-nitrophenol.

Additionally genes coding for glucose transporters decreased in expression. Interestingly it has been reported that the expression of glucose transporters are under the control of the PDR genes as glucose transporters have been found to facilitate the transport of xenobiotics across membranes (Boles *et al* 1997; Nourami *et al* 1997; Santos *et al* 2009). Osmotic stress also results in glycogen binding to glucose sensors inhibiting the transcription of glucose transporters (Mangat *et al* 2009)

7.7. Osmotic stress leads to oxidative stress which leads to reduced respiration in *S. cerevisiae*

Osmotic stress frequently occurs alongside oxidative stress resulting from the production of free radicals. SNQ2, which mediates response to free radicals, was increased in expression alongside genes involved in the pentose phosphate pathway after two hours exposure to 39 mg/l 4-nitrophenol indicating cells were under oxidative stress (Cui *et al* 1998; Shanmuganathan *et al* 2004). Oxidative stress results in increased Fenton reactions which convert soluble iron (Fe^{3+}) to less soluble iron (Fe^{2+}), decreasing the availability of iron to the cell (van Bakel *et al* 2005; Protchenko *et al* 2001; De Freitas *et al* 2004) . This may explain why after two hours exposure to 39 mg/l 4-nitrophenol fourteen genes involved in iron uptake and metabolism were increased in expression, indicative of an iron deficiency or an increased requirement for iron. Additionally Environmental Stress Response (ESR) genes decreased in expression after one hour of exposure to 39 mg/l 4-nitrophenol are transcribed by an initiation factor that has a requirement for iron and their decreased expression further indicates an iron deficiency / lack of soluble iron. The ESR genes are repressed by Iron Responsive Elements (IREs) and code for genes involved in iron and oxidative metabolism (Cano *et al* 2010; Khoshnevis *et al* 2010; Regupathy & Nair 2010; Goforth *et al* 2010; Hohmann 2008 p.115). This constitutes a logical feedback loop, where decreased iron availability inhibits the expression of genes that produce proteins that have a requirement for iron. Genes coded for by ESR genes have been reported to act to balance energy generation under stress conditions. A large number of the proteins encoded by genes that decreased in expression after two hours of exposure to 39 mg/l 4-nitrophenol have a requirement for iron and are involved in energy generation via oxidative phosphorylation. Additionally some genes involved in mitochondria biogenesis were also decreased in expression alongside genes involved in cell division. Proceeding cell division mitochondria are duplicated and daughter cells acquire their mitochondria from mother cells, decreased mitochondrial biogenesis may reflect decreased cell biogenesis or alternatively it may act to reduce cell division in response to oxidative stress (Lai *et al* 2008; Iwaki *et al* 2005; Miao *et al* 2008; Sakaki *et al* 2003). The biogenesis of mitochondria is under the control of the heme activated protein and thus iron concentrations affect mitochondrial biogenesis also (Ocampo *et al* 2010; Miao *et al* 2008). Previous biochemical studies have implicated 4-nitrophenol in damaging mitochondrial membranes and inhibiting electron transport, and it is a known un-coupler of oxidative phosphorylation (Williamson *et al* 1995; TenBrook *et al* 2005). The gene expression analysis reported here provides additional information on this process showing that decreased cellular iron concentrations resulting from hypo-osmotic and oxidative stress lead to reduced oxidative phosphorylation. Additionally the increased expression of RIM2, which rescues respiration

deficient cells by transporting pyrimidine nucleotides for mitochondrial DNA synthesis, shows that cells are attempting to address respiration deficiencies (Marobbio *et al* 2006). Additionally gene expression analysis has confirmed that 4-nitrophenol damages cell walls and membranes, and this is most likely the primary cause of toxicity in that it leads to both osmotic, and oxidative stress. RIM2 was increased in expression after two hours exposure to 10 mg/l 4-nitrophenol and it rescues respiration deficient cells by transporting pyrimidine nucleotides for mtDNA synthesis (Marobbio *et al* 2006)

7.8   *S. cerevisiae* adapt to the effects of 4-nitrophenol after three hours exposure

After three hours exposure to 10 mg/l 4-nitrophenol genes involved in the synthesis of mitochondrial proteins were increased in expression, alongside YHB1 which regulates expression of some components of the electron transport chain. However most of the genes increased in expression were Environmental Stress Response genes. The ESR genes function to rebalance energy supply and demand as well as coordinating cell growth rates (Lai *et al* 2008). Increased expression of these genes indicate changes occurring one and two hours after 4-nitrophenol addition such as; alterations in cell membrane and wall composition, increased ergosterol production, and increased import of pyrimidine nucleotides, increased iron uptake and 4-nitrophenol export have successfully addressed respiration deficiencies and oxidative phosphorylation has resumed. The ESR genes code for oxidoreductase proteins involved in energy generation via oxidative phosphorylation that have a requirement for iron and oxygen. Consistent with this observation expression of genes involved in the production of ATP increased alongside ATP requiring PDR pumps and genes involved in cell wall and membrane maintenance. Genes encoding chaperone proteins decreased in expression. Chaperones assist in protein folding it has been suggested that decreased expression of chaperone proteins occurs when ESR genes are increased in expression because RNA accumulation decreases the expression of chaperone proteins (Horton *et al* 2001; Izawa 2010). Other genes which decreased in expression in response to 4-nitrophenol included genes involved in the synthesis of NADPH for biosynthesis via the pentose phosphate pathway and lipid metabolism. These results indicated that *S. cerevisiae* treated with 39 mg/l 4-nitrophenol for three hours still have a requirement for lipids.

7.9   Comparing genes affected by 4-nitrophenol with those affected by other xenobiotics

4-nitrophenol is classed as a lipophilic organic acidic (pKa 7.2) compound. Results from the current study indicate that 4-nitrophenol damaged cell membranes, and caused hypo-osmotic stress; this resulted in oxidative stress, reduced iron availability and altered respiration. Genes increased in expression that were associated with toxicity included the drug resistance genes

(YPL088W, YLR346C, PDR3, PDR15, PDR16, PDR5 & YOR1) which have previously been shown to increase in expression in response to the oxidants: Diamide and Menadione (Gasch *et al* 2000). Most are general drug response genes that are increased in expression in response to a wide range of chemicals, and serve to protect cells from chemical insult.

Increased expression of drug resistance genes resulting from exposure to xenobiotics in the environment therefore has the potential to select for drug resistant microbial populations. The results also seem to suggest these genes are increased in expression more specifically in response to oxidants. Consistent with this all of the genes increased in expression are known to be involved in the Unfolded Protein Response (UPR) (Leber *et al* 2004). The UPR involves HAC1 (two fold and significantly (p<0.01) increased in expression at 39 mg/l confirmed by qRT-PCR). The UPR is activated by the accumulation of misfolded proteins in the Endoplasmic Reticulum (ER), and this often occurs alongside oxidative stress. Genes that respond to oxidative stress are differentially expressed alongside genes involved in the unfolded protein response because ER stress causes oxidative stress, and HAC1 is thought to be capable of transcribing oxidative stress response genes (Kimata *et al* 2006). Additionally increased uptake of iron occurred upon treatment with 4-nitrophenol, and previous studies have shown that iron plays a role in resistance to xenobiotics (Tuttle *et al* 2003; Rojas *et al* 2008; Kimura *et al* 2007; Fairn *et al* 2007; Almeida *et al* 2008), this is supported by observations that decreased iron availability results in increased sensitivity to xenobiotics (Emerson *et al* 2001; Rojas *et al* 2008). The results therefore suggest that increased iron availability leads to increased resistance to xenobiotics and this has many potential implications. A recent bioremediation study by Lu *et al* (2010) found that soil contaminated with petroleum could be treated with slurry kept at neutral pH and treated with chelated iron, in these conditions organic xenobiotics were reduced six fold. In the presence of iron chelators, or when iron uptake genes are deleted *S. cerevisiae* are more sensitive to chloroquine (Emerson *et al* 2001). Previous studies have identified that iron deficiency increases membrane fluidity and this is a probable cause of increased sensitivity (Prasad *et al* 2006). Changes in membrane fluidity inhibit electron transport resulting in increased generation of oxygen radicals increasing sensitivity of *S. cerevisiae* to Farnesol (Fairn *et al* 2007). These results show that iron plays important roles in drug resistance, and implies that decreasing iron availability to microbes has the potential to control microbial populations, i.e. it may have medical implications. Iron chelators have been used in the treatment of *Candida albicans* and *Candida vini*, pathogenic *C. albicans* is inhibited by stronger chelators than the non pathogenic *C. vini* (Holbein & Orduna 2010). Additionally this approach has been considered for the treatment of malaria (Emerson *et al* 2001). Genes involved in iron homeostasis are increased in expression in response to zinc pyrithione, chloroquine, saxitoxin and cryptolene (Yasokawa *et al* 2010; Emerson *et al* 2001;

Cusick *et al* 2009; Rojas *et al* 2008) also. These investigations identify that iron plays an important role in resistance to chemicals.

7.5 Summary & Conclusions

The gene expression results indicate that exposure to 4-nitrophenol leads to cell envelope damage which leads to osmotic stress which leads to oxidative stress. This was implied from increased expression of genes whose protein products are involved in cell wall and membrane re-structuring, activation of the hypo-osmotic response pathway, and increased expression of genes whose products are involved in responding to oxidative stress. Additionally the results show that the expression of iron homeostasis genes and drug resistance genes are correlated. The results of this and other toxicogenomics studies suggest that iron plays an important role in drug resistance.

Both low (10 mg/l) and high (39 mg/l) concentrations of 4-nitrophenol chosen for investigation in Chapter 2 significantly affected gene expression. The results of this study have highlighted an interesting avenue of further study i.e. whether increasing iron availability increases microbial resistance to xenobiotics. As well as establishing that gene expression can be successfully used to identify effects of toxicity and move the toxicogenomics agenda forward. A number of genes have been identified as differentially expressed; any combination of the genes discussed could theoretically be used to identify the presence of xenobiotics in damaging concentrations. It does not appear that specific changes in gene expression have occurred in response to 4-nitrophenol, this technique cannot be used to specifically detect 4-nitrophenol because at these concentrations it induces a wide range of responses. This study examined the effects of 4-nitrophenol at damaging concentrations on gene expression in *S. cerevisiae*, future work could examine the effects of lower non-inhibitory concentrations i.e. the legal limit of 0.01 mg/l 4-nitrophenol.

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Appendix I

Table 2.1 Constituents of YEPD media

Constituent	Amount (g/l)	Supplier
Bacteriological Peptone	20	Sigma-Aldrich
D-Glucose	20	Sigma-Aldrich
Yeast Extract	10	Sigma-Aldrich
De-ionised H_2O	Fill to 1 litre	N/A
Agar (only for plates)	15	Sigma-Aldrich

Table 2.2 constituents of SC media

Constituent	Amount (g/l)	Supplier
Yeast Nitrogen Base with amino acids	6.9	Formedium
D-Glucose	20	Sigma-Aldrich
Yeast Complete Supplement Mixture	0.79	Formedium
De-ionised H_2O	Fill to 1 litre	N/A
Agar (only for plates)	15	Sigma-Aldrich

Table 2.3 constituents of SD minimal media

Constituent	Amount (g/l)	Supplier
Yeast Nitrogen Base with amino acids	6.9	Formedium
D-Glucose	20	Sigma-Aldrich
K_2HPO_4	0.001	Sigma-Aldrich
De-ionised H_2O	Fill to 1 litre	N/A
Agar (only for plates)	15	Sigma-Aldrich

Table 2.4 Amount (mg/l) of 4-nitrophenol used in each experimental flask

Amount of test compound (mg/l)	Amount in g/l	Concentration of 4-nitrophenol required (µmoles/l)	Amount of 4-nitrophenol stock solution (µl) in 50ml	Amount of water (µl) needed, to make a total volume of 1ml
Untreated	0	0	0	1000
10	0.0100	71.94	45.4	954.6
20	0.0200	143.88	90.9	909.1
32	0.0320	230.22	145.5	854.5
39	0.039	280	177.8	822.2
64	0.064	460	290.9	709.1
112	0.112	805.76	509.1	490.9

Calculation = required concentration mg/l / concentration mg/ml of stock solution = amount of stock solution per l * 0.05 = amount of stock solution in 50ml

Table 2.5 Standard curve for glucose concentration

Tube	Water (µl)	Sample (µl)	Glucose standard (µl)
Reagent Blank	500	-------	--------
Standard # 1	245	-------	5
Standard # 2	240	-------	10
Standard # 3	235	-------	15
Standard # 4	230	-------	20
Test	125	125	-------

The percentage inhibition of *S. cerevisiae* exposed to increasing concentrations of 4-nitrophenol in each of the media (YEPD, SC, & SD) was calculated using the formula $(1-(OD_{600test} / OD600_{control}))*100$. The percentage inhibition at each concentration was then plotted in individual bar charts for each media.
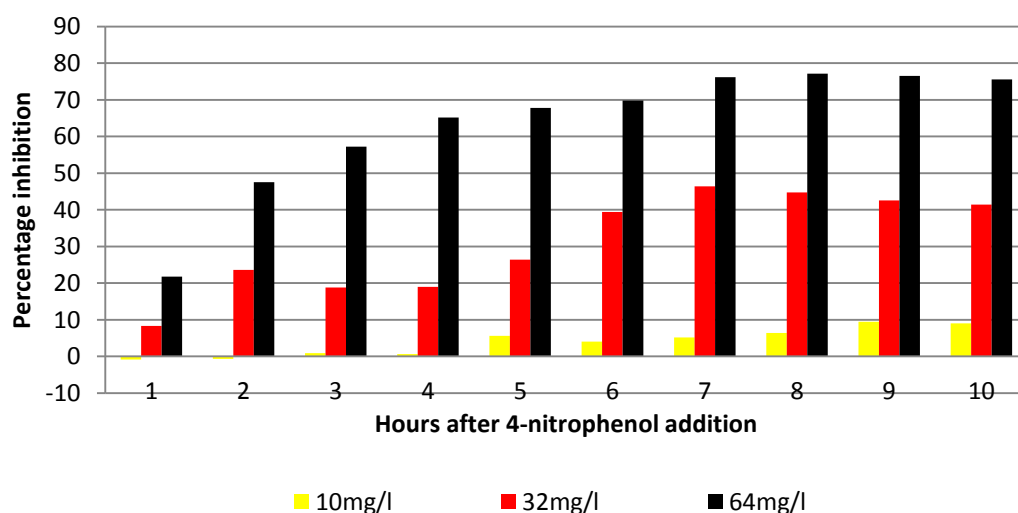

■ 10mg/l　■ 32mg/l　■ 64mg/l

Figure 2.6 percentage inhibition of increasing concentrations of 4-nitrophenol to *S. cerevisiae* in YEPD growth media

Figure 2.7 percentage inhibition of increasing concentrations of 4-nitrophenol to *S. cerevisiae* in SC growth media
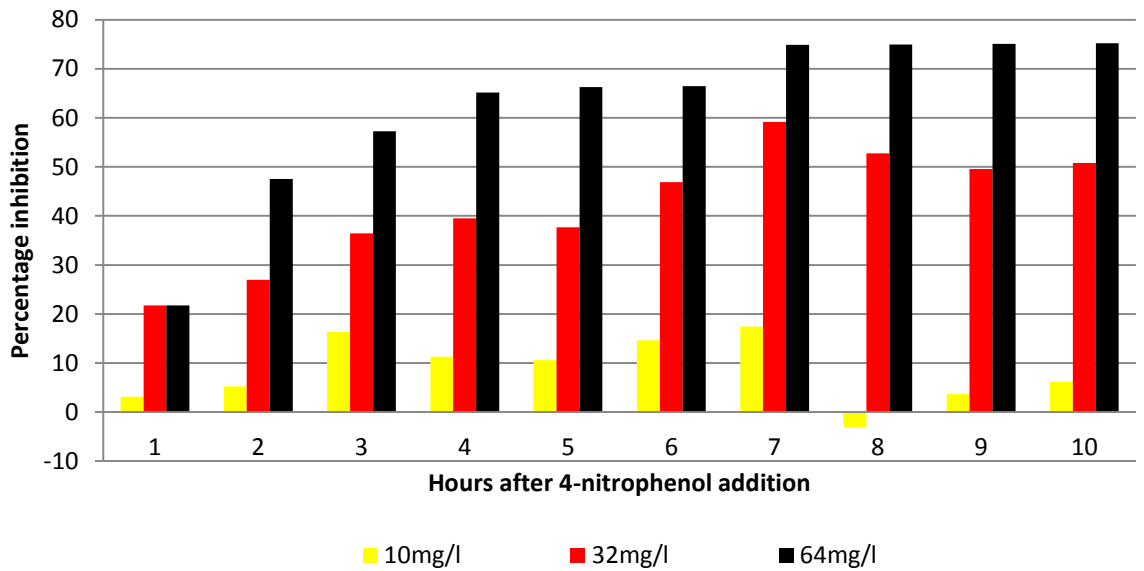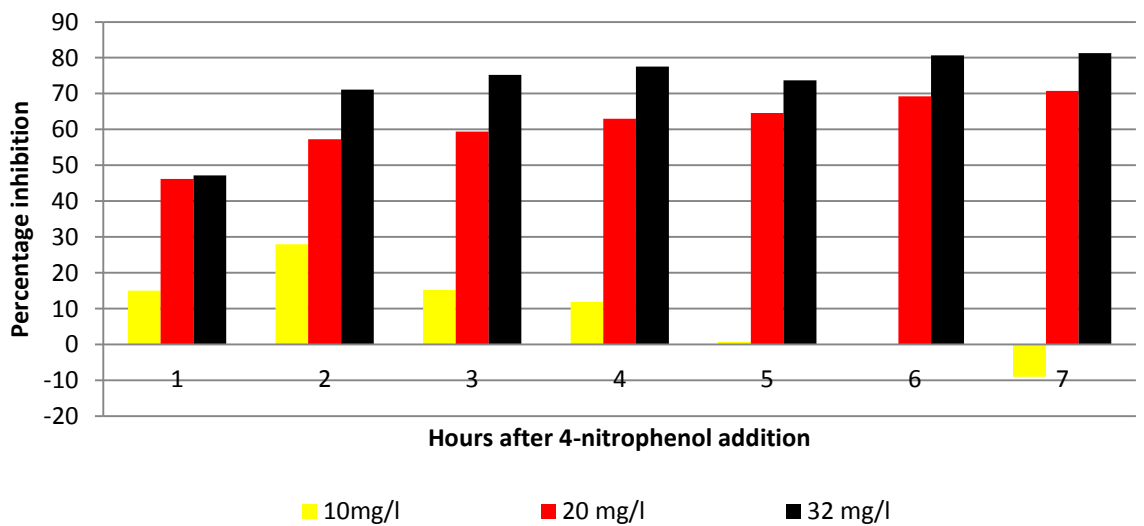

Figure 2.8 percentage inhibition of increasing concentrations of 4-nitrophenol to *S. cerevisiae* in SD growth media
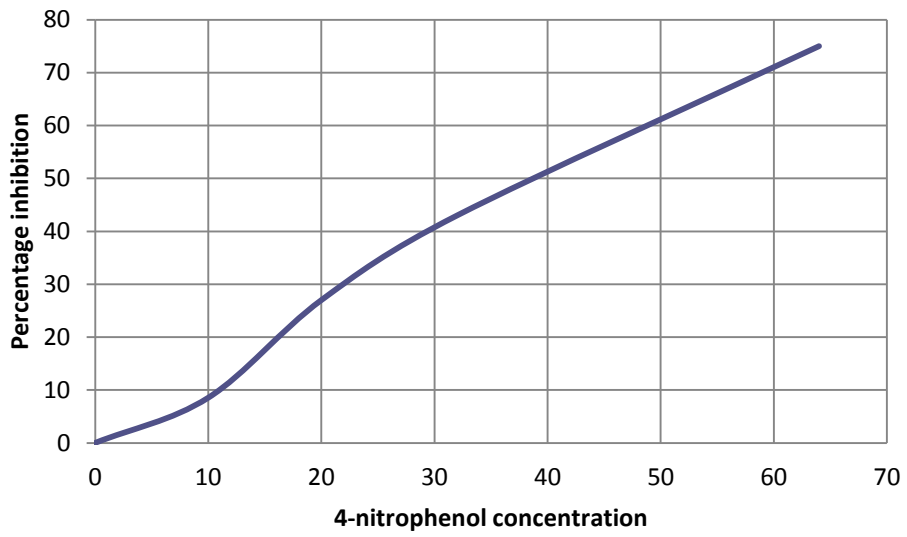
Figure 2.9 Percentage inhibition of *S. cerevisiae* exposed to increasing concentrations of 4-nitrophenol in SC media used to calculate the EC_{50}

Appendix II

Table 3.1 constituents of 12X MES Stock

Component	Amount
MES Free Acid Monohydrate	64.61g
MES sodium salt	193.3g
Molecular biology grade water	Adjust volume to 1000ml
pH	6.5-6.7
Filter through 0.2µm filter	

Table 3.2 constituents of 2X MES Hybridisation buffer (50ml)

Component	Amount
12X MES stock	8.3ml
5M NaCl	17.7ml
EDTA	4.0ml
10% Tween 20	0.1ml
Molecular biology grade water	19.9ml

Table 3.3 constituents of Stringent Buffer B

Component	Amount
12X MES stock	83.3ml
5M NaCl	5.2ml
10% Tween 20	1.0ml
Molecular Biology grade water	910.5ml
Filter 0.2µm filter	
Store at 2-8ºC in dark	

Table 3.4 constituents of 20X SSPE Buffer

Component	Amount
NaCl	175.3g
Na2H2PO4	27.6g
EDTA	9.4g
Molecular biology grade water	Volume up to 800mls
pH 7.4 with NaOH	
Autoclave 20 minutes	

Table 3.5 constituents of Non Stringent Buffer A

Component	Amount
20X SSPE	300ml
10% Tween 20	1.0ml
Molecular biology grade water	699ml
Filter through a 0.2µm filter	

Table 3.6 constituents of 2X stain buffer

Component	Amount
12X MES stock solution	41.7ml
5M NaCl	92.5ml
10% Tween 20	2.5ml
Molecular biology grade water	112.8ml
Filter through a 0.2µm filter	

10mg/ml goat IgG stock

50mg of IgG was re-suspended in 5ml of NaCl and stored at 4ºC

Anti-streptavidin biotinylated

Biotinylated Anti-streptavidin was re-suspended in PBS to a final concentration of 0.5mg/ml and stored at 4ºC

Making up the stain and antibody solutions

Table 3.7 constituents of SAPE solutions

Components	Volume (µl)	Final Concentration
2X MES Stain buffer	600	1X
50mg/ml acetylated BSA	48	2mg/ml
1mg/ml SAPE	12	10µg/ml
dH2O	540	
Total	1200	

The SAPE solution prepared above was enough for one microarray, it was divided into 2 aliquots to be used for stains 1 and 3 respectively.

Table 3.8 constituents of Antibody Solution

Components	Volume (µl)	Final Concentration
2X MES stain buffer	300	1X
50mg/ml acetylated BSA	24	2mg/ml
10mg/ml Normal Goat IgG	6	0.1mg/ml
0.5mg/ml biotinylated antibody	3.6	3mg/ml
dH2O	266.4	
Total	600	

Sample hybridisation with arrays

Arrays were left for 30 minutes to warm up to room temperature. The hybridisation cocktails were prepared for hybridisation by heating it 99ºC for 5 minutes. After which it was transferred to 45ºC for 5 minutes. The arrays were filled with 100µl of 1x hybridisation buffer and incubated at 45ºC for 10 minutes. The hybridisation cocktail was spun at full speed for 5 minutes to remove insoluble material. The hybridisation buffer was then removed from the probe arrays and the arrays were re-filled with 100µl of the hybridisation cocktail. The probe arrays were then placed in a rotisserie box and rotated at 60rpm and 45ºC for 16 hours.

The arrays were removed from the hybridisation oven and run through the fluidics station 3 at a time. To do this the hybridisation cocktail was removed from the arrays and placed in a

centrifuge tube, the arrays were then filled with Non-Stringent wash buffer. The fluidics station was firstly primed for all used modules on the setting Prime_450, where intake buffer reservoir contained Non-stringent wash buffer, and intake reservoir contained stringent wash buffer. In GCOS the experiment was set up and the type of microarray set as Yeast 2.0, and the washing and staining protocol was set to the default Micro 1v1. The fluidics protocol followed the steps outlined in table 3.11 and the pre-prepared SAPE and antibody solutions were placed in holders 1,3 & 2 respectively.

Table 3.9 Hybridisation programme

Post Hyb wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 25ºC
Post Hyb wash #2	8 cycles of 15 mixes/cycle with wash buffer B at 50ºC
Stain	Stain the probe array for 10 minutes in SAPE solution at 25ºC
Post stain wash	10 cycles of 4 mixes/cycle with wash buffer A at 30ºC
2nd Stain	Stain the probe array for 10 minutes in antibody solution at 25ºC
3rd Stain	Stain the probe array for 10 minutes in SAPE solution at 25ºC
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 35ºC. Holding temperature 25ºC

Mini_euk2v3 fluidics protocol

Appendix III

Table 4.1 The number of genes significantly, and both significantly and two fold increased and decreased in expression in response to 4-nitrophenol at each concentration and exposure time.

Time Hours	Number of genes significantly (p<0.01) affected by 10 mg/l 4-nitrophenol		Number of genes significantly (p<0.01) and two fold affected by 10 mg/l 4-nitrophenol	
	Increased	**Decreased**	**Increased**	**Decreased**
1	670	687	6	59
2	667	690	28	71
3	639	718	116	102
1, 2 & 3	489	533	0	19
Time Hours	**Number of genes significantly (p<0.01) affected by 39 mg/l 4-nitrophenol**		**Number of genes significantly (p<0.01) and two fold affected by 39 mg/l 4-nitrophenol**	
	Increased	**Decreased**	**Increased**	**Decreased**
1	698	659	91	88
2	719	638	82	96
3	641	716	161	140
1, 2 & 3	232	222	20	3