Universidade do Minho
Escola de Ciências

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Toxicogenomics - based tests for hazard assessment

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Toxicogenomics - based tests for hazard assessment

Dissertação de Mestrado
Genética Molecular

Trabalho efetuado sob a orientação da
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Professora Doutora Maria João Marques Ferreira Sousa Moreira

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II. Agradecimentos

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IV. Resumo

Como resultado do crescimento populacional, houve a necessidade de aumentar a produção alimentar e, para isso, houve um aumento na utilização de agroquímicos. Adicionalmente, com os avanços na indústria farmacêutica e química, foram introduzidas no mercado novas classes de medicamentos e químicos, muitas das quais não possuem dados toxicológicos ou possuem uma classificação toxicológica imprecisa. A produção destes compostos tem vindo a aumentar ao longo das últimas décadas e a tendência é que esse aumento se prolongue para os próximos anos. No entanto, estes produtos podem contaminar o meio aquático e nos alimentos, tornando-os perigosos para o Homem e para o ambiente.

De facto, existe pouca informação sobre o modo de ação de muitos contaminantes e os seus potenciais efeitos em organismos não alvo. Um excelente exemplo é o cimoxanil, um fungicida sistémico da família das amidas que é amplamente usado para controlar o míldio em vinhas. Foi já descrito que o cimoxanil inibe o crescimento, a produção de biomassa e a respiração das células de *S. cerevisiae*. No entanto, o modo específico de ação do cimoxanil permanece desconhecido.

O presente trabalho teve como objetivo analisar as respostas celulares produzidas pelo cimoxanil na levedura *S. cerevisiae* e, então, desenvolver um rastreio de alto débito para tentar compreender o mecanismo de ação deste composto. Para já, verificamos que para além de inibir o crescimento, o cimoxanil induz morte celular de uma forma dose-dependente e origina alterações funcionais e estruturais em células de levedura. Os nossos dados sugerem que as mitocôndrias serão um dos alvos do cimoxanil, embora outros alvos não possam ser excluídos e mais ensaios sejam necessários.

Palavras-chave: cimoxanil; modo de ação; *S. cerevisiae*; *screening*; toxicogenómica.

V. Abstract

As a result of population growth, there was a need to increase food production. To accomplish this goal, there was an increase in the use of agrochemicals. Also, with advances in the pharmaceutical and chemical industries, new classes of drugs have been introduced in the market, many of which do not have toxicological data or have an inaccurate classification. The production of these compounds has been increasing over the years, and the trend is that increases will continue for the following years. However, these products can endanger the aquatic environment and food, making them dangerous for humanity and the environment.

Indeed, there is little information on the mode of action of many contaminants and their potential off-target effects. One excellent example is cymoxanil, a systemic fungicide of the amide family widely used to control downy mildew in vineyards. It has been reported that cymoxanil inhibits the growth, biomass production, and respiration of *S. cerevisiae* cells. However, the specific mode of action of cymoxanil remains unknown.

The present work aimed to analyze the cellular responses produced by cymoxanil in *S. cerevisiae* and then develop a genome-wide screen to uncover the mechanism of action of this compound. We found that it leads to both growth inhibition and cell death of yeast cells in a dose-dependent manner and induces cell functional and structural alterations. Our data so far suggest that mitochondria are one of the targets of cymoxanil, although other targets cannot be excluded.

Keywords: cymoxanil; mechanism of action; *S. cerevisiae*; screening; toxicogenomics.

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BCECF, AM	2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester
catD	Cathepsin D
CFUs	Colony Formation Units
CHX	Cycloheximide
CMAC	7-amino-4-chloromethylcoumarin
CYM	Cymoxanil
DHE	Dihydroethidium
DMSO	Dimethyl Sulfoxide
E. coli	*Escherichia coli*
ECHA	European Chemicals Agency
ER	Endoplasmic Reticulum
EU	European Union
FAO	Food and Agriculture Organization
HOP	Homozygous Deletion Profiling
Isc1p	Inositol phosphosphingolipid Phospholipase C
LB	Luria Broth
LD	Lethal Dose
MoA	Mechanism of Action
NCR	National Research Council
OD	Optical Density
ORFs	Open Reading Frames
PCR	Polymerase chain reaction
Pep4p	Proteinase A

Pgk1p	Phosphoglycerate Kinase
P. infestans	*Phytophthora infestans*
PI	Propidium Iodide
Por1p	Porin
ROS	Reactive Oxygen Species
S. cerevisiae	*Saccharomyces cerevisiae*
SC-Gal	Synthetic Complete Galactose medium
SCpro-Glu	Synthetic Complete Glucose medium containing proline
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOC	Super Optimal broth with Catabolite repression
WHO	World Health Organization
WT	Wild Type
WWTPs	Wastewater Treatment Plants
YKO	Yeast Knockout
YNB	Yeast Nitrogen Base
YPD	Yeast Extract Peptone Dextrose

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1. Introduction

1.1. Human development and chemical substances used

According to the United Nations, the world population increased from 2.5 billion in 1950 to 7.8 billion in 2020. For the year 2090, the prediction for the world population is about 11 billion (between 7.9 and 14.6 billion, depending on the estimate). Therefore, the number of people on Earth in the last 70 years has tripled and will continue to increase (Figure 1).

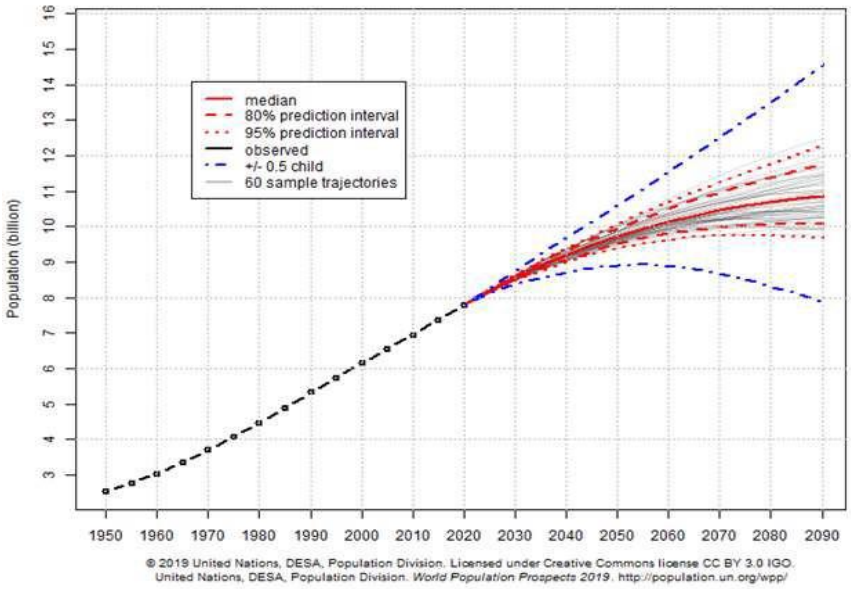

Figure 1 - Estimated population growth until the year 2090. Adapted from United Nations, 2020.

With the successive population increase, there was a demand to maintain and even increase the production of essential goods, particularly food. Therefore, there has been industrialization of agriculture to enhance food production and preservation. As a result, there has been an increase in the use of agrochemicals, particularly two large groups of compounds, chemical fertilizers and pesticides (Balbus *et al.,* 2013; Carvalho, 2006; Liu *et al.,* 2015).

Agrochemicals, namely pesticides, are mainly used in agriculture and urban green areas to protect plants from pests/diseases and human vector-borne diseases, such as malaria and dengue (Nicolopoulou-Stamati, Maipas, Kotampasi, Stamatis, & Hens, 2016). However, although these chemical substances have increased food production, their indiscriminate use has become a risk to the environment and humans (Mensah *et al.,* 2014), since many of these pesticides can appear in food, which may have consequences for the final consumer. Pesticides used in agriculture can also be leached by rains, causing groundwater contamination (Alewu & Nosiri, 2011; Liu *et al.,* 2015).

Figure 2 shows the global use of pesticides over the years (insecticides, herbicides, fungicides, plant growth regulators, and rodenticides), particularly in Europe and Eastern Asia, according to FAO, 2019. Although the use of pesticides tends to be constant in Europe, it has been increasing since 1990 in Asia and the rest of the world, and the trend is that this increase will continue in the following years (Carvalho, 2006; Liu *et al.,* 2015; Mensah *et al.,* 2014).

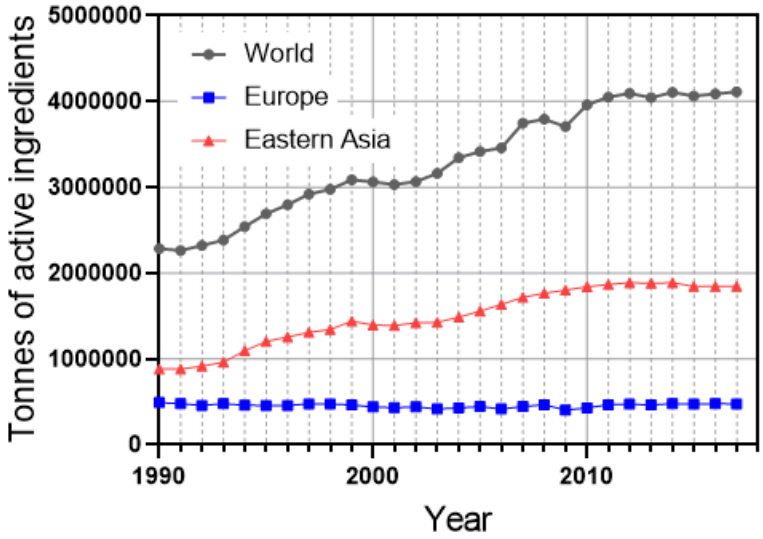

Figure 2 - Use of the major pesticides' groups (insecticides, herbicides, fungicides, plant growth regulators, and rodenticides) and relevant chemical families in Europe, Eastern Asia, and the world, from 1990 to 2017. Data report the quantities (in tonnes of active ingredients) of pesticides used in or sold to the agricultural sector for crops and seeds. Data from (FAO, 2019).

Apart from agrochemicals, pharmaceutical manufacturing facilities and sectors that use these products, for example, the livestock industry, can also be a significant source of contaminants in the environment (Phillips *et al.,* 2010). One example is anticancer drugs, which have been increasingly used over the years due to increased cancer incidence (Ferrando-Climent *et al.,* 2014). It has been shown that the effluents from hospitals possess a higher level of contamination. However, these drugs are released from domestic wastewater as well (Ferrando-Climent *et al.,* 2013; Ferrando-Climent *et al.,* 2014). The process of decontaminating them is not easy, being a challenge for the traditional water treatment methods (Zhang *et al.,*2013). It was also demonstrated that this type of substances has "cytotoxic, genotoxic, mutagenic, carcinogenic, endocrine disruptor and/or teratogenic effects in numerous organisms," which would be expected as they are meant to disrupt and prevent cellular proliferation (Ferrando-Climent *et al.,* 2014; Zhang *et al.,* 2013; Zounkova *et al.,*2010). In another study, effluents from two wastewater treatment plants (WWTPs) that receive discharges from pharmaceutical manufacturing facilities displayed concentrations 10 to 1000 times higher of pharmaceutical products than the effluents from the WWTPs that do not receive such discharges. It was also found that the water

released from these two WWTPs was discharged into streams, where the measured pharmaceutical products could be tracked 30 km downstream from the emission source (Phillips *et al.,* 2010).

Legislative bodies regulate the use of many of the chemical substances; for example, the European Union (EU) has the most "comprehensive and protective regulations", and it is up to the European Commission to oversee the "approval, restriction and cancellation of pesticides in the EU following Regulations 1107/2009 and 396/2005" (Donley, 2019; European Parliament Council of the European Union, 2009). The European Chemicals Agency (ECHA) works for the "safe use of chemicals, implementing EU chemicals legislation, benefiting human health and the environment" (Bjorn, 2007). Although there is control by governments, it has now been found that some substances already introduced into the market and considered as "safe" have a potential risk to the environment and human health because some of these products are recalcitrant in wastewater treatment procedures resulting in bioaccumulation, becoming a serious concern (Noutsopoulos *et al.,* 2019; Rosales *et al.,* 2018). The risks associated with chemical substances will depend on their type and concentration. Their adverse effects can occur at different levels, from interfering with DNA structure and function and leading to gene expression disruption (Figure 3) to inducing reproductive damage and inhibition of cell proliferation, among others (Phillips *et al.,* 2010; Ueda, 2009).

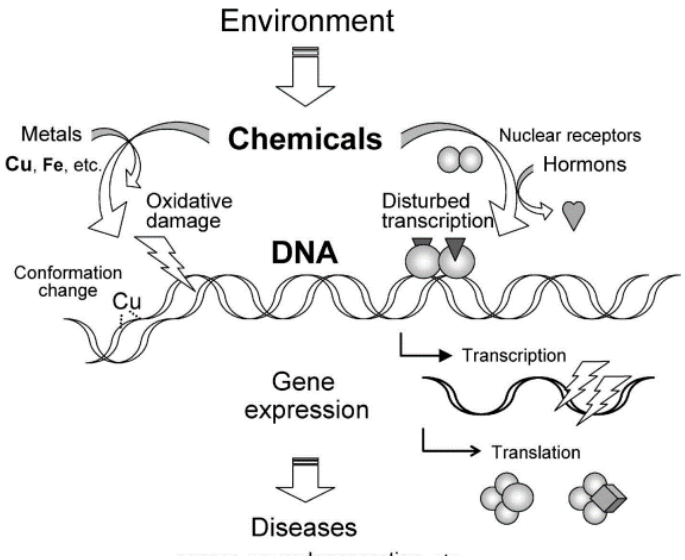

Figure 3 - Effects of chemicals on gene expression. Metals can catalyze oxidative and/or conformational damage and transcription disturbance. These actions on the structure and function of DNA disrupt the regulation of gene expression, leading to various diseases. Retrieved from (Ueda, 2009).

Because of the potential deleterious effects, it is necessary to carry out studies to identify the adverse effects that chemical substances can bring. Indeed, the mechanism of action (MoA) of many pesticides is not well described and sometimes there is no information on their toxicological profile (Hillebrand *et al.,* 2019). Therefore, it is not just chemicals newly introduced in the market that are getting a second look, but also those that are already in use but which have a poor or even no characterization of their MoA (Beaman *et al.,* 2008). A complete characterization of toxicological profiles and a better understanding of the MoAs is thus essential to improve the efficiency of hazard identification and risk assessment (Hamadeh *et al.,* 2002), to carry out preventative and mitigation actions. An excellent example of a chemical compound lacking information on the MoA is cymoxanil.

1.1.1. Cymoxanil

Cymoxanil (CYM) [2-cyano-*N*-[(ethylamino)carbonyl]-2-(methoxyimino)acetamide] (Figure 4) is the only fungicide that belongs to the chemical class of cyanohydroxyiminoacetamides (Genet & Vincent, 1999; Hillebrand *et al.,* 2019). Its sale started in 1997 and it is used to control fungus-like pathogens in a wide variety of cultures like downy mildew diseases induced by *Plasmopara viticola* in grapes and late blight caused by *Phytophthora infestans* in tomatoes and potatoes (Fidente *et al.,* 2005). This fungicide is applied as a foliar spray and designated as a foliar fungicide. The level of protection will depend on climate conditions after application and crop growth (European Commission, 2020).

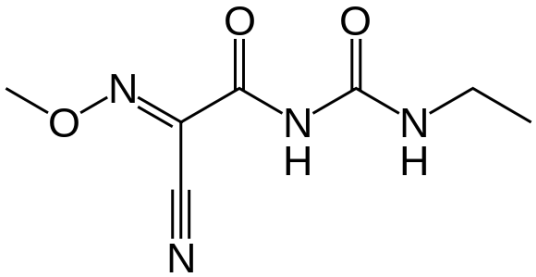

Figure 4 - Chemical structure of cymoxanil.

The preventive effect of CYM is short-lived (2 - 4 days), and this fungicide has been applied in mixtures with other contact and/or systemic fungicides, such as mancozeb, propamocarb, and famoxadone, which are known to have a longer-lasting protective effect (European Commission, 2020; Genet & Vincent, 1999; Hillebrand et al., 2012; Toffolatti et al., 2015). CYM has a rapid degradation in plants (Belasco *et al.,* 1981), animals, and the environment and has acute oral toxicity in rats: 50% lethal dose (LD_{50}) = 960 mg/kg/day. Because of that, it is classified by the World Health Organization (WHO) as slightly toxic (Belasco *et al.,* 1981; Hillebrand *et al.,* 2012). Although CYM has been used for the past 23 years, its MoA remains unknown. It has just been shown that CYM is metabolized to glycine as the

major and last metabolite formed via the intermediates 2-cyano-2-methoxyimino-acetic acid and 2-cyano-2-hydroxyimino-acetic acid formed in plants and animals (Belasco *et al.,* 1981; Gisi & Sierotzki, 2008; Hillebrand *et al.,* 2012). These two metabolites were tested in *Botrytis cinerea* strain Cya S_1 (*B. cinerea*) to determine if they could be the active principle of CYM, however, they did not display any fungitoxic activity in the tested model, contrary to CYM. Ziogas & Davidse (1987) reported that CYM does not affect respiration and energy production of *Phytophthora infestans* (*P. infestans*) strain 85371 at concentrations above 100 µg/ml, at which the citric acid and glyoxylate cycles remain operative in zoospores. Instead, they demonstrate that CYM inhibits mycelial growth by 50% and germ tube formation by sporangia at a concentration below 1 µg/ml. On the other hand, up to 100 µg/ml of CYM did not affect the sporangia and zoospore release, demonstrating that the processes inhibited by CYM are not essential for zoospore release. The same study also showed that 100 µg/ml CYM did not inhibit the uptake of radiolabelled precursors of DNA [*metyl*-^{3}H]thymidine, RNA [^{3}H]uridine, or protein [^{14}C]phenylalanine by the *P. infestans* mycelium. However, in *P. infestans* sporangia, thymidine incorporation was reduced, uridine incorporation was slightly affected, and phenylalanine incorporation was unaffected. This finding indicates that DNA synthesis inhibition might be a secondary effect, since 10 µg/ml CYM completely inhibited mycelial growth and germ tube formation but failed to inhibit thymidine incorporation as well as RNA synthesis, since CYM failed to inhibit endogenous RNA polymerase activity of isolated nuclei. In contrast to the previous report, Ribeiro *et., al* (2000) have shown that CYM inhibits growth and biomass production in *S. cerevisiae* IGC 3507 cells at concentrations from 5 - 100 µg/ml, and respiration in the concentration range from 5 – 25 µg/ml in a mineral medium with vitamins and glucose or acetic acid as the carbon source. These findings were corroborated by Estève *et al* (2009) that used *S. cerevisiae* var. *bayanus* wild type as a model organism. Additionally, Lum et al, (2004) demonstrated that *S. cerevisiae* strains with heterozygous deletions in *LCB1,* a gene involved in sphingolipid synthesis (Buede *et al.,* 1991) and in *FMP30*, a protein with a role in maintaining cardiolipin levels and mitochondrial morphology (Kuroda *et al.,* 2011), were sensitive to CYM. Regarding the MoA of CYM in combination with other substances, Huang et al, (2020) demonstrated that cymoxanil plus famoxadone leads to decreased heart rate, pericardial edema, heart shape changes, and reduced body length in zebrafish embryos as the model organism. They also demonstrated that famoxadone-cymoxanil causes oxidative stress, affects cell proliferation, and decreases ATPase activity. Overall, CYM is an example of chemical substances in use that have a poorly characterized toxicological profile.

1.2. Toxicology

Toxic substances can be classified into two different classes, exposure class or use class. In the first case, toxicants are classified as food, air, water, or soil occurrences. In the second case, drugs are classified as drugs of abuse, therapeutic drugs, agricultural chemicals, additives in food, pesticides, toxins from plants, and cosmetics (Parasuraman, 2011). The study of the adverse effects of these toxic substances belongs to toxicology (Alewu & Nosiri, 2011; Hamadeh *et al.,* 2002).

The tests currently available to assess the effectiveness, quality, and adverse effects of products such as drugs and biological compounds are based on testing animals *in vivo*. For instance, acute toxicity testing is carried out by determining the effect of a single dose on an animal species, and the use of two different animal species (one rodent and one nonrodent) is recommended (Parasuraman, 2011). The tested substance is administered to an animal at different dose levels. Acute toxicity testing can determine the LD_{50} of the assessed substance. It is recognized that there is a need to perform experiments on animals to make scientific and medical advances. However, it is necessary to use many animals to obtain this value, and the mortality ratio is high (Parasuraman, 2011). Besides, this type of experiment brings several limitations, such as being relatively expensive and time-consuming, taking 2 - 3 years to determine the toxicity of only one compound (Judson *et al.,* 2009; Nuwaysir *et al.,* 1999). With the new political guidelines, the indiscriminate use of animals for research purposes was considered "irrational, unacceptable and immoral" (Singh *et al.,* 2018) and their use in laboratory assessments also has ethical implications, and it decreased through the implementation of the 3R's (Replace – animal studies with non-animal methods; Reduce – as few animal studies as required and necessary; and Refine – minimize the stress of study animals) (Webster *et al.,* 2010).

As mentioned above, tens of thousands of chemicals are used annually, most of which do not have toxicological data available (North & Vulpe, 2010). The trend is that this number will increase with population, industry, and scientific development (Judson *et al.,* 2009). For instance, advances in nanotechnology have led to the introduction of new compounds for general use, bringing with it an additional challenge for hazard assessment (North & Vulpe, 2010). Given the growing number of possibly toxic substances, it is of great importance to develop new and faster approaches to characterize toxicological profiles of a high number of compounds. One of the solutions can be toxicogenomics.

1.2.1. Toxicogenomics: a new tool for *hazard* assessment

Toxicogenomics has emerged as a new transdisciplinary field that complements traditional toxicological studies with the global analysis, omics, of modifications induced by a toxicant among any

biomolecules (genome, transcriptome, metabolome, and proteome) (Hamadeh *et al.,* 2002; Vachon *et al.,* 2018). As shown in Figure 5, the number of publications in the field of toxicogenomics has been increasing over the years, with a total of 2528 articles on PubMed. Toxicogenomics can be used to analyze several thousand genes to identify changes associated with drug-induced toxicity (Chavan-Gautam *et al.,* 2017). Through omics tools, it is possible to identify response patterns to a specific toxicant at the genomic, transcriptomic, proteomic, and/or metabolomic level, leading to the characterization of the metabolic pathways involved in this response (dos Santos & Sá-Correia, 2015; Hamadeh *et al.,* 2002; Harrill, 2008).

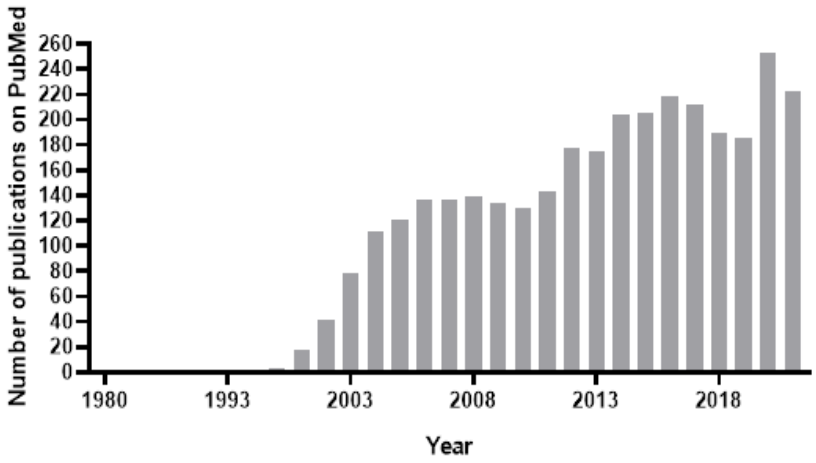

Figure 5 – Number of publications in the field of toxicogenomics, updated on the PubMed platform from 1980 to 2021. Data from (PubChem Compound Summary for CID 5361250, 2021).

Transcriptomics can be defined as studying the complete set of RNA transcripts produced by the genome. Quantification of the transcriptome can be carried out using methods such as qRT-PCR, microarrays, and RNA-seq. Subsequently, comparisons between different expression results can be made to identify different types of gene expression in response to various compounds (Alexander-Dann *et al.,* 2018; Vatakuti, 2016a). Proteomics aims to characterize the expression of all proteins in the cell, tissue, or organism. This omic thus allows understanding the function of proteins in response to toxic stress (Vatakuti, 2016b). Metabolomics, in turn, aims to characterize the metabolic profile in the cell, tissue, or organism under a given condition (Vatakuti, 2016b). The toxicogenomics approach can focus on multiple levels of the molecular cascade that can be impacted by a toxicological challenge. When changes occur in one of the levels, it can lead to changes in other levels, being necessary study the effects on these. For instance, the use of gene expression alone is not adequate to understand the action of a toxicant in the cell since abnormalities in the production and/or function of proteins are also predictable to occur, and

thus the integration of the different omics is recommended to understand these changes (dos Santos *et al.,* 2012; Martins *et al.,*2019).

1.2.2. *In vitro* toxicity detection tests – The *yeast S. cerevisiae* as a tool for toxicogenomics studies

Along with omics tools, high-throughput screening based on cells is one of the methods that can accelerate toxicity tests. The assessment of the stress response through these tests can lead to the identification of toxic pathways, that is, "cellular response pathways that, when sufficiently perturbed in an intact animal, are expected to result in adverse health effects" (North & Vulpe, 2010; Singh *et al.,* 2018). The use of yeast *S. cerevisiae* as an experimental model has several advantages such as: (1) it is a unicellular non-pathogenic microorganism with (2) rapid and inexpensive growth; (3) it is amenable to genetic manipulation; (4) it possesses a strikingly high-level of functional conservation within the human genome and other higher eukaryotes; (5) it has the unique advantage of possessing functional information available for most genes (dos Santos *et al.,* 2012) and genome-wide analyses are easily implemented due to the collection of *S. cerevisiae* strains, in which each known or suspected open reading frame (ORF) is deleted and replaced with the marker *KanMX* (e.g., EUROSCARF)) (Giaever *et al.,* 2002; Sousa *et al.,* 2013). Although many cytotoxic compounds act on their target organisms through physiological mechanisms that do not exist in yeasts, many basic mechanisms underlying toxicity, adaptation, and resistance to chemical and environmental stresses are conserved between yeasts and higher organism cells (North & Vulpe, 2010).

Identifying variations in gene expression and proteins in *S. cerevisiae* exposed to toxics allows the identification of pathways and cellular components that are important and are involved in the toxicological response (dos Santos *et al.,* 2012). *S. cerevisiae* provides an integrated assessment and a comprehensive view of the mechanisms of toxicity throughout the genome, with the combination of omics (Figure 6).

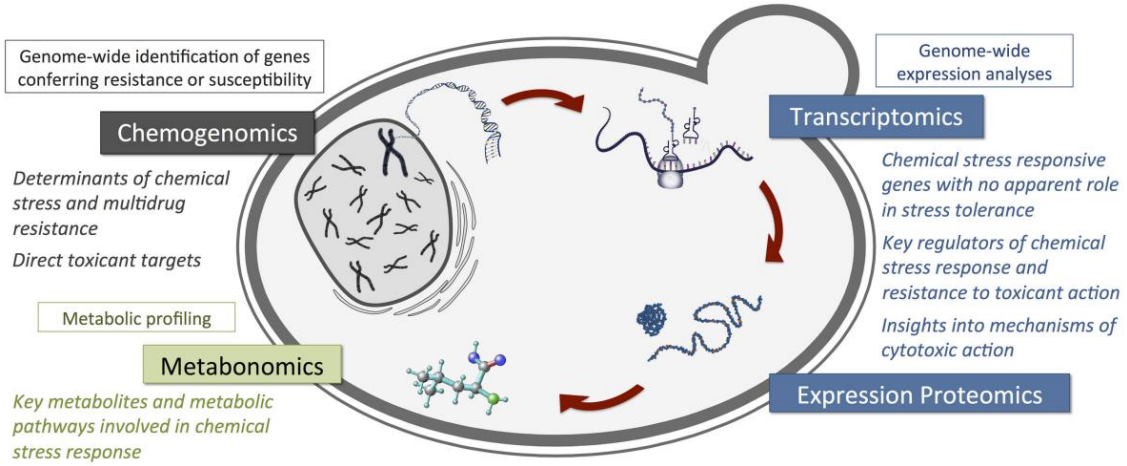

Figure 6 – Application of different omics to understand and predict the mechanisms and profiles of toxicity in yeasts. Transcriptomics and proteomics provide an assessment of changes that occur in response to toxicants. Metabolomics provides the metabolic profile of small-cell molecules in response to toxicity, and chemogenomics identifies molecular targets for cellular toxicity. Retrieved from (dos Santos *et al.,*, 2012).

1.2.3. The *Saccharomyces* Genome Deletion Project

The *Saccharomyces* Genome Sequencing Project identified more than 6000 ORFs in the *S. cerevisiae* genome. However, about a third of these ORFs have no known function (Engel *et al.,* 2014; Nislow *et al.,* 2016). An advantageous method to determine the role of genes is the creation of strains with loss of function, allowing to observe the resulting phenotype. In 1998, 16 laboratories began work on the *Saccharomyces* Genome Deletion Project (Nislow *et al.,* 2016). The main objective of this project was to generate a complete set of yeast deletion strains. To achieve this objective, a PCR-based gene deletion strategy was used to create a start- to -stop- codon deletion of each ORF. Each gene interruption was replaced with a KanMX module (Figure 7) (Yeast Deletion Project, n.d.). This method made it possible to generate a complete set of deletion strains and yeast knockout (YKO) collections, including haploid

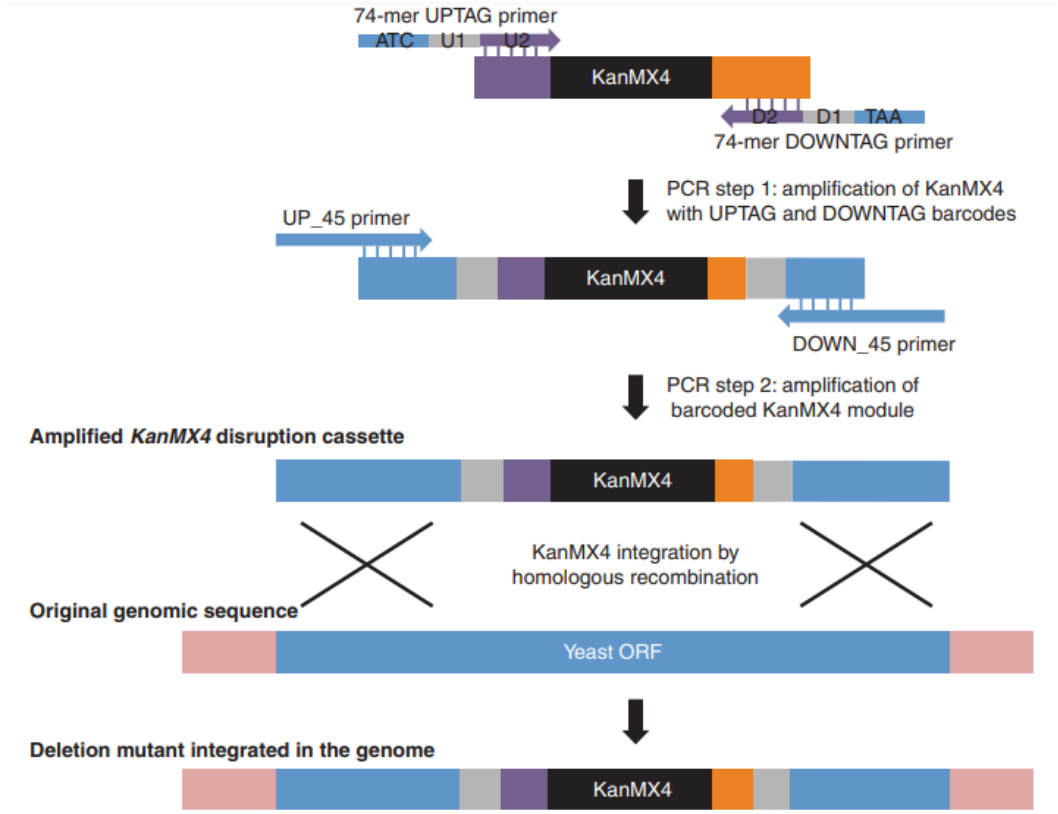

Figure 7 – Construction of single-gene deletions in *S. cerevisiae*. The gene disruption KanMX cassette was constructed using two-step PCR. In the first PCR, 74 bp UPTAG and DOWNTAG primers, which flank the upstream 5′ and downstream 3′ end of the KanMX ORF, respectively, were used to amplify the KanMX gene from the pFA6-kanMX4 template. UPTAG (U) and DOWNTAG (D) are primers with a sequence common to all open reading frame (ORF) disruptions (U1: 5′ -GATGTCCACGAGGTCTCT-3′ or D1: 5′ -CGGTGTCGGTCTCGTAG-3′), a 20-bp unique "molecular barcode" TAG sequence, and a sequence homologous to the KanMX4 cassette (U2: 5′ -CGTACGCTGCAGGTC GAC-3′ or D2: 5′ -ATCGATGAATTCGAGCTCG-3′). In the second PCR, amplification was performed with two 45-mer primers that have specific homology with 45 bp upstream and downstream from each ORF start and stop codon (UP_45 and DOWN_45, respectively). Retrieved from (Nislow *et al.,*, 2016).

strains of both yeast mating types and homozygous/heterozygous deletions (dos Santos *et al.,* 2012). Moreover, each deletion has a unique 20 bp sequence, bar-code, which serves as a strain identifier (Baudin *et al.,* 1993; Nislow *et al.,* 2016).

1.2.3.1. Functional toxicogenomics using gene deletions

Functional toxicogenomics is the study of the biological function of genes in response to an adverse external event, such as the presence of chemicals, environmental stressors, drugs, or toxins. As previously mentioned, gene and protein expression can be influenced by exposure to a toxicant, being dependent on the dose and the time of exposure (dos Santos *et al.,* 2012; Hamadeh *et al.,* 2002; North & Vulpe, 2010). Therefore, functional toxicogenomics can provide a direct link between genes and toxicants. Based on this, it is possible to use cells containing individual gene deletions to determine the MoA of potential contaminants. When a cell with a deletion in a given gene is treated with a particular chemical substance and shows sensitivity in relation to the wild-type (WT) strain, it means that gene is responsible for conferring resistance to the cell, implying that this gene is involved in response to that chemical substance, as exemplified in Figure 8.

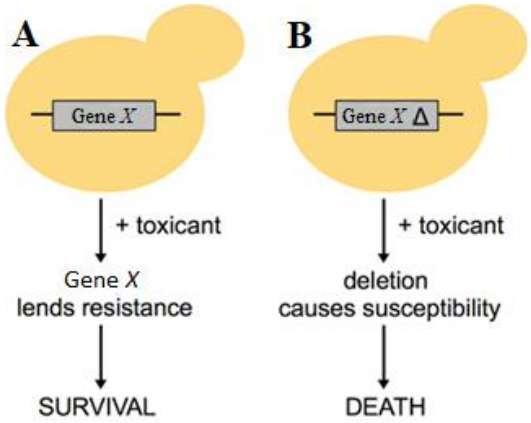

Figure 8 - Concept for determination of the biological function of a gene when exposed to a particularly toxic compound, using the gene deletion. When the cell that expresses the X gene is placed in the middle with the toxic compound, the cell remains viable **(A)**. However, when the cell that has the X gene deletion is set in the medium with the same toxic compound, the cell dies **(B)**. With this, it is possible to conclude that the X gene is involved in response to the drug, giving it resistance. Adapted from (Gaytán & Vulpe, 2014)

Any assayable phenotype can be measured in response to a toxicant; however, viability/fitness are the most conventional endpoints (Gaytán & Vulpe, 2014). For this type of test, screens can be performed on homozygous or haploid deletions (total loss of the gene function, gene dosage = 0%), heterozygous deletions (partial loss of gene function, gene dosage = 50%), and overexpression (gene dosage > 100%) and the fitness of strains upon chemical treatment can be assessed in non-competitive arrays (Figure 9

B) or in competitive "bar-coded" pools (Figure 9 C). For the first test, 96-well plates are used, and each of the mutant strains occupies a different well upon a chemical treatment, making it possible to determine the fitness directly when compared with WT strains. In the second test, all mutant strains, which have a unique "bar-coded" tag, grow together in the presence of the chemical substance. The fitness value is determined by the abundance of the different strains, for which microarrays are used mutually with PCR, where the bar-codes of each mutant strain will be amplified (dos Santos *et al.,* 2012).

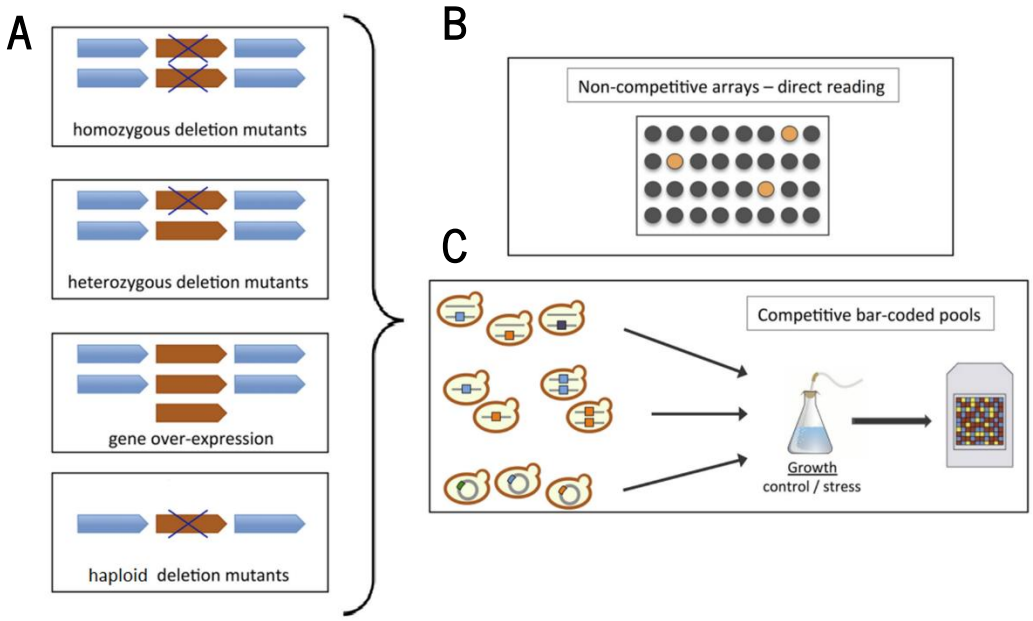

Figure 9 - Schematic representation of methodologies and cell libraries available for chemogenomics testing in *S. cerevisiae.* Gene deletion mutant collections **(A)** The fitness of strains upon chemical treatment is usually assessed in non-competitive arrays **(B)**, where the toxicant is added to a well plate, and each mutant occupies a separate well and treated with the drug, the growth is monitored by optical density; or in competitive barcoded pools **(C)**, in this case, gene deletion mutants are pooled together and subject to drug treatment. Adapted from (dos Santos *et al.,*, 2012).

Several functional toxicological screens have been performed in yeast (Table 1) and organisms other than yeasts, such as *E. coli* (Wang *et al.,* 2018), *H. sapiens* (Wang *et al.,* 2014).

Table 1 - Examples of functional toxicological screens performed in yeasts. Adapted from (Gaytán & Vulpe, 2014).

Chemical class	Description	Organism	References
Solvents	Butanol	*S. cerevisiae*	(González-Ramos, Van Den Broek, Van Maris, Pronk, & Daran, 2013)
	Dimethylsulfoxide	*S. cerevisiae*	(Gaytán, *et al.,* 2013; Zhang *et al.,* 2013)
Metals	Aluminum	*S. cerevisiae*	(Tun *et al.,* 2013)
	Gold nanoparticles	*S. cerevisiae*	(Smith *et al.,* 2013)
	Cobalt	*S. pombe*	(Ryuko, Ma, Ma, Sakaue, & Kuno, 2012)
	Cadmium	*S. pombe*	(Kennedy *et al.,* 2008)
Pollutants	Dieldrin	*S. cerevisiae*	(Gaytán *et al.,* 2013)
	Various conozeles	*S. cerevisiae*	(Guan, Xia, Tian, Chen, & Zhang, 2020)
	Formaldehyde	*S. cerevisiae*	(North *et al.,* 2016)
	Menadione and hydrogen peroxide	*S. cerevisiae*	(Daskalova *et al.,* 2021)
Antimicrobials	2,4-diacetylphloroglucinol	*S. cerevisiae*	(Troppens, Dmitriev, Papkovsky, O'Gara, & Morrissey, 2013)
	Antimicrobial peptides	*S. cerevisiae*	(Lis *et al.,* 2013)
	Curcumin	*S. cerevisiae*	(Azad, Singh, Golla, & Tomar, 2013)
	Chitosan	*S. cerevisiae*	(Galván Márquez *et al.,* 2013)
	Eugenol	*S. cerevisiae*	(Darvishi, Omidi, Bushehri, Golshani, & Smith, 2013b)
	Thymol	*S. cerevisiae*	(Darvishi, Omidi, Bushehri, Golshani, & Smith, 2013a)
	Polyalkyl guanidiniums	*S. cerevisiae*	(Bowie, Parvizi, Duncan, Nelson, & Fyles, 2013)
	TA-289	*S. cerevisiae*	(Quek *et al.,* 2013)
	Micafungin	*S. pombe*	(Zhou *et al.,* 2013)
	Various antifungals	*S. pombe*	(Fang *et al.,* 2012)
Drugs	Chloroquine	*S. cerevisiae*	(Islahudin *et al.,* 2013)
	Edelfosine	*S. cerevisiae*	(Cuesta-Marbán *et al.,* 2013)
	Porphyrin TMpyP4	*S. cerevisiae*	(Andrew *et al.,* 2013)
	FK506	*S. cerevisiae*	(Ma, Jiang, Liu, Ryuko, & Kuno, 2011)
	Caffeine	*S. pombe*	(Calvo *et al.,* 2009)
	Cisplatin	*S. cerevisiae*	(Costa *et al.,* 2019)
Genotoxicants	Methyl methanesulfonate	*S. cerevisiae*	(Huang *et al.,* 2013)
	Various	*S. cerevisiae*	(Svensson *et al.,* 2013)
	Various	*S. cerevisiae*	(Torres, Lee, Giaever, Nislow, & Brown, 2013)
	Various	*S. cerevisiae*	(Guan *et al.,* 2022)
	Various	*S. pombe*	(Pan *et al.,* 2012)
Other	Lanthanide series	*S. cerevisiae*	(Pallares *et al.,* 2021)
	Acetic acid	*S. cerevisiae*	(Sousa *et al.,* 2013)
	Hydrolysate	*S. cerevisiae*	(Skerker *et al.,* 2013)

4-Methylcyclohexanemethanol	*S. cerevisiae*	(Pupo, Ku, & Gallagher, 2019)
Neonicotinoids	*S. cerevisiae*	(Mattiazzi Ušaj, Kaferle, Toplak, Trebše, & Petrovič, 2014)
Manzamine A	*S. cerevisiae*	(Kallifatidis, Hoepfner, Jaeg, Guzmán, & Wright, 2014)
NaCl diversity/ mechanistic sets	*S. cerevisiae/ pombe*	(Kapitzky *et al.,* 2010)

Of the types of assays that can be performed in yeast, homozygous profiling (HOP assay) is one of the most used. It provides an overview of the entire genome of synthetic growth interactions between the chemical and loss of function (Hoepfner *et al.,* 2014; Smith *et al.,* 2010). Despite having similar mechanisms of action, many chemical compounds will give origin to a unique profile of sensitivity and resistance of the non-essential mutants. However, because of multiple similarities, these profiles can be grouped and compared to infer the mechanism of action (Parsons *et al.,* 2004; Smith *et al.,* 2010). For example, Hoepfner *et., al* (2014) used these and other assays as the basis for the construction of networks to identify a target for CMB1257, a compound with antimicrobial activity but not previously characterized. A similar strategy can be used to uncover the MoA of CYM.

2. Aim

The use of chemical substances has been increasing over the years. Therefore, there is a continued increase in the introduction of new chemical compounds on the market. Although there are legislative bodies that regulate the use of such chemical substances, there has been a concern in recent years about chemicals that were once considered safe. Indeed, many are now getting a second look because they have a poorly characterized toxicological profile. These chemicals can result in water and food contamination, where man will be the final consumer, with potentially hazardous consequences. The assays currently used to identify the MoA of the different compounds are often animal-based and are therefore slow and expensive. It is, therefore, crucial to develop new methods to elucidate the mechanisms of action in a fast and more economical manner. The present work aimed to evaluate the effect of CYM on *S. cerevisiae* cells and then develop a genome-wide to understand its MoA. To achieve this, we set out to:

I. Analyze the cellular processes affected in response to cymoxanil at structural and biochemical levels. For that purpose, we analyzed the effects of CYM on cell growth and viability. To characterize cell death process, we assessed several cell death markers such as mitochondrial fragmentation, alterations on mitochondrial potential, and mass and ROS production.

II. Based on results obtained in I, develop a method to screen an *S. cerevisiae* collection of mutants deleted in non-essential genes (≅5000) for sensitivity or resistance to cymoxanil.

By understanding the mechanism of the action of CYM, it will be possible to infer its toxicity profile and infer the adverse consequences that it can bring, not only for the human population but also for the environment.

3. Materials and methods

3.1. Yeast strains

The *S. cerevisiae* strains used in this work are listed below in Table 2. BY4741 was used as the WT strain. BY4741 WT, BY4741 *Δerg6*, BY4741 rho^0 were used in viability assays. For fluorescence microscopy assays, the strains BY4741 pUG35-Nhp6A-GFP, BY4741 pRS413-Pep4-mCherry, BY4741 pYX242-mt-GFP, and BY4741 pER.Isc1-mCherry were used.

Table 2 – List of the BY4741 strains used in this work.

Yeast Strains	Genotype	Source
BY4741	MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*	EUROSCARF
BY4741 pER.ISC1-mCherry	MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0,* pER.ISC1-mCherry (LEU2)	This study
BY4741 pRS413- Pep4-mCherry	MATa; ura3-52; leu2-3, 112; his3-Δ1, pRS413-Pep4-mCherry (HIS3)	(Terra-Matos et al., 2022)
BY4741 pUG35-nhp6a-GFP	MATa, *his3Δ1*, *leu2Δ0*, *met15Δ0*, *ura3Δ0*, pUG35-nhp6a-GFP (URA3)	(Canossa, 2017)
BY4741 pYX-mt-GFP.	MATa, *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*, pYX-mt-GFP (URA3)	This study
BY4741 rho^0	MATa, *his3Δ1*, *leu2Δ0, met15Δ0, ura3Δ0 [rho^0]*	(Carvalho, 2018)
BY4741 *Δerg6*	MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0; YML008C::kanMX4*	EUROSCARF

3.2. *Escherichia coli* transformation and plasmid extraction

pER.Isc1-mCherry was transformed and amplified in *Escherichia coli (E. coli)*. 100 ng of plasmid DNA were added to 100 µL of competent *E. coli* XL-1 Blue cells and incubated on ice for 30 minutes, followed by heat shock at 42 °C for 45 seconds. Then, the cells were incubated on ice for 10 minutes and 900 µL of SOC [2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 20 mM glucose] medium was added. Cells were then incubated for 1 hour at 37 °C with agitation at 200 rpm. Afterwards, cells were collected, centrifuged at 3000 rpm for 3 minutes. 800 µl of supernatant was discarded, and the pellet was resuspended in the remaining supernatant. At the end, the cells were plated on solid LB medium [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 2% (w/v) agar] containing 100 µg/ml ampicillin, overnight at 37 °C. The next day, one colony was

selected and incubated in liquid SOC medium containing 100 µg/ml ampicillin for plasmid extraction. Plasmids were extracted from cultures grown overnight using the GenElute Plasmid Miniprep Kit according to the manufacturer's instructions (Sigma Aldrich). Extracted plasmid DNA was quantified in a NanoDrop spectrophotometer (Nanodrop ND1000) (pER.Isc1-mCherry = 221.4 µg/µL).

3.3. Yeast pER.ISC1-mCherry transformation

In the Table 3 are listed the plasmid used in this study that was first extracted from *E. coli* using GenElute Plasmid Miniprep Kit (Sigma Aldrich).

Table 3 - List of plasmids used in this study.

Plasmid	Description	Source
p*ER.ISC1*-mCherry	CEN/ARS, *LEU2*, $P_{tet0\text{-}CYC1,}$ Endoplasmatic reticulum targeted ISC1-mCherry	(Rego, 2017)

Strain BY4741 was cultivated overnight in YPD medium (1% (v/w) yeast extract, 2% (v/w) bactopeptone and 2% (v/w) glucose). The next day, cells were diluted in YPD containing 4% (v/w) glucose to an OD_{640nm} = 0.2 and incubated at 30 °C with agitation at 200 rpm until reaching an OD_{640nm} = 0.6-0.8. Then, cells were collected, centrifuged at 5000 rpm for 3 minutes, the supernatant was discarded, and cells were washed with deionized sterile water, centrifuged again at maximum speed for 1 minute, and the supernatant was discarded. For each transformation, 100 µL of deionized sterile water was added to resuspend the pellet. 100 µL of cell suspension were transferred to new microtubes, centrifuged again, the supernatant discarded and resuspended in 369 µL of transformation mix, as presented in Table 4, and incubated at 42 °C for 40 minutes. Cells were then centrifuged at maximum speed, the supernatant discarded, and the pellet resuspended in 50 µL of deionized sterile water. In the end, cells were plated on a medium lacking leucine and incubated at 30 °C for 2 days.

Table 4 - Transformation mix.

Reagents	Negative control (µL)	Transformation (µL)
PEG3350 (50%)	250	250
Lithium acetate (LiAc) (1M)	36	36
Boiled ssDNA (carrier 10 mg/ml)	50	50
H_2O	33	32
Plasmid DNA	-	1

3.4. Growth conditions and treatment

Cells of the *S. cerevisiae* WT strain were grown overnight on Synthetic Complete (SC) medium [0.17% (w/v) YNB, 0.14% (w/v) DROP-out, 2% (w/v) galactose, 0.04% (w/v) leucine, 0.008% (w/v) histidine, 0.008% uracil] at 30 °C, 200 rpm. The yeast strains expressing plasmids were grown in SC medium lacking histidine for Pep4-mcherry [0.17% (w/v) YNB, 0.14% (w/v) DROP-out, 2% (w/v) galactose, 0.04% (w/v) leucine, 0.008% uracil], uracil for Nhp6A-GFP and mt-GFP [0.17% (w/v) YNB, 0.14% (w/v) DROP-out, 2% (w/v) galactose, 0.04% (w/v) leucine, 0.008% (w/v) histidine] and leucine for Isc1-mCherry [0.17% (w/v) YNB, 0.14% (w/v) DROP-out, 2% (w/v) galactose, 0.008% (w/v) histidine, 0.008% uracil]. Then, cells were centrifuged and resuspended diluted in fresh medium to an OD_{640nm} = 0.1. After, cells were treated with the desired concentrations of CYM during different time periods for the different assays performed throughout this work.

A 38000 µg/ml stock solution of CYM (Sigma Aldrich) was prepared every week by diluting the compound in dimethyl sulfoxide (DMSO). The treatment was carried out by adding CYM to the diluted cells to a final concentration of 12.5 µg/ml, 25 µg/ml, 50 µg/ml, or 100 µg/ml. The same volume of DMSO (≈ 0.13%) was added to another tube as a negative control. After that, cells were incubated at 30 °C with agitation at 200 rpm for 24 hours. Cells were collected after 4, 8, and 24 hours of treatment for the different assays. At 0 hours, cells were collected before adding the compound. When used, 100 µg/ml cycloheximide (CHX) was added to the cell suspensions at the same time as CYM.

3.5. Viability assays

50 µL of culture samples were washed in sterile deionized water and diluted 10^{-4} in sterile deionized water. 5 µL of each condition and time point were spotted directly from the cell culture and cell suspension at four different dilutions (10^{-1}, 10^{-2}, $10^{-3,}$ and 10^{-4}) and plated on YPD plates for the spot assay. Five drops of 40 µL from the 10^{-4} dilution were plated on YPD plates to assess cell viability by counting colony formation units (CFUs). The plates were incubated for 2 days at 30 °C.

3.6. Oxygen consumption quantification

To estimate oxygen consumption, a Clark electrode connected to a recorder (Kipp & Zonen) was used. The electrode was immersed in a water chamber with magnetic stirring. 4.5 ml of deionized water and 0.5 ml of yeast suspension (concentrated to an OD_{640nm} = 50) were added to the chamber, and a baseline was obtained. Subsequently, 100 µl of 5 M galactose was added, and the oxygen consumption

was followed in the recorder. The oxygen consumption values were calculated based on O_2 saturation at 25 °C as 23.6 mmol/ml.

3.7. Epifluorescence microscopy analyses

The yeast strains expressing Pep4-mcherry, Nhp6A-GFP, Isc1-mCherry, and pYX-mt-GFP were grown in SC medium lacking the appropriate components and treated with CYM or with DMSO (negative control). Cells were then collected for visualization by epifluorescence microscopy to evaluate the localization of the proteins. In the case of the strain expressing Pep4-mcherry, cells were stained with Celltracker™ Blue CMAC (Molecular Probes Eugene, OR) at a final concentration of 2 μM and incubated for 20 minutes at room temperature, to assess vacuole membrane permeabilization. In the case of the strain expressing Nhp6A-GFP, cells were also incubated with 2 μg/ml of propidium iodide (PI) to assess plasma membrane integrity.

Cells were visualized with a Leica Microsystems DM-5000B microscope with appropriate filter settings (red, green, blue, and Differential Interference Contrast (DIC)) with a 100x oil immersion objective. Images were obtained with a Leica DFC350 FX Digital Camera and processed with LAS X Microsystems software.

3.8. Evaluation of mitochondrial protein degradation by SDS gel electrophoresis/Western Blot

S. cerevisiae BY4741 pYX-mt-GFP cells were treated with CYM or with DMSO (negative control), and after 4, 8, and 24 hours of treatment, 1 ml of cells were collected to prepare the cell extracts.

3.8.1. Cell extracts preparation

Cells were resuspended in 500 μl of water, followed by the addition of 50 μl of 7.4% (v/v) β-mercaptoethanol in 2 M NaOH. Then, cells were vortexed and incubated on ice for 15 minutes. After that, 50 μl of 50% (w/v) TCA were added, followed by a 15-minute incubation on ice. The samples were then centrifuged at 14800 rpm for 5 minutes at 4 °C and the pellets were resuspended in 30 μl of 1x *Laemmli* buffer (0.0625 M Tris-HCl, 2.3% (w/v) SDS, 10% (w/v) glycerol, 1.25% (w/v) β-mercaptoethanol, 0.125% (w/v) bromophenol blue). Then, the extracts were denatured at 100 °C for 5 minutes and stored at -20 °C until SDS-PAGE analysis.

3.8.2. SDS gel electrophoresis/Western Blot

The protein lysates obtained as described above were separated by SDS gel electrophoresis on a 12.5% SDS-poly-acrylamide gel, in a Mini-Protean III electrophoresis system (Bio-Rad) at 25 mA per gel, using 1x running buffer (0.025 M Tris base, 0.192 M Glycine, 46.1% SDS). The separated proteins were then transferred to a nitrocellulose membrane (Hybond-ECL, GE Healthcare) at 60 mA for 90 minutes in a semi-dry transfer unit (TE77X Hoefer) using transfer buffer (0.025 M Tris-Base, 0.192 M glycine). Then, the membranes were blocked in 5% (w/v) non-fat milk in 1x PBS containing 0.05% (v/v) Tween 20 for 30 minutes at room temperature with agitation. Then, the membranes were incubated with the primary antibodies overnight at 4 °C. For phosphoglycerate kinase (Pgk1p) detection, a mouse monoclonal antibody anti-yeast phosphoglycerate kinase (1:5000, Molecular Probes) was used. For mitochondrial porin (Por1p) detection, a mouse monoclonal anti-yeast porin (1:10000, Molecular Probes) was used. Finally, the membranes were incubated with an anti-mouse peroxidase-coupled secondary antibody (1:5000) for 60 minutes at room temperature. Chemiluminescence detection was performed using the ECL detection system in an automatic reveal machine (Agfa, Curix 60).

3.9. Flow cytometry analysis

Flow cytometry analysis was performed in a CytoFlex System B4-R2-V0 (Beckman Coulter) flow cytometer. Ten thousand cells were analyzed per sample at a low-medium flow rate. Flow cytometry analyses were performed with CytExpert software version 2.4.0.28 (Beckman Coulter, Inc.).

3.9.1. Assessment of membrane potential and plasma membrane integrity

Cells were collected, washed, and resuspended in resuspension buffer (0,1 mM $MgCl_2$, 10 mM MES (2(N-Morpholino)ethanesulfonic acid), and 2% (w/v) glucose, pH 6.0 set with NaOH). Then, to assess mitochondrial membrane potential, they were incubated with $DiOC_6(3)$ (Molecular Probes Eugene, OR) at a final concentration of 1 nM for 30 minutes at 30 °C in the dark, in duplicate. One of the duplicates was incubated with 2 μg/ml of PI to assess plasma membrane integrity.

3.9.2. Assessment of mitochondrial mass and ROS accumulation

Cells were collected, washed, and resuspended in 1x PBS. For mitochondrial mass assessment, cells were incubated with MitoTracker™ Green FM (Invitrogen™) at a final concentration of 0.4 μM for 30 minutes at 37 °C in the dark. For ROS accumulation assessment cells were incubated with MitoTracker™ Red CM-H_2Xros (Invitrogen™) at a final concentration of 0.8 μg/ml for 30 minutes at 37 °C in the dark.

3.9.3. Assessment of pH alterations

For pH alteration assessment, BCECF, AM (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) (Invitrogen™) was used. The treated cells were collected, washed, and resuspended in SC-Gal medium. Then, they were incubated with the probe at a final concentration of 18 μM for 20 minutes at 30 °C in the dark.

3.9.4. Statistical analysis of the results

The results obtained are represented by mean and standard deviation (SD) values of at least two independent experiments. Statistical analyses were carried out using GraphPad Prism Software v8.00 (GraphPad Software, California, USA).

4. Results and discussion

As the goal of this project was to optimize a phenotypic genome-wide screen of the response to cymoxanil, first we set out to identify conditions where the cells displayed a higher sensitivity to this compound, as well cellular alterations that could be used as a readout. In this way, it would be possible to proceed with the screening of the mutant collection in order to find resistance and sensitive mutants in response to CYM and, thus, to characterize the MoA of the CYM.

4.1. Cymoxanil inhibits cell growth and oxygen consumption

To select the conditions where the cells displayed more sensitivity to CYM, we tested different media and growth conditions. We first inoculated cells in YPD medium and, after overnight growth, diluted the culture in SC-Gal media containing 0.5% proline as a nitrogen source (SCpro-Glu). The addition of proline as the only source of nitrogen and SDS was already described as a membrane permeabilizer for toxic compounds (McCusker & Davis, 1991; Pannunzio *et al.,* 2004). Half the culture was exposed to CYM immediately, and another was allowed to grow in SCpro-Glu plus 0.003% SDS for another 3 hours before exposure (Figure 10). CYM treatment inhibited growth (Figure 11 A) and decreased the viability of cells (Figure 11 B) regardless of the treatment medium. However, cell viability loss in response to CYM

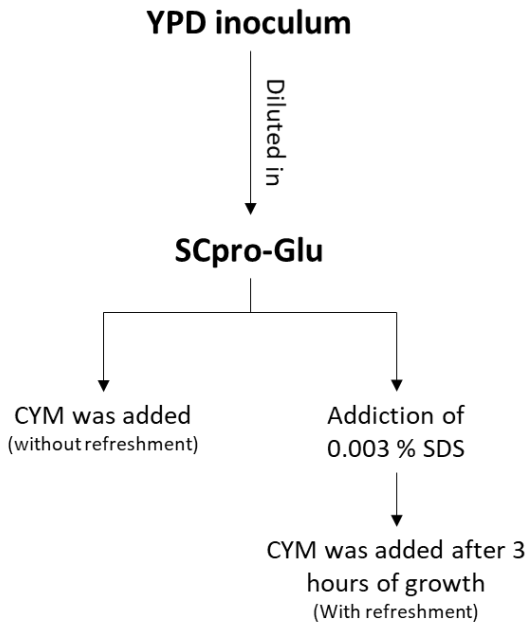

Figure 10 – Schematic representation of the assay in the presence of SDS. An inoculum was made in YPD medium, then diluted in SCpro – Glu medium. Half of the culture was immediately exposed to 100 µg/ml CYM (without refreshment), and the other grew in the presence of 0.003% SDS, and 100 µg/ml CYM was only added after 3 hours of growth (with refreshment).

was higher without refreshment, even though cells grew more in these culture conditions, both with and without CYM treatment (Figure 11 A and B). Next, we assessed the effect of CYM when the inoculum was

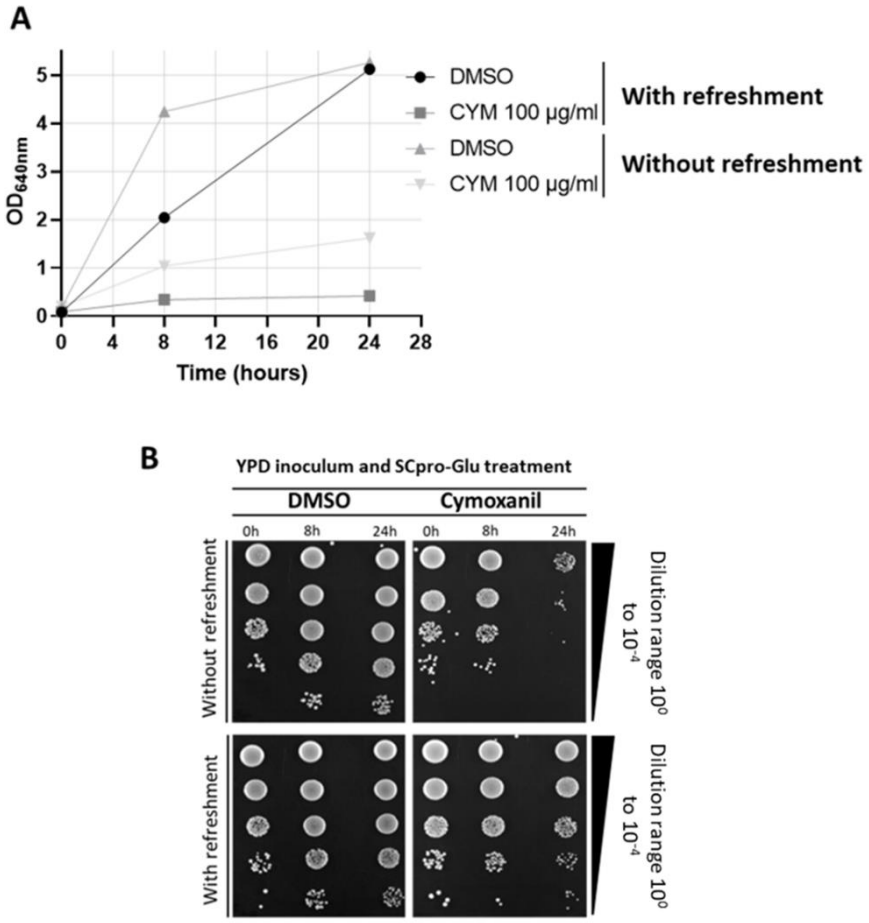

Figure 11 - Sensitivity of *S. cerevisiae* BY4741 WT cells to 100 µg/ml of cymoxanil without and with culture refreshment. A) Cells were grown overnight in YPD medium. The following day cells were diluted to an OD_{640nm} = 0.1, and 0,003% SDS was added to medium SCpro-Glu (with refreshment) and let it grow for 3 hours. After, 100 µg/ml CYM was added and incubated for 24 hours in YPD or SCpro-Glu media with or without refreshment of 3 hours of cells. OD_{640nm} was read at different time points until 24 hours of treatment. **B)** Viability of cells treated as in A) assessed at different time points by spot assay.

performed in YPD or SCpro-Glu and then the cells treated in YPD or SCpro-Glu. Regardless of the inoculum medium, CYM led to a higher inhibition of cell growth and decrease of viability when cells were exposed to CYM in SCpro-Glu medium (Figure 12 A and B). Therefore, we proceeded with assays with both inoculum and treatment in SCpro-Glu medium.

As previously referred, it was already described that CYM leads to an inhibition of biomass production and oxygen consumption in yeast, but in a different strain background (Ribeiro *et al.,* 2000). To confirm

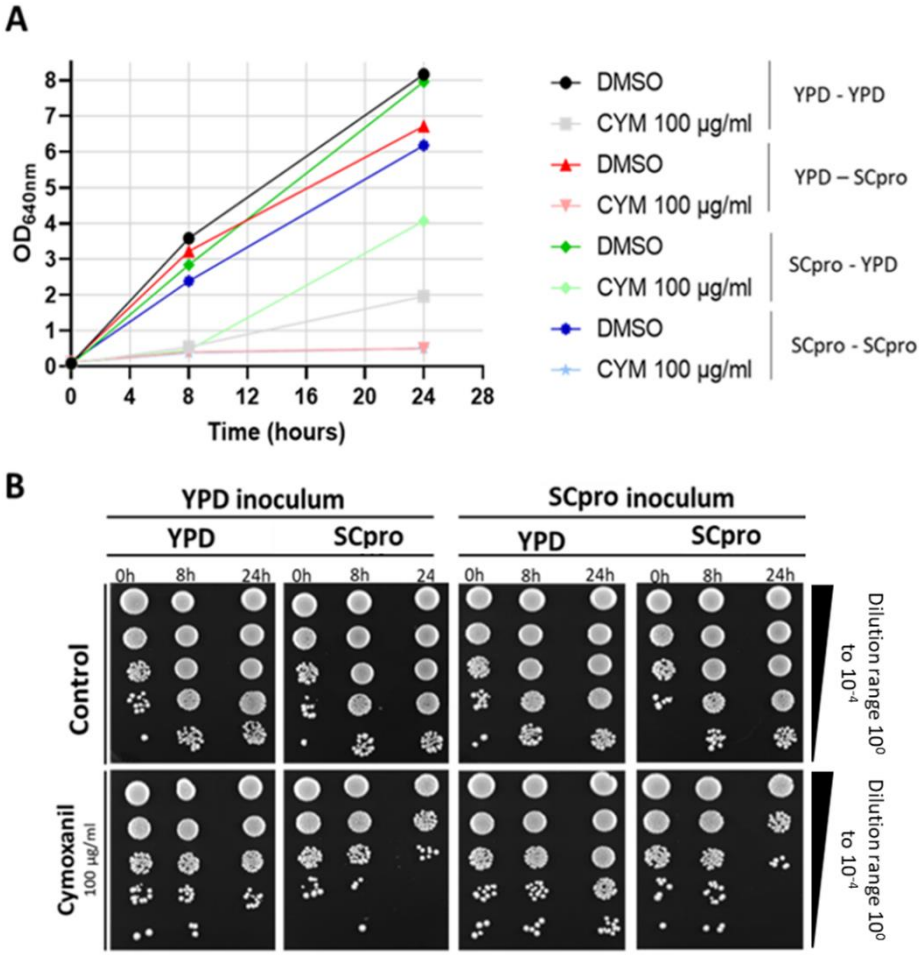

Figure 12 - Sensitivity of *S. cerevisiae* BY4741 WT cells to 100 µg/ml of cymoxanil with different inoculum/media combinations. A) Cells were grown overnight in YPD or SCpro-Glu medium. The following day cells were diluted to an OD_{640nm} = 0.1, exposed to 100 µg/ml CYM for 24 hours in YPD or SCpro-Glu media. OD_{640nm} was read at different time points until 24 hours of treatment. **B)** Viability of cells treated as in A) assessed at different time points by spot assay. Results from one experiment.

that phenotype, we assessed the effect of the compound on growth, viability, and oxygen consumption in BY4741 WT cells. We observed that CYM leads to inhibition of cell growth (Figure 13 A) and loss of viability (Figure 13 B) in a dose-dependent manner, as well as to a decrease in oxygen consumption (Figure 13 C) under our experimental conditions.

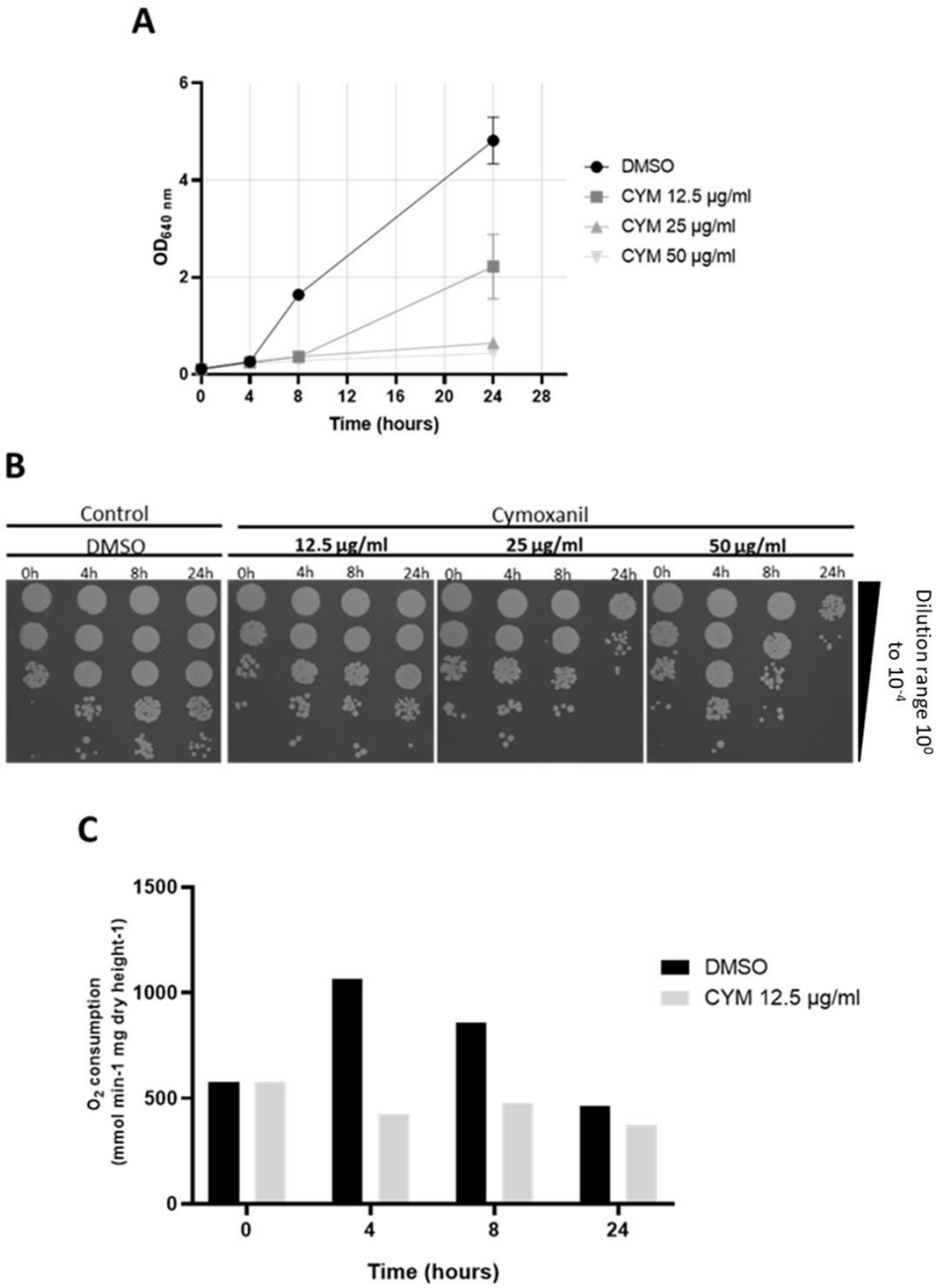

Figure 13 - Response of *S. cerevisiae* BY4741 WT cells to different concentrations of cymoxanil. A) Cells were diluted to an OD_{640nm} = 0.1, exposed to 12.5, 25, or 50 µg/ml CYM for 24 hours in SCpro-Glu media. OD_{640nm} was read at different time points until 24 hours of treatment. Values are mean ± SD of two independent experiments. **B)** Viability of cells treated as in A) assessed at different time points by spot assay. A representative image is shown from two independent experiments. **C)** O_2 consumption measured by Clark electrode. Cells were treated as in A) a B). Results from one experiment.

4.2. Assessment of the effect of cymoxanil on different organelles

To further characterize the effect of CYM, we next proceeded with a morphological characterization of different organelles of cells treated with the compound, to try to identify possible targets involved in its mode of action.

4.2.1. Assessment of vacuole membrane permeabilization and Pep4p localization

The vacuole plays a vital role in pH and ion homeostasis and functions as a storage compartment, (Bryant & Stevens, 1998). Indeed, one of the distinguishing exciting characteristics of this organelle is its acidification, which is caused by the vacuolar proton pumping ATPase (Thumm, 2000).

To assess if CYM treatment affects the vacuole, we treated a *S. cerevisiae* strain expressing Pep4p fused with mCherry with CYM and DMSO (negative control) and assessed the cytosolic translocation of Pep4p. Pep4p, an ortholog of human cathepsin D (CatD), is a pepsin-like aspartic protease (proteinase A) found in yeast vacuole. The release of this protease from the yeast vacuole to the cytosol plays a role in mitochondrial degradation during acetic acid-induced regulated cell death (Pereira *et al.,* 2010). This translocation of the vacuole to the cytosol was also observed during hydrogen peroxide-induced apoptotic cell death (Mason *et al.,* 2005), suggesting that Pep4p might play a role in apoptotic cell death. We also transformed BY4741 cells expressing Pep4p-mCherry with Vba1p-GFP, to visualize the vacuolar membrane, but this strain did not grow well and thus was not used (not shown). To assess vacuole permeabilization, the cells were also stained with the fluorescence dye CMAC blue, which is sequestered within the vacuole lumen if the vacuolar membrane is intact (Stewart and Deacon, 1995). As shown in Figure 14, we could not observe the translocation of Pep4-mCherry to the cytosol, and the cells present red fluorescence in the vacuole lumen, even with 24 hours of treatment with the higher concentration of CYM tested. We also did not observe vacuole permeabilization, characterized by a dispersed blue fluorescence through the cell with the probe blue CMAC. Considering these results, we could conclude that the compound, in the conditions tested, does not seem to affect vacuole morphology or permeability.

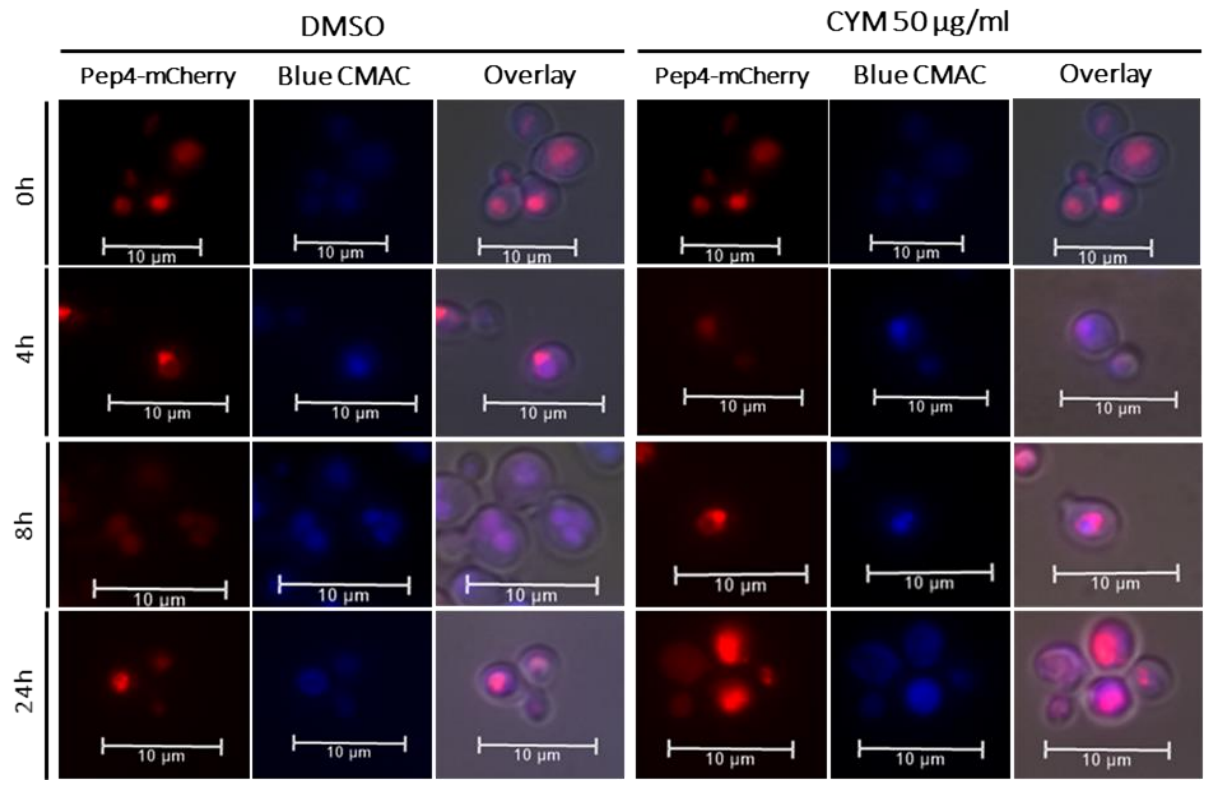

Figure 14 – Assessment of Pep4-mCherry localization and vacuolar membrane permeabilization in *S. cerevisiae* BY4741 WT cells. Cells were treated in the absence or presence of CYM for 24 hours and then stained with CMAC to assess vacuole membrane integrity. Samples were collected at different time points, before (time 0) and after 4, 8, and 24 hours of treatment, then visualized by epifluorescence microscopy with a 100x oil immersion objective. Representative epifluorescence microscopy images of WT of two independent experiments.

4.2.2. Assessment of endoplasmic reticulum morphology

Next, we assessed the effect of CYM on the endoplasmic reticulum (ER). The ER participates in protein translocation and folding, lipid synthesis, and calcium homeostasis (Zimmermann *et al.,* 2011). It is also the leading site for synthesizing sphingolipids, phospholipids, and sterols (Carman & Henry, 2007; Henry *et al.,* 2012). For that purpose, a *S. cerevisiae* strain expressing Isc1p fused with mCherry was treated with CYM and DMSO (negative control) and visualized by epifluorescence microscopy (not shown). We could not detect an effect on ER morphology with CYM treatment, even with 24 hours of treatment and for the higher concentration of CYM tested, the ER remains intact.

4.2.3. Assessment of mitochondria morphology

Mitochondria is a dynamic organelle. In healthy cells, mitochondria form an interconnected network that continually divides (fission) and fuses (fusion), and this process is essential to maintaining functional mitochondria when cells are under stress (Youle & Van Der Bliek, 2012). To assess if CYM leads to mitochondrial fragmentation, we used the BY4741 pYX-mt-GFP strain, which expresses a mitochondrial protein fused with GFP. After 4 and 8 hours of treatment, we observed that the compound does not affect

the mitochondrial network. However, after 24 hours of treatment, the cells treated with the compound lose their fluorescence (Figure 15 A). We, therefore, assessed the levels of a mitochondrial protein (Por1p) by western blot to determine if cells lose the fluorescence as a result of mitochondrial protein degradation. Western blot analysis demonstrated that 4, 8 and 24 hours of CYM treatment did not lead to Por1p degradation, which indicates that the loss of fluorescence observed in the epifluorescence microscopy wasn't caused by generalized mitochondrial protein degradation (Figure 15 B).

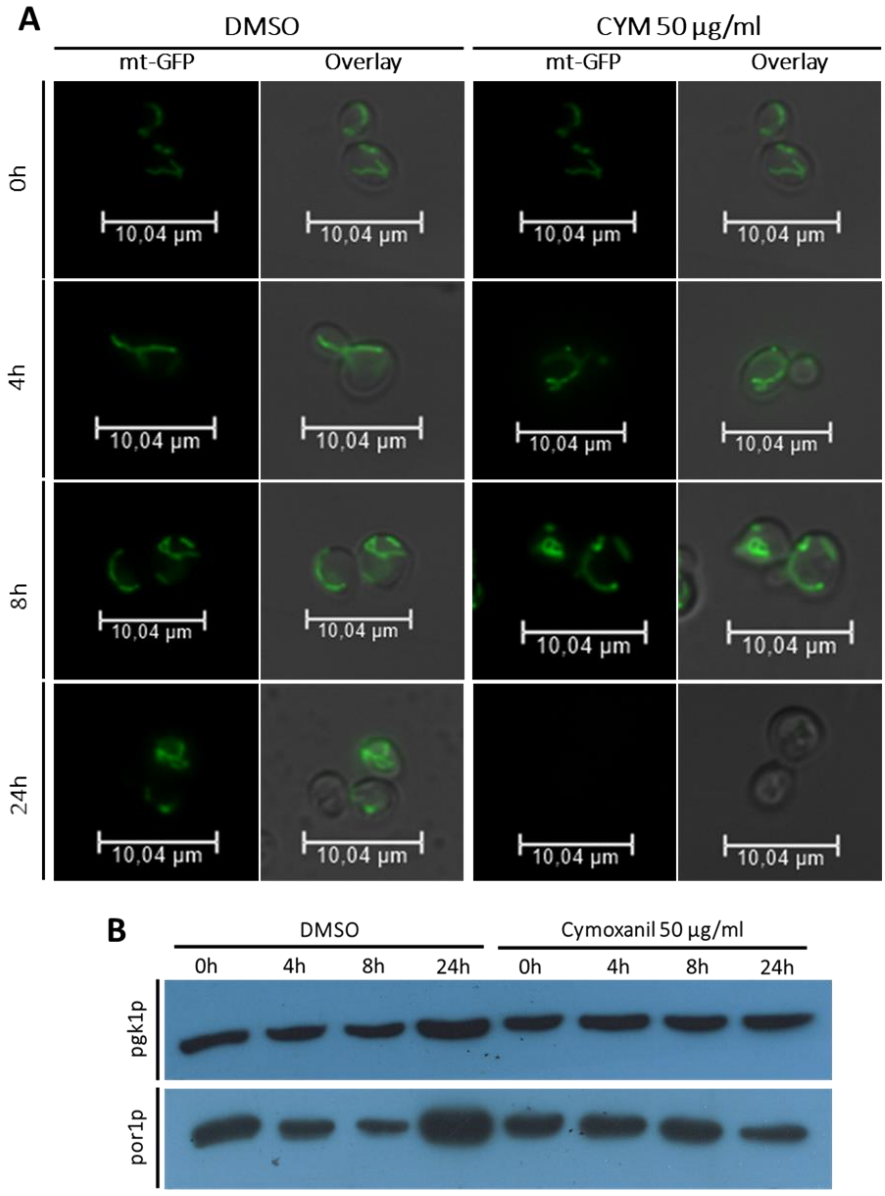

Figure 15 - Effect of cymoxanil on mitochondria. **A)** Epifluorescence microscopy images of BY4741 WT pYX-mt-GFP cells after treatment in the presence or absence of CYM in SCpro-Glu medium. Samples were collected at different time points, before (time 0) and after 4, 8, and 24 hours of treatment, then visualized by epifluorescence microscopy with a 100x oil immersion objective. **B)** Western-blot analysis of Por1p levels in BY4741 WT cells. Cells were treated as in A). Pgk1p was used as a loading control. Results from one experiment.

4.3. Assessment of cell death markers

Cell death processes are important for development and homeostasis and have been classified into multiple different forms (Galluzzi *et al.,* 2018). These can be mainly divided into regulated (RCD), of which apoptosis is the most characterized, or accidental (ACD), usually by necrosis. Many of these processes are conserved in yeasts, and there is a great number of cell death markers that can be used to characterize them (Carmona-Gutierrez *et al.,* 2010; Galluzzi *et al.,* 2018). For instance, reactive oxygen species (ROS) accumulation mediates cell apoptosis and, at very high concentrations, leads to necrosis (Farrugia & Balzan, 2012; Yakes & Van Houten, 1997). In addition, loss of mitochondrial membrane potential signals cell stress and can lead to the release of apoptotic factors such as cytochrome *c* (Gutiérrez *et al.,* 2017). On the other hand, loss of plasma membrane integrity is characteristic of a necrosis process, either primary or secondary (Eisenberg *et al.,* 2010). The nuclear release of Nhp6Ap to the cytosol has also been used as a necrosis marker (Eisenberg *et al.,* 2010). In order to identify the cell death process induced by CYM, we, therefore, assessed several of these markers.

To assess Nhp6Ap translocation, we exposed BY4741 pUG35-Nhp6A-GFP cells to CYM or DMSO (negative control) and observed the protein localization by epifluorescence microscopy. We also stained the cells with PI. Cells with an intact plasma membrane are impermeable to PI, so it can only enter cells with a compromised membrane. Once entering in the cell, PI binds to nucleic acids and red fluorescence is emitted. Therefore, PI can be used to differentiate dead cells (plasma membrane compromised) from live cells (plasma membrane intact) (Bang *et al.,* 2000). We did not observe Nhp6Ap nuclear translocation, characterized by a diffuse fluorescence in the cell, at any time. Instead, we observed that when cells lose plasma membrane integrity, stained with red (PI-positive cells), cells lose the green fluorescence of Nhp6A-GFP. These findings were more evident at 24 hours of treatment (Figure 16 A). The process may therefore not be necrotic, but since the cells lose the GFP fluorescence it is not possible to have a definitive conclusion.

Since we obtained a considerable number of PI-positive cells by epifluorescence microscopy, we proceeded with quantification of the cells that lose their plasma membrane integrity by flow cytometry. After 8 hours of treatment, we observed that about half the cells are PI-positive, and after 24 hours practically all cells had lost plasma membrane integrity (Figure 16 B).

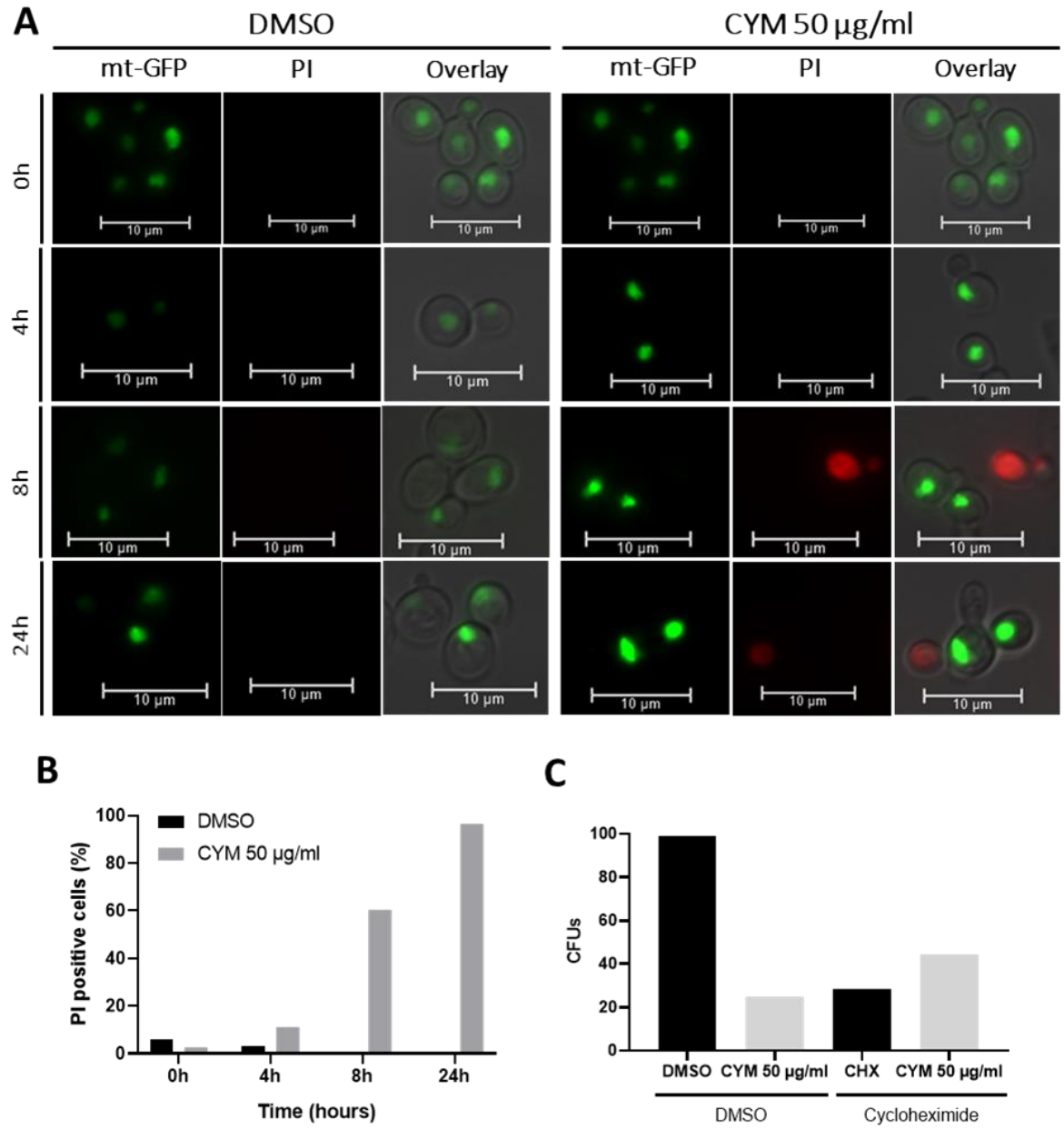

Figure 16 – Effect of CYM on *S. cerevisiae* Nhp6Ap nuclear release and plasma membrane integrity. A) Epifluorescence microscopy of Nhp6Ap cellular localization after treatment in the presence or absence of 50 µg/ml CYM in SCpro-Glu medium. Samples were collected at different time points, before (time 0) and after 4, 8, and 24 hours of treatment, then visualized by epifluorescence microscopy with a 100x oil immersion objective. **B)** Percentage of cells that lose the plasma membrane integrity assessed by flow cytometry of PI-stained cells. **C)** Effect of CYM on BY4741 WT cells treated with 100 µg/mL CHX for 4 hours. Results were obtained from one experiment.

Considering that most cells lose their plasma membrane integrity, but do not show Nhp6A in the cytosol, we addressed whether cell death induced by CYM was an active process. For that purpose, BY4741 WT cells were treated with CYM in the presence or absence of 100 µg/ml of CHX. CHX is a protein synthesis inhibitor utilized in studies of cell death (Mattson & Furukawa, 1997) and has been used

to prevent apoptosis (Madeo *et al.,* 1999; Sanchez *et al.,* 1997). However, incubation with cycloheximide alone for 4h resulted in high toxicity, and thus it was not possible to obtain a conclusion (Figure 16 C).

Among the organelles that we assessed, CYM seems to affect just the mitochondria so we considered utilizing a carbon source where mitochondria may be more active. We, therefore, tested the growth of BY4741 WT in media containing lactate, raffinose, or galactose (not shown). We selected galactose to proceed with further assays since it was the carbon source where cells grew better in the absence of CYM.

4.4. Cymoxanil inhibits cell growth and oxygen consumption in a medium containing galactose

Cells treated in a medium containing galactose as a carbon source (SC-Gal) exhibit a similar phenotype to that obtained previously, with inhibition of growth (Figure 17 A) and loss of cell viability (Figure 17 B). In this medium, exposure to 50 µg/ml CYM leads to inhibition of oxygen consumption in

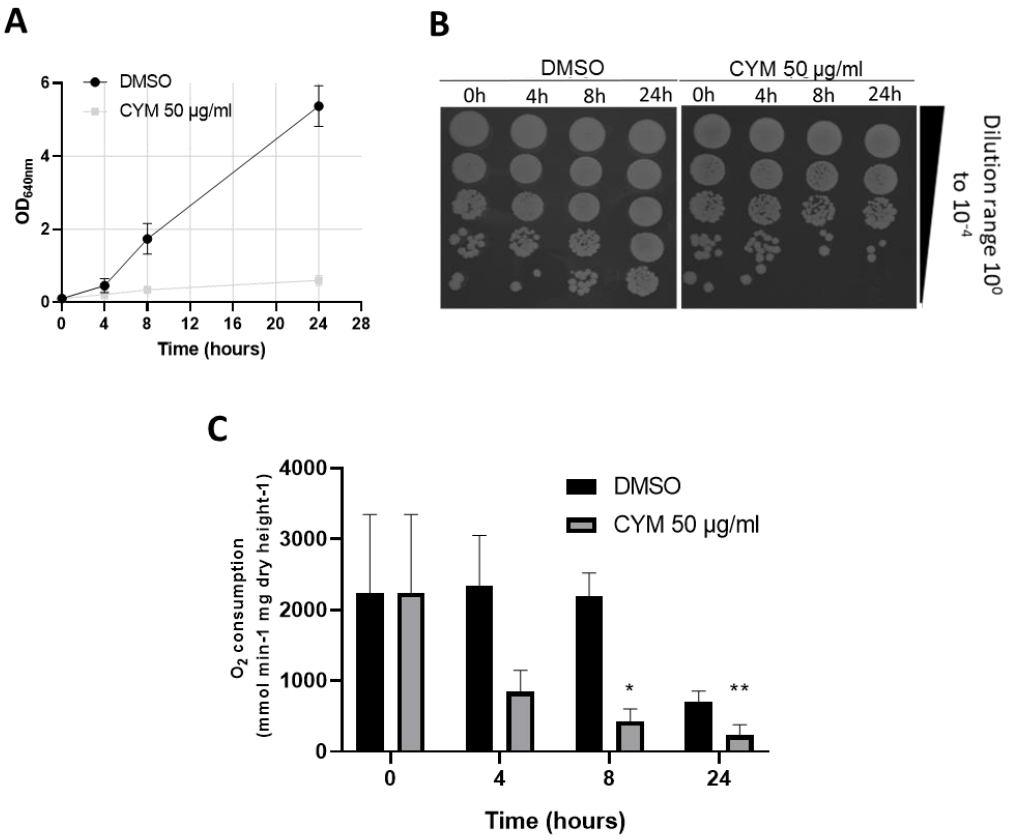

Figure 17 - Response of *S. cerevisiae* BY4741 WT cells to CYM in galactose media. A) Cells were diluted to an OD_{640nm} = 0.1, exposed to 50 µg/ml cymoxanil for 24 hours in SC-Gal media. OD_{640nm} was read at different time points until 24h of treatment. Values are mean ± SD of three independent experiments. **B)** Viability of cells treated as in A) assessed at different time points by spot assay. A representative image is shown from three independent experiments. **C)** O_2 consumption measured with Clark electrode. Cells were treated as in A) and B). Results were obtained from at least 3 independent experiments. Statistical analysis was performed by two-way ANOVA. *P<0.05, **P<0.01.

comparison with the control (Figure 17 C), indicative of the effect of CYM on mitochondria. We also found that inhibition of oxygen consumption does not occur immediately. In fact, inhibition occurs when cells are incubated with the compound for at least 3 hours (see 7. Annexes Figure A1).

4.5. Assessment of mitochondria fragmentation in medium containing galactose

Since we altered the carbon source, we also assessed the effect of CYM on cells exposed to 50 µg/ml of CYM in SC-Gal for 24 hours. In this case, we observed that, after 4 hours of treatment, some cells display a fragmented mitochondrial network. This effect on mitochondria is more evident after 8 hours. It was possible to observe mitochondrial network fragmentation in a higher number of cells after 24 hours of treatment, where we saw multiple spots and smaller mitochondria, in contrast with the intact mitochondrial network of the control (Figure 18).

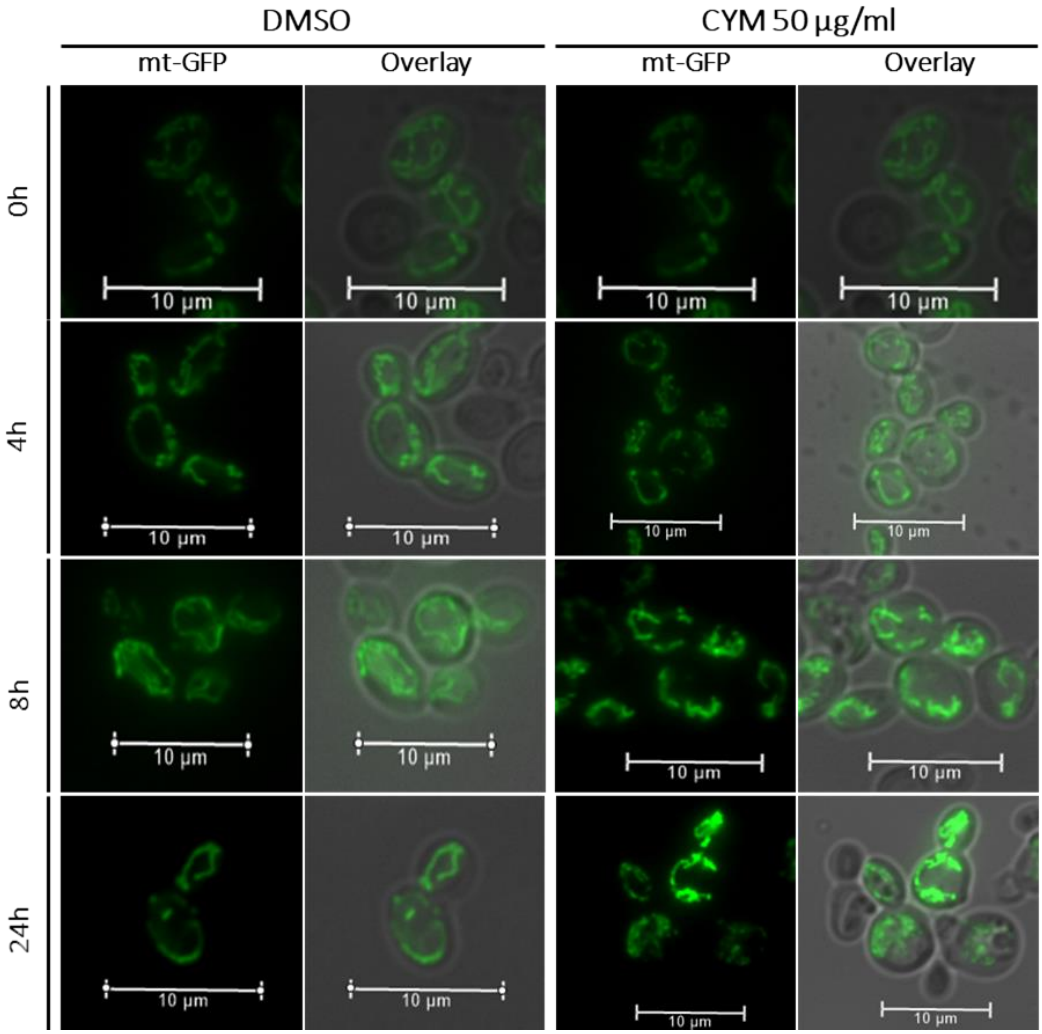

Figure 18 - Effect of CYM on mitochondrial network. Epifluorescence microscopy images of BY4741 WT pYX-mt-GFP cells after treatment in the presence or absence of CYM in SC-Gal medium. Samples were collected at different time points, before (time 0) and after 4, 8 and 24 hours of treatment, and then visualized by epifluorescence microscopy with a 100x oil immersion objective.

4.6. Functional characterization

Considering the previous observations regarding the effect of CYM on mitochondrial network fragmentation, it seems that CYM in some way is affecting the mitochondria. To assess if this effect is not only morphologic but also functional, we evaluated ROS production, changes in mitochondrial membrane potential, and mitochondrial mass.

4.6.1. Assessment of mitochondrial membrane potential and mass

The mitochondrial membrane potential ($\Delta\Psi_m$) is an indicator of cell health. To assess changes in mitochondrial membrane potential, we used the dye $DiOC_6(3)$, which accumulates in mitochondria depending on its membrane potential (Pringle *et al.,* 1989). Cells were stained with $DiOC_6(3)$ and analyzed by flow cytometry. Cells were also stained with PI in order to certify that the analysis of $\Delta\Psi_m$ is performed in cells that still did not suffer any alteration in the integrity of the plasma membrane.

After 4 hours of treatment, it is possible to observe a hyperpolarization of the mitochondrial membrane, followed by a depolarization at 8 hours of treatment (Figure 19 A and B). However, to be sure

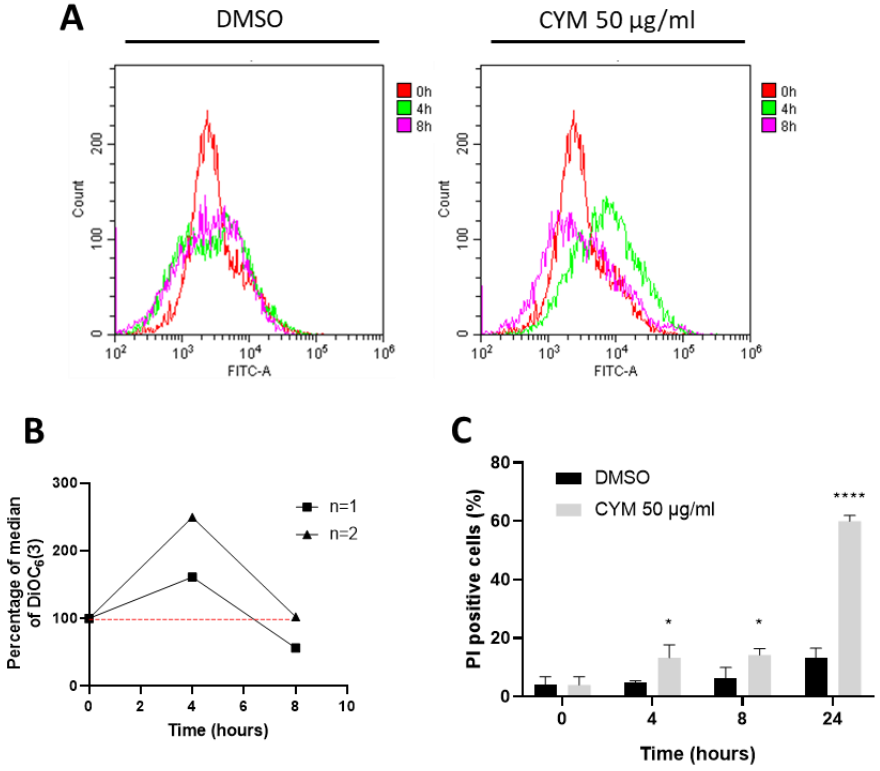

Figure 19 – Mitochondrial membrane potential assessment. Cells were treated in the presence or absence of CYM in the SC-Gal medium. Samples were collected at different time points, before (time 0) and after 4, 8, and 24 hours of treatment, and analyzed by flow cytometry. **A)** Representative image of three independent experiments of the $DiOC_6(3)$ quantification. **B)** Percentage of a median of $DiOC_6(3)$. The percentage was calculated considering the control, in all time points, as 100% (red line). **C)** Loss of plasma membrane integrity assessed by PI staining. Statistical analysis was performed by two-way ANOVA. *P<0.05, ****P<0.0001.

of the results, it is necessary repeat this assay or even utilize $DiOC_5(3)$, JC-1, or TMRM, which can also be used for mitochondrial membrane potential assessments. The 24h time point was not analyzed due to the high number of cells that had lost their plasma membrane integrity (Figure 19 C).

Figure 20 shows the mean signal of Mitrotracker Green fluorescence intensity representing the mitochondrial content during the 24 hours of treatment. The mitochondrial content slightly increased after 4 and 8 hours of treatment, but not significantly. After 24 hours of treatment, there was a more evident increase in the fluorescence, but this may be derived from the loss of plasma membrane integrity of cells thus unspecific staining of cells. To test this hypothesis, co-staining with PI was performed, but due to the high contamination of Mitrotracker Green on the PI channel (red), these results were not considered (not shown).

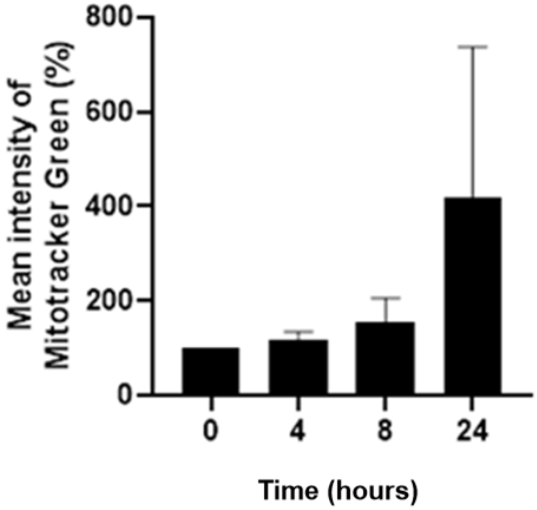

Figure 20 - Mitochondrial mass assessment through flow cytometry using the Mitrotracker Green probe. Cells were treated in the presence or absence of CYM in SC-Gal medium. Samples were collected at different time points, before (time 0) and after 4, 8, and 24 hours of treatment, and then analyzed by flow cytometry.

4.6.2. Quantification of ROS production

Next, we assessed if the compound leads to ROS accumulation. ROS accumulation has been shown to cause oxidative damage on nucleic acids, proteins, and lipids. Untreated cells and cells treated with CYM were labeled with MitoTracker Red CM-H_2XRos and Dihydroethidium (DHE). The reduced form of MitoTracker Red CM-H_2XRos only fluoresces when oxidized by ROS, emitting red fluorescence (Cottet-Rousselle *et al.,* 2011). DHE reacts with superoxide to form 2-hydroxyethidium and has been used to assess superoxide levels (Carmona-Gutierrez *et al.,* 2010; Liao *et al.,* 2020).

When MitoTracker Red CM-H_2XRos was used as a probe, the percentage of stained cells after 4 and 8 hours of CYM treatment was very low. However, after 24 hours of treatment, there was a higher percentage of ROS-positive cells (Figure 21 A). When DHE was used as a probe, staining was also low after 4 and 8 hours of treatment with CYM compared with the control (Figure 21 B). DHE-positive staining was only observed at 24 hours of treatment. However, the percentage of DHE-positive cells was very similar to that of PI-positive cells. It is, therefore, possible that the production of ROS accessed by DHE is not accurate, being affected by unspecific staining. However, since the percentage of MitoTracker Red CM-H_2XRos -positive cells was higher, it seems there is at least some increase in ROS production after 24 hours of exposure to CYM.

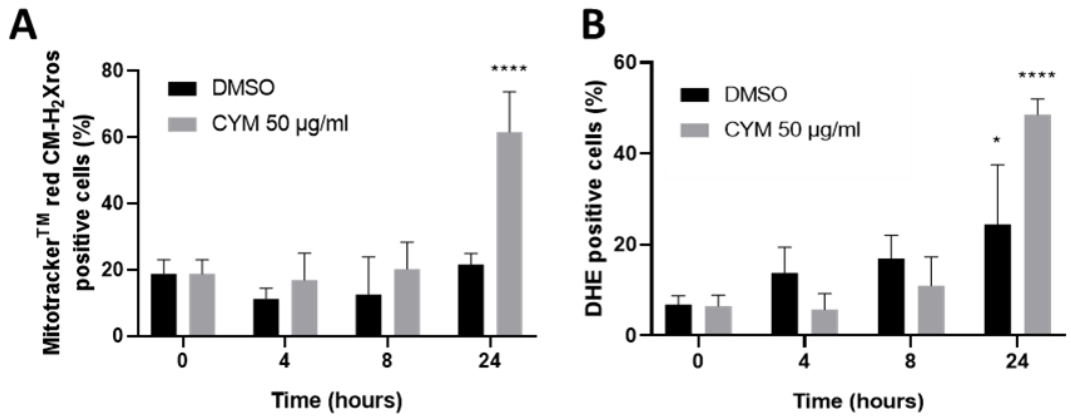

Figure 21 - Levels of mitochondrial ROS and superoxide anion in *S. cerevisiae* strains. Cells were treated in the presence or absence of 50 µg/ml CYM in SC-Gal medium. Samples were collected at different time points, before (time 0) and after 24 hours of treatment and stained with MitoTracker Red CM-H_2XRos **(A)** and DHE **(B)** and then analyzed by flow cytometry. Values are mean ± SD of at least three independent experiments. Cells were treated in the presence or absence of CYM in the SC-Gal medium. Statistical analysis was performed by two-way ANOVA. *P<0.05, ****P<0.0001.

4.6.3. Assessment of pH alterations

Several biological processes are dependent on the regulation of intracellular pH. For instance, protein structures and enzyme activity depend on this factor. In the secretory pathway, the pH is more acidic starting from the ER to secretory vesicles. This acidification is essential to activate some enzymes involved in post-translational modifications and degradation processes, as some proteases are activated in the acidic vacuole (Deschamps *et., al* 2013). In contrast, mitochondrial pH is higher, as a membrane potential and pH gradient are required to drive production of ATP (Brand & Lehningert, 1977). Given the importance of maintaining pH homeostasis, it is relevant to assess pH alterations that can be caused by CYM.

To analyze intracellular pH changes, we used BCECF, AM, a fluorescein derivative that has been used to measure the pH. BCECF, AM accumulates in the vacuole where hydrolytic enzymes cleave the acetoxy methyl ester from BCECF-AM and lead to vacuolar retention (Plant *et al.,* 1999). Changes in the pH were monitored by assessing changes in the green/red fluorescence intensity ratio. This ratio value is independent of the probe concentration and only dependent on pH, where the ratio increases as the pH decreases (Johnson *et al.,* 2010; Ozkan & Mutharasan, 2002). After 4 and 8 hours, no alteration was observed in the green/red ratio on untreated cells, while it decreased after 24h under both conditions. However, after 4 and 8 hours of treatment, we observed that CYM led to a slight increase in the ratio, indicative of acidification, while control cells were not affected (Figure 22). To confirm whether this increase is significant, it will be necessary to use a positive control in subsequent experiments.

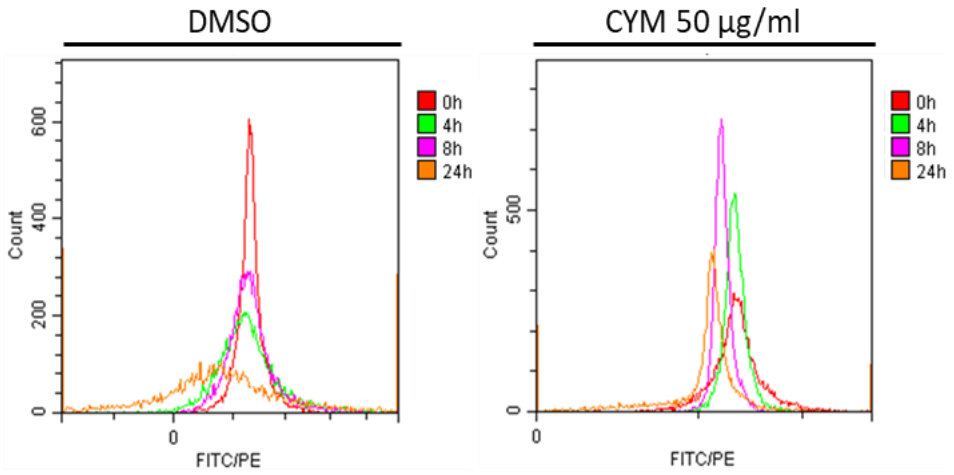

Figure 22 – Cymoxanil induces intracellular acidification in BY4741 WT cells. Representative histograms of the BCECF-AM cells displaying intracellular acidification were assessed through the decrease of FITC-A/PE-A fluorescence intensity ratio compared to negative control cells (DMSO).

4.7. Optimizing a genome-wide screen

The results obtained show that CYM leads to cell death, and likely targets the mitochondria, though more evidence of the involvement of the mitochondria in the response to CYM is needed. However, the molecular target remains unknown. To uncover which specific cellular processes can be affected by CYM, we proceed with optimization of a genome-wide screen to find strains that demonstrate sensitivity and/or resistance to the compound. First, it was necessary to optimize the exposure conditions, since the effect of any drug on microplates can be different from assays performed in tubes, due to low agitation and aeration. We first inoculated SC-Gal medium in 96-well plates with BY4741 WT cells using a pin replicator. The next day, we diluted the cells in fresh medium and added different concentrations of CYM, and

assessed growth and loss of plasma membrane integrity by PI staining, which resulted in the clearest phenotype from the initial characterization.

As shown in Figure 23 A, 100 µg/ml, and 200 ug/ml CYM partially inhibited growth of BY4741 cells, already after 8 hours of exposure. However, an increase in PI-positive cells was evident mostly after 24 hours (Figure 23 B). We, therefore, proceed with an optimization of the assay with two mutants that could be used as controls with increased sensitivity: rho^0, a strain characterized by the absence of mitochondrial DNA (mtDNA) (Dirick *et al.,* 2014) and Δ*erg6,* deleted in the gene encoding for a C-24 sterol methyltransferase, an enzyme that acts in the late steps of ergosterol biosynthesis, which has been characterized as hypersensitive to many drugs, since the cells are more permeable to the diffusion of drugs across the membrane, increasing drug uptake (Bard *et al.,* 1978; Emter *et al.,* 2002; Welihinda *et al.,* 1994).

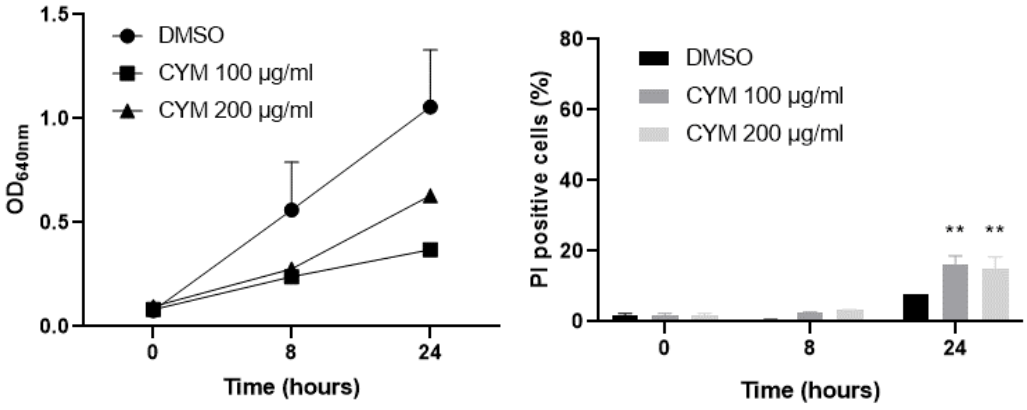

Figure 23 – Conditions optimization of the screening. A) Optical density of the BY4741 WT cells and **B)** Loss of plasma membrane integrity of the cells assessed by PI staining. Cells were treated in the presence or absence of 100 µg/ml, or 200 µg/ml CYM in the SC-Gal medium. Samples were collected at different time points, before (time 0) and after 8 and 24 hours of treatment, and then analyzed by flow cytometry. Values are mean ± SD of at least three independent experiments. Statistical analysis was performed by two-way ANOVA. *P<0.05, ****P<0.0001.

After 8 hours of treatment, we found that CYM-treated cells do not significantly lose plasma membrane integrity compared with the control. However, after 24 hours of treatment, there was a significant increase of PI-positive cells and the rho^0 strain was slightly more sensitive when compared with the WT strain. Still, it was the Δ*erg6* mutant that was most sensitive to the compound, with approximately 80 % of the cells losing their plasma membrane integrity (Figure 24).

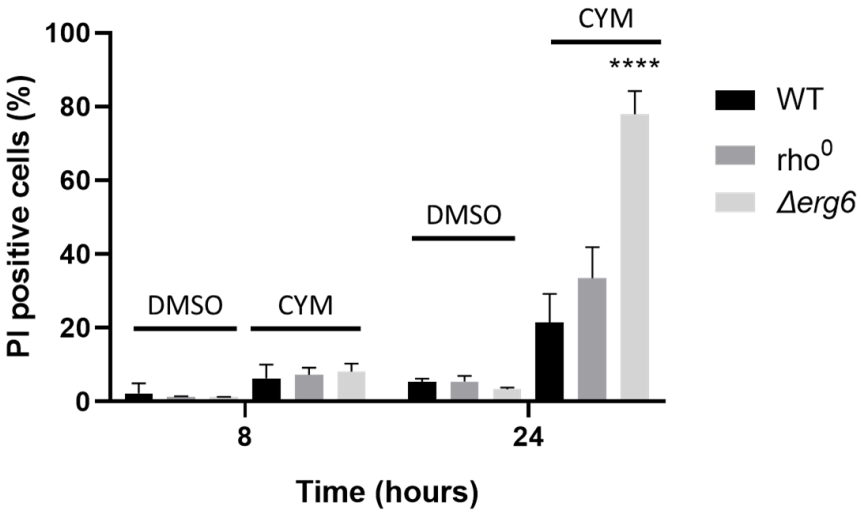

Figure 24 – Loss of plasma membrane integrity of selected mutants assessed by PI staining. BY4741 WT, Δ*erg6*, and rho◦ were treated in the presence or absence of 200 μg/ml CYM in the SC-Gal medium. Samples were collected at different time points, before (time 0) and after 8 and 24 hours of treatment, and then analyzed by flow cytometry. Values are mean ± SD of at least three independent experiments. Cells were treated in the presence or absence of CYM in the SC-Gal medium. Statistical analysis was performed by two-way ANOVA. ****P<0.0001.

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5. Conclusions and future perspectives

Cymoxanil is a fungicide that has been used for the past 24 years. However, its mechanism of action remains largely unknown (Belasco *et al.,* 1981; Fidente *et al.,* 2005; Gisi & Sierotzki, 2008; Hillebrand *et al.,* 2012). There are only a few reports regarding the effect of cymoxanil in the inhibition of mycelial growth, germ tube formation by sporangia as well the inhibition of DNA and RNA synthesis as a secondary effect in the *P. infestans* model, the causal organism of late blight in potato and tomato cultures (Ziogas & Davidse, 1987). It was also demonstrated that cymoxanil inhibits growth and respiration in the model organism *S. cerevisiae* (Estève et al., 2009; Ribeiro et al., 2000). Like other compounds that are widely used, it is necessary to understand its mode of action, to better understand the target and potential of effects, and carry out preventative and mitigation actions. One method that can increase our understanding on the cymoxanil mechanism of action in a comprehensive way is to screen the phenotype of a *S. cerevisiae* mutant collection, in which each strain has been deleted in one of the non-essential yeast genes.

In the past, our lab had already attempted to optimize a screen with cymoxanil based on growth and optical density, but no reproducible results were obtained (Carvalho, 2018). Therefore, it was necessary to perform more optimizations and uncover another read-out for a cellular phenotype. First, we proceeded with a structural and functional characterization to determine how cymoxanil affects cells. We found that cymoxanil leads to an inhibition of both cell growth and respiration, as observed by Ribeiro *et., al* (2000). The cymoxanil effect on respiration may be explained by the presence and hypothetic release of the cyan group. We didn't find information about the release of this group in yeast, but it was already described that low concentrations of cyanide, about 100 and 300 µM, lead to respiratory inhibition in *S. cerevisiae* (Peña *et., al* 2015), and thus this hypothesis should be tested. For instance, we could treat the cells with 50 µg/ml of cymoxanil for four hours (time where oxygen consumption displayed the highest inhibition), and then lyse the cells, filtrate the suspension and perform HPLC analyses. Our results also suggest that cymoxanil does not affect organelles like the vacuole and endoplasmic reticulum, but affects mitochondria, agreeing with previous indications that the mechanism of action of cymoxanil may be related to the mitochondria (Estève *et., al* (2009) and Ribeiro *et., al* (2000)).

As we found that cymoxanil leads to cell death, we sought to characterize this process. The functional characterization shows that cymoxanil leads to hyperpolarization of the mitochondrial membrane after 4 hours of treatment, followed by a depolarization after 8 hours of treatment, without alteration in mitochondrial mass. However, it will be necessary to repeat the assay or even utilize another

probe to confirm the results obtained for mitochondria membrane potential alterations. Cymoxanil also leads to loss of plasma membrane integrity and ROS accumulation after 24 hours of treatment. We also assessed the nuclear release of the Nhp6Ap, a well-known necrotic marker. However, we observed a different release pattern from the one described for necrotic processes. Instead of a uniformly dispersed localization of Nhp6Ap, cells that lose plasma membrane integrity also lose the green fluorescence of Nhp6A-GFP. So, the results obtained were inconclusive. We were also not able to conclude whether cycloheximide, a protein synthesis inhibitor, reverts cymoxanil-induced cell death, which would indicate if this process is not dependent on protein synthesis. We therefore could not definitively conclude if cymoxanil leads to a necrotic or an apoptotic-like regulated process. For this reason, it would be interesting to analyze other cell death markers like cytochrome *c* release from mitochondria, since this release is considered a critical initial step in the apoptotic process (Ott *et al.,*2002). Another marker would be the co-staining of Annexin V (AnnV) and propidium iodide (PI), where it will be possible to discriminate between early apoptotic cells ($AnnV^+$, PI^-), primary necrotic ($AnnV^-$, PI^+), and late apoptotic/secondary necrotic cells ($AnnV^+$, PI^+).

The identification of genes, in a genome-wide screen, that confer resistance or sensitivity induced by cymoxanil could be a powerful method to uncover the mechanism of action of this compound. For this reason, a screening was optimized to assess the effect of cymoxanil on *S. cerevisiae* cells. To understand the mechanism of action of cymoxanil, we took advantage of the reproducible increase in PI staining of cells treated with this compound to proceed with the optimization of a genome-wide screen. We selected two mutants as controls that could show increased sensitivity to cymoxanil: *Δerg6*, more permeable to small molecules, and rho^0, defective in mitochondrial respiration. We found that the *Δerg6* mutant was much more sensitive to the compound, likely because the cells were more permeable to small molecules. These findings reinforce the idea that the target of cymoxanil is intracellular. In contrast, rho^0 cells do not display a large difference in the percentage of cells that lose their plasma membrane integrity when compared with the control, so it's possible that cymoxanil has other molecular targets inside the cells or its mode of action can be related with respiratory processes that are not compromised in rho^0 cells. In this way, other targets cannot be excluded, and more assays need to be carried out. In a first optimization, we were able to find conditions that may be used in a larger genome-wide screen in *S. cerevisiae*, as well as a strain sensitive to the compound that can be a promising control.

The present work provides several insights regarding the mechanism of action of cymoxanil and the optimized conditions for the screening in the entire collection of *S. cerevisiae* strains deleted in non-essential genes. After that, the genes whose deletion results in sensitive or resistant phenotypes should

be clustered according to their conserved biological function to assess cellular functions affected by cymoxanil and finally uncover its mechanism of action.

6. Bibliography

Alewu, B., & Nosiri, C. (2011). Pesticides and Human Health. In M. Stoytcheva (Ed.), *Pesticides in the Modern World - Effects of Pesticides Exposure* (pp. 231–250). InTech. https://doi.org/10.5772/18734

Alexander-Dann, B., Pruteanu, L. L., Oerton, E., Sharma, N., Berindan-Neagoe, I., Módos, D., & Bender, A. (2018). Developments in toxicogenomics: Understanding and predicting compound-induced toxicity from gene expression data. *Molecular Omics*, *14*(4), 218–236. https://doi.org/10.1039/c8mo00042e

Andrew, E. J., Merchan, S., Lawless, C., Banks, A. P., Wilkinson, D. J., & Lydall, D. (2013). Pentose Phosphate Pathway Function Affects Tolerance to the G-Quadruplex Binder TMPyP4. *PLoS ONE*, *8*(6), 1–10. https://doi.org/10.1371/journal.pone.0066242

Azad, G. K., Singh, V., Golla, U., & Tomar, R. S. (2013). Depletion of Cellular Iron by Curcumin Leads to Alteration in Histone Acetylation and Degradation of Sml1p in *Saccharomyces cerevisiae*. *PLoS ONE*, *8*(3). https://doi.org/10.1371/journal.pone.0059003

Balbus, J. M., Boxall, A. B. A., Fenske, R. A., McKone, T. E., & Zeise, L. (2013). Implications of global climate change for the assessment and management of human health risks of chemicals in the natural environment. *Environmental Toxicology and Chemistry*, *32*(1), 62–78. https://doi.org/10.1002/etc.2046

Bang, K.-H., Lee, D.-W., Park, H.-M., & Rhee, Y.-H. (2000). Inhibition of Fungal Cell Wall Synthesizing Enzymes by trans -Cinnamaldehyde. *Bioscience, Biotechnology, and Biochemistry*, *64*(5), 1061–1063. https://doi.org/10.1271/bbb.64.1061

Bard, M., Lees, N. D., Burrows, L. S., & Kleinhans, F. W. (1978). Differences in crystal violet uptake and cation-induced death among yeast sterol mutants. *Journal of Bacteriology*, *135*(3), 1146–1148. https://doi.org/10.1128/jb.135.3.1146-1148.1978

Baudin, A., Ozier-kalogeropoulos, O., Denouel, A., Lacroute, F., & Cullin, C. (1993). A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Research*, *21*(14), 3329–3330. https://doi.org/10.1093/nar/21.14.3329

Beaman, J., Co-chair, W., Eignor, D., & Huff, L. (2008). *Aquatic life criteria for contaminants of emerging concern*.

Belasco, I. J., Han, J. C., Chrzanowski, R. L., & Baude, F. J. (1981). Metabolism of [14C] Cymoxanil in Grapes , Potatoes and Tomatoes. 355–364. https://doi.org/https://doi.org/10.1002/ps.2780120402

Bjorn, H. (2007). European Chemicals Agency (ECHA). Retrieved October 2, 2021, from https://european-union.europa.eu/institutions-law-budget/institutions-and-bodies/institutions-and-bodies-profiles/echa_en

Bowie, D., Parvizi, P., Duncan, D., Nelson, C. J., & Fyles, T. M. (2013). Chemical-genetic identification of the biochemical targets of polyalkyl guanidinium biocides. *Organic and Biomolecular Chemistry*, *11*(26), 4359–4366. https://doi.org/10.1039/c3ob40593a

Brand, M. D., & Lehningert, A. L. (1977). H + / ATP ratio during ATP hydrolysis by mitochondria. *Modification of the chemiosmotic theory*. *74*(5), 1955–1959. https://doi.org/https://doi.org/10.1073/pnas.74.5.1955

Bryant, N. J., & Stevens, T. H. (1998). Vacuole Biogenesis in *Saccharomyces cerevisiae*: Protein Transport Pathways to the Yeast Vacuole . *Microbiology and Molecular Biology Reviews*, *62*(1), 230–247. https://doi.org/10.1128/mmbr.62.1.230-247.1998

Buede, R., Rinker-Schaffer, C., Pinto, W. J., Lester, R. L., & Dickson, R. C. (1991). Cloning and characterization of LCB1, a *Saccharomyces* gene required for biosynthesis of the long-chain base component of sphingolipids. *Journal of Bacteriology*, *173*(14), 4325–4332. https://doi.org/10.1128/jb.173.14.4325-4332.1991

Calvo, I. A., Gabrielli, N., Iglesias-Baena, I., García-Santamarina, S., Hoe, K. L., Kim, D. U., ... Hidalgo, E. (2009). Genome-wide screen of genes required for caffeine tolerance in fission yeast. *PLoS ONE*, *4*(8). https://doi.org/10.1371/journal.pone.0006619

Canossa, J. (2017). *Characterization of vacuole permeabilization and HMGB1 nuclear release in the yeast cell death induced by a benzo[a]phenoxazine derivative*. Universidade do Minho.

Carman, G. M., & Henry, S. A. (2007). Special issue: Regulation of lipid metabolism in yeast. *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, *1771*(3), 239–240. https://doi.org/10.1016/j.bbalip.2006.11.001

Carmona-Gutierrez, D., Eisenberg, T., Büttner, S., Meisinger, C., Kroemer, G., & Madeo, F. (2010). Apoptosis in yeast: Triggers, pathways, subroutines. *Cell Death and Differentiation*, *17*(5), 763–773. https://doi.org/10.1038/cdd.2009.219

Carvalho, C. (2018). *Novel yeast biosensors for environmental monitoring*. University of Minho.

Carvalho, F. P. (2006). Agriculture, pesticides, food security and food safety. *Environmental Science and Policy*, *9*(7–8), 685–692. https://doi.org/10.1016/j.envsci.2006.08.002

Chavan-Gautam, P., Shah, T., & Joshi, K. (2017). Transcriptomics and Epigenomics. In *Innovative Approaches in Drug Discovery: Ethnopharmacology, Systems Biology and Holistic Targeting*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-801814-9.00008-8

Costa, A. R., Machado, N., Rego, A., Sousa, M. J., Côrte-Real, M., & Chaves, S. R. (2019). Proteasome inhibition prevents cell death induced by the chemotherapeutic agent cisplatin downstream of DNA damage. *DNA Repair*, *73*(October 2018), 28–33. https://doi.org/10.1016/j.dnarep.2018.10.006

Cottet-Rousselle, C., Ronot, X., Leverve, X., & Mayol, J. F. (2011). Cytometric assessment of mitochondria using fluorescent probes. *Cytometry Part A*, *79 A*(6), 405–425. https://doi.org/10.1002/cyto.a.21061

Cuesta-Marbán, Á., Botet, J., Czyz, O., Cacharro, L. M., Gajate, C., Hornillos, V., … Mollinedo, F. (2013). Drug uptake, lipid rafts, and vesicle trafficking modulate resistance to an anticancer lysophosphatidylcholine analogue in yeast. *Journal of Biological Chemistry*, *288*(12), 8405–8418. https://doi.org/10.1074/jbc.M112.425769

Darvishi, E., Omidi, M., Bushehri, A. A., Golshani, A., & Smith, M. L. (2013a). Thymol antifungal mode of action involves telomerase inhibition. *Medical Mycology*, *51*(8), 826–834. https://doi.org/10.3109/13693786.2013.795664

Darvishi, E., Omidi, M., Bushehri, A. A. S., Golshani, A., & Smith, M. L. (2013b). The Antifungal Eugenol Perturbs Dual Aromatic and Branched-Chain Amino Acid Permeases in the Cytoplasmic Membrane of Yeast. *PLoS ONE*, *8*(10). https://doi.org/10.1371/journal.pone.0076028

Daskalova, A. V., Tomova, A. A., Kujumdzieva, A. V., Velkova, L. G., Dolashka, P. A., & Petrova, V. Y. (2021). Menadione and hydrogen peroxide trigger specific alterations in RNA polymerases profiles in quiescent *Saccharomyces cerevisiae* cells. *Biotechnology and Biotechnological Equipment*, *35*(1), 1190–1199. https://doi.org/10.1080/13102818.2021.1941255

Deschamps, A., Colinet, A. S., Zimmermannova, O., Sychrova, H., & Morsomme, P. (2020). A new pH sensor localized in the Golgi apparatus of *Saccharomyces cerevisiae* reveals unexpected roles of Vph1p and Stv1p isoforms. *Scientific Reports*, *10*(1), 1–11. https://doi.org/10.1038/s41598-020-58795-w

Dirick, L., Bendris, W., Loubiere, V., Gostan, T., Gueydon, E., & Schwob, E. (2014). Metabolic and environmental conditions determine nuclear genomic instability in budding yeast lacking mitochondrial DNA. *G3: Genes, Genomes, Genetics*, *4*(3), 411–423. https://doi.org/10.1534/g3.113.010108

Donley, N. (2019). The USA lags behind other agricultural nations in banning harmful pesticides. *Environmental Health: A Global Access Science Source*, *18*(1), 1–12. https://doi.org/10.1186/s12940-019-0488-0

dos Santos, S. C., & Sá-Correia, I. (2015). Yeast toxicogenomics: Lessons from a eukaryotic cell model and cell factory. *Current Opinion in Biotechnology*, *33*, 183–191. https://doi.org/10.1016/j.copbio.2015.03.001

dos Santos, S. C., Teixeira, M. C., Cabrito, T. R., & Sá-Correia, I. (2012). Yeast toxicogenomics: Genome-wide responses to chemical stresses with impact in environmental health, pharmacology, and biotechnology. *Frontiers in Genetics*, *3*(APR), 1–17. https://doi.org/10.3389/fgene.2012.00063

Eisenberg, T., Carmona-Gutierrez, D., Büttner, S., Tavernarakis, N., & Madeo, F. (2010). Necrosis in yeast. *Apoptosis*, *15*(3), 257–268. https://doi.org/10.1007/s10495-009-0453-4

Emter, R., Heese-Peck, A., & Kralli, A. (2002). ERG6 and PDR5 regulate small lipophilic drug accumulation in yeast cells via distinct mechanisms. *FEBS Letters*, *521*(1–3), 57–61. https://doi.org/10.1016/S0014-5793(02)02818-1

Engel, S. R., Dietrich, F. S., Fisk, D. G., Binkley, G., Balakrishnan, R., Costanzo, M. C., … Cherry, J. M. (2014). The Reference Genome Sequence of *Saccharomyces cerevisiae*: Then and Now. *G3: Genes, Genomes, Genetics*, *4*(3), 389–398. https://doi.org/10.1534/g3.113.008995

Estève, K., Poupot, C., Dabert, P., Mietton-Peuchot, M., & Milisic, V. (2009). A *Saccharomyces cerevisiae*-based bioassay for assessing pesticide toxicity. *Journal of Industrial Microbiology and Biotechnology*, *36*(12), 1529–1534. https://doi.org/10.1007/s10295-009-0649-1

European Commission. (2020). Cymoxanil. *Pharmaceuticals Policy and Law*, *1*.

European Parliament Council of the European Union, C. regulation 1107/2009. (2009). Concerning the placing of plant Protection products on the market and repealing council directives 79/117/EEC and 91/414/EEC; 2009. *Official Journal of the European Union*, *309*(1), 1–50.

Fang, Y., Hu, L., Zhou, X., Jaiseng, W., Zhang, B., Takami, T., & Kuno, T. (2012). A genomewide screen

in *Schizosaccharomyces pombe* for genes affecting the sensitivity of antifungal drugs that target ergosterol biosynthesis. *Antimicrobial Agents and Chemotherapy*, *56*(4), 1949–1959. https://doi.org/10.1128/AAC.05126-11

FAO. (2019). Food and Agriculture Organization. Retrieved June 5, 2020, from http://www.fao.org/faostat/en/#data/RP

Farrugia, G., & Balzan, R. (2012). Oxidative stress and programmed cell death in yeast. *Frontiers in Oncology*, *2 JUN*(June), 1–21. https://doi.org/10.3389/fonc.2012.00064

Ferrando-Climent, L., Rodriguez-Mozaz, S., & Barceló, D. (2013). Development of a UPLC-MS/MS method for the determination of ten anticancer drugs in hospital and urban wastewaters, and its application for the screening of human metabolites assisted by information-dependent acquisition tool (IDA) in sewage samples. *Analytical and Bioanalytical Chemistry*, *405*(18), 5937–5952. https://doi.org/10.1007/s00216-013-6794-4

Ferrando-Climent, L., Rodriguez-Mozaz, S., & Barceló, D. (2014). Incidence of anticancer drugs in an aquatic urban system: From hospital effluents through urban wastewater to natural environment. *Environmental Pollution*, *193*, 216–223. https://doi.org/10.1016/j.envpol.2014.07.002

Fidente, P., Di Giovanni, C., Seccia, S., & Morrica, P. (2005). Determination of cymoxanil in drinking water and soil using high-performance liquid chromatography. *Biomedical Chromatography*, *19*(10), 766–770. https://doi.org/10.1002/bmc.513

Galluzzi, L., Vitale, I., Aaronson, S. A., Abrams, J. M., Adam, D., Agostinis, P., … Kroemer, G. (2018). Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death and Differentiation*, *25*(3), 486–541. https://doi.org/10.1038/s41418-017-0012-4

Galván Márquez, I., Akuaku, J., Cruz, I., Cheetham, J., Golshani, A., & Smith, M. L. (2013). Disruption of protein synthesis as antifungal mode of action by chitosan. *International Journal of Food Microbiology*, *164*(1), 108–112. https://doi.org/10.1016/j.ijfoodmicro.2013.03.025

Gaytán, B. D., Loguinov, A. V., De La Rosa, V. Y., Lerot, J. M., & Vulpe, C. D. (2013). Functional genomics indicates yeast requires Golgi/ER transport, chromatin remodeling, and DNA repair for low dose DMSO tolerance. *Frontiers in Genetics*, *4*(AUG), 1–9. https://doi.org/10.3389/fgene.2013.00154

Gaytán, B. D., Loguinov, A. V., Lantz, S. R., Lerot, J. M., Denslow, N. D., & Vulpe, C. D. (2013). Functional profiling discovers the dieldrin organochlorinated pesticide affects leucine availability in yeast.

Toxicological Sciences, *132*(2), 347–358. https://doi.org/10.1093/toxsci/kft018

Gaytán, B. D., & Vulpe, C. D. (2014). Functional toxicology: Tools to advance the future of toxicity testing. *Frontiers in Genetics*, *5*(MAY), 1–11. https://doi.org/10.3389/fgene.2014.00110

Genet, J. L., & Vincent, O. (1999). Sensitivity of European *Plasmopara viticola* populations to cymoxanil. *Pesticide Science*, *55*(2), 129–136. https://doi.org/10.1002/(SICI)1096-9063(199902)55:2<129::AID-PS884>3.0.CO;2-H

Giaever, G., Chu, A. M., Ni, L., Connelly, C., Riles, L., Véronneau, S., … Johnston, M. (2002). Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature*, *418*(6896), 387–391. https://doi.org/10.1038/nature00935

Gisi, U., & Sierotzki, H. (2008). Fungicide modes of action and resistance in downy mildews. *European Journal of Plant Pathology*, *122*(1), 157–167. https://doi.org/10.1007/s10658-008-9290-5

González-Ramos, D., Van Den Broek, M., Van Maris, A. J. A., Pronk, J. T., & Daran, J. M. G. (2013). Genome-scale analyses of butanol tolerance in *Saccharomyces cerevisiae* reveal an essential role of protein degradation. *Biotechnology for Biofuels*, *6*(1), 1–18. https://doi.org/10.1186/1754-6834-6-48

Guan, M., Xia, P., Tian, M., Chen, D., & Zhang, X. (2020). Molecular fingerprints of conazoles via functional genomic profiling of *Saccharomyces cerevisiae*. *Toxicology in Vitro*, *69*(July), 104998. https://doi.org/10.1016/j.tiv.2020.104998

Guan, M., Zhu, Z., Jiang, Y., Tian, M., Yan, L., Xu, X., … Zhang, X. (2022). Assessment of genotoxic chemicals using chemogenomic profiling based on gene-knockout library in *Saccharomyces cerevisiae*. *Toxicology in Vitro*, *79*(July 2021), 105278. https://doi.org/10.1016/j.tiv.2021.105278

Gutiérrez, L., Stepien, G., Gutiérrez, L., Pérez-Hernández, M., Pardo, J., Pardo, J., … de la Fuente, J. M. (2017). Nanotechnology in drug discovery and development. In *Comprehensive Medicinal Chemistry III* (Vol. 1–8). https://doi.org/10.1016/B978-0-12-409547-2.12292-9

Hamadeh, H. K., Amin, R. P., Paules, R. S., & Afshari, C. A. (2002). An overview of toxicogenomics. *Current Issues in Molecular Biology*, *4*(2), 45–56. https://doi.org/10.21775/cimb.004.045

Harrill, A. H. (2008). *Toxicogenomic analysis of risk factors that predict sensitivity to acetaminophen-induced liver injury using a mouse model of the human population*. University of North Carolina.

Henry, S. A., Kohlwein, S. D., & Carman, G. M. (2012). Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. *Genetics*, *190*(2), 317–349. https://doi.org/10.1534/genetics.111.130286

Hillebrand, S., Tietjen, K., & Zundel, J.-L. (2019). Fungicides with unknown mode of action. in *modern crop protection compounds* (vol. 1, pp. 911–932). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527699261.ch23

Hillebrand, S., Zundel, J.-L., & Tietjen, K. (2012). Fungicides with unknown mode of action. in *modern crop protection compounds* (vol. 1, pp. 865–885). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA. https://doi.org/10.1002/9783527644179.ch23

Hoepfner, D., Helliwell, S. B., Sadlish, H., Schuierer, S., Filipuzzi, I., Brachat, S., ... Movva, N. R. (2014). High-resolution chemical dissection of a model eukaryote reveals targets, pathways and gene functions. *Microbiological Research*, *169*(2–3), 107–120. https://doi.org/10.1016/j.micres.2013.11.004

Huang, D., Piening, B. D., & Paulovich, A. G. (2013). The preference for error-free or error-prone postreplication repair in *saccharomyces cerevisiae* exposed to low-dose methyl methanesulfonate is cell cycle dependent. *Molecular and Cellular Biology*, *33*(8), 1515–1527. https://doi.org/10.1128/mcb.01392-12

Huang, Y., Chen, Z., Meng, Y., Wei, Y., Xu, Z., Ma, J., ... Lu, H. (2020). Famoxadone-cymoxanil induced cardiotoxicity in zebrafish embryos. *Ecotoxicology and Environmental Safety*, *205*(September), 111339. https://doi.org/10.1016/j.ecoenv.2020.111339

Islahudin, F., Khozoie, C., Bates, S., Ting, K. N., Pleass, R. J., & Avery, S. V. (2013). Cell wall perturbation sensitizes fungi to the antimalarial drug chloroquine. *Antimicrobial Agents and Chemotherapy*, *57*(8), 3889–3896. https://doi.org/10.1128/AAC.00478-13

Johnson, R. M., Allen, C., Melman, S. D., Waller, A., Young, S. M., Sklar, L. A., & Parra, K. J. (2010). Identification of inhibitors of vacuolar proton-translocating ATPase pumps in yeast by high-throughput screening flow cytometry. *Analytical Biochemistry*, *398*(2), 203–211. https://doi.org/10.1016/j.ab.2009.12.020

Judson, R., Richard, A., Dix, D. J., Houck, K., Martin, M., Kavlock, R., ... Smith, E. (2009). The toxicity data landscape for environmental chemicals. *Environmental Health Perspectives*, *117*(5), 685–695. https://doi.org/10.1289/ehp.0800168

Kallifatidis, G., Hoepfner, D., Jaeg, T., Guzmán, E. A., & Wright, A. E. (2014). Correction: Kallifatidis, G. et al. The marine natural product manzamine a targets vacuolar atpases and inhibits autophagy in pancreatic cancer cells (Marine Drugs (2013) 11, (3500-3516)). *Marine Drugs*, *12*(4), 2305–2307. https://doi.org/10.3390/md12042305

Kapitzky, L., Beltrao, P., Berens, T. J., Gassner, N., Zhou, C., Wüster, A., ... Krogan, N. J. (2010). Cross-species chemogenomic profiling reveals evolutionarily conserved drug mode of action. *Molecular Systems Biology*, *6*(451), 1–13. https://doi.org/10.1038/msb.2010.107

Kennedy, P. J., Vashisht, A. A., Hoe, K. L., Kim, D. U., Park, H. O., Hayles, J., & Russell, P. (2008). A genome-wide screen of genes involved in cadmium tolerance in *Schizosaccharomyces pombe*. *Toxicological Sciences*, *106*(1), 124–139. https://doi.org/10.1093/toxsci/kfn153

Kuroda, T., Tani, M., Moriguchi, A., Tokunaga, S., Higuchi, T., Kitada, S., & Kuge, O. (2011). FMP30 is required for the maintenance of a normal cardiolipin level and mitochondrial morphology in the absence of mitochondrial phosphatidylethanolamine synthesis. *Molecular Microbiology*, *80*(1), 248–265. https://doi.org/10.1111/j.1365-2958.2011.07569.x

Liao, P. C., Yang, E. J., & Pon, L. A. (2020). Live-cell imaging of mitochondrial redox state in yeast cells. *STAR Protocols*, *1*(3). https://doi.org/10.1016/j.xpro.2020.100160

Lis, M., Bhatt, S., Schoenly, N. E., Lee, A. Y., Nislow, C., & Bobek, L. A. (2013). Chemical genomic screening of a *Saccharomyces cerevisiae* genomewide mutant collection reveals genes required for defense against four antimicrobial peptides derived from proteins found in human saliva. *Antimicrobial Agents and Chemotherapy*, *57*(2), 840–847. https://doi.org/10.1128/AAC.01439-12

Liu, Y., Pan, X., & Li, J. (2015). A 1961–2010 record of fertilizer use, pesticide application and cereal yields: a review. *Agronomy for Sustainable Development*, *35*(1), 83–93. https://doi.org/10.1007/s13593-014-0259-9

Lum, P. Y., Armour, C. D., Stepaniants, S. B., Cavet, G., Wolf, M. K., Butler, J. S., ... Shoemaker, D. D. (2004). Discovering modes of action for therapeutic compounds using a genome-wide screen of yeast heterozygotes. *Cell*, *116*(1), 121–137. https://doi.org/10.1016/S0092-8674(03)01035-3

Ma, Y., Jiang, W., Liu, Q., Ryuko, S., & Kuno, T. (2011). Genome-wide screening for genes associated with FK506 sensitivity in fission yeast. *PLoS ONE*, *6*(8). https://doi.org/10.1371/journal.pone.0023422

Madeo, F., Fröhlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H., & Fröhlich, K. U. (1999). Oxygen stress: A regulator of apoptosis in yeast. *Journal of Cell Biology*, *145*(4), 757–767. https://doi.org/10.1083/jcb.145.4.757

Martins, C., Dreij, K., & Costa, P. M. (2019). The state-of-the art of environmental toxicogenomics: Challenges and perspectives of "omics" approaches directed to toxicant mixtures. *International Journal of Environmental Research and Public Health*, *16*(23), 1–18. https://doi.org/10.3390/ijerph16234718

Mason, D. A., Shulga, N., Undavai, S., Ferrando-May, E., Rexach, M. F., & Goldfarb, D. S. (2005). Increased nuclear envelope permeability and Pep4p-dependent degradation of nucleoporins during hydrogen peroxide-induced cell death. *FEMS Yeast Research*, *5*(12), 1237–1251. https://doi.org/10.1016/j.femsyr.2005.07.008

Mattiazzi Ušaj, M., Kaferle, P., Toplak, A., Trebše, P., & Petrovič, U. (2014). Determination of toxicity of neonicotinoids on the genome level using chemogenomics in yeast. *Chemosphere*, *104*, 91–96. https://doi.org/10.1016/j.chemosphere.2013.10.063

Mattson, M. P., & Furukawa, K. (1997). Anti-apoptotic actions of cycloheximide: Blockade of programmed cell death or induction of programmed cell life? *Apoptosis*, *2*(3), 257–264. https://doi.org/10.1023/A:1026433019210

McCusker, J. H., & Davis, R. W. (1991). The use of proline as a nitrogen source causes hypersensitivity to, and allows more economical use of 5FOA in *Saccharomyces cerevisiae*. *Yeast*, *7*(6), 607–608. https://doi.org/10.1002/yea.320070608

Mensah, P. K., Palmer, C. G., & Muller, W. J. (2014). Lethal and sublethal effects of pesticides on aquatic organisms: the case of a freshwater shrimp exposure to roundup®. In *Pesticides - Toxic Aspects*: *Vol. i* (p. 13). InTech. https://doi.org/10.5772/57166

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical pesticides and human health: The urgent need for a new concept in agriculture. *Frontiers in Public Health*, *4*(July), 1–8. https://doi.org/10.3389/fpubh.2016.00148

Nislow, C., Wong, L. H., Lee, A. H. Y., & Giaever, G. (2016). Functional genomics using the *Saccharomyces cerevisiae* yeast deletion collections. *Cold Spring Harbor Protocols*, *2016*(9), 751–756. https://doi.org/10.1101/pdb.top080945

North, M., Gaytán, B. D., Romero, C., De La Rosa, V. Y., Loguinov, A., Smith, M. T., ... Vulpe, C. D.

(2016). Functional toxicogenomic profiling expands insight into modulators of formaldehyde toxicity in yeast. *Frontiers in Genetics*, *7*(NOV), 1–11. https://doi.org/10.3389/fgene.2016.00200

North, M., & Vulpe, C. D. (2010). Functional toxicogenomics: Mechanism-centered toxicology. *International Journal of Molecular Sciences*, *11*(12), 4796–4813. https://doi.org/10.3390/ijms11124796

Noutsopoulos, C., Koumaki, E., Sarantopoulos, V., & Mamais, D. (2019). Analytical and mathematical assessment of emerging pollutants fate in a river system. *Journal of Hazardous Materials*. https://doi.org/https://doi.org/10.1016/j.jhazmat.2018.10.033

Nuwaysir, E. F., Bittner, M., Trent, J., Barrett, J. C., & Afshari, C. A. (1999). Microarrays and toxicology: The advent of toxicogenomics. *Molecular Carcinogenesis*, *24*(3), 153–159. https://doi.org/10.1002/(SICI)1098-2744(199903)24:3<153::AID-MC1>3.0.CO;2-P

Ott, M., Robertson, J. D., Gogvadze, V., Zhivotovsky, B., & Orrenius, S. (2002). Cytochrome *c* release from mitochondria proceeds by a two-step process. *Proceedings of the National Academy of Sciences*, *99*(3), 1259–1263. https://doi.org/10.1073/pnas.241655498

Ozkan, P., & Mutharasan, R. (2002). A rapid method for measuring intracellular pH using BCECF-AM. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1572*(1), 143–148. https://doi.org/10.1016/S0304-4165(02)00303-3

Pallares, R. M., Faulkner, D., An, D. D., Hébert, S., Loguinov, A., Proctor, M., ... Abergel, R. J. (2021). Genome-wide toxicogenomic study of the lanthanides sheds light on the selective toxicity mechanisms associated with critical materials. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(18). https://doi.org/10.1073/pnas.2025952118

Pan, X., Lei, B., Zhou, N., Feng, B., Yao, W., Zhao, X., ... Lu, H. (2012). Identification of novel genes involved in DNA damage response by screening a genome-wide *Schizosaccharomyces pombe* deletion library. *BMC Genomics*, *13*(1), 1–15. https://doi.org/10.1186/1471-2164-13-662

Pannunzio, V. G., Burgos, H. I., Alonso, M., Mattoon, J. R., Ramos, E. H., & Stella, C. A. (2004). A simple chemical method for rendering wild-type yeast permeable to brefeldin A that does not require the presence of an *erg6* mutation. *Journal of Biomedicine and Biotechnology*, *2004*(3), 150–155. https://doi.org/10.1155/S1110724304308077

Parasuraman, S. (2011). Toxicological screening. *Journal of Pharmacology and Pharmacotherapeutics*, *2*(2), 74–79. https://doi.org/10.4103/0976-500X.81895

Parsons, A. B., Brost, R. L., Ding, H., Li, Z., Zhang, C., Sheikh, B., ... Boone, C. (2004). Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. *Nature Biotechnology*, *22*(1), 62–69. https://doi.org/10.1038/nbt919

Peña, A., Sánchez, N. S., González-López, O., & Calahorra, M. (2015). Mechanisms involved in the inhibition of glycolysis by cyanide and antimycin a in *Candida albicans* and its reversal by hydrogen peroxide: A common feature in candida species. *FEMS Yeast Research*, *15*(8), 1–11. https://doi.org/10.1093/femsyr/fov083

Pereira, C., Chaves, S., Alves, S., Salin, B., Camougrand, N., Manon, S., ... Côrte-Real, M. (2010). Mitochondrial degradation in acetic acid-induced yeast apoptosis: The role of Pep4 and the ADP/ATP carrier. *Molecular Microbiology*, *76*(6), 1398–1410. https://doi.org/10.1111/j.1365-2958.2010.07122.x

Phillips, P. J., Smith, S. G., Kolpin, D. W., Zaugg, S. D., Buxton, H. T., Furlong, E. T., ... Stinson, B. (2010). Pharmaceutical formulation facilities as sources of opioids and other pharmaceuticals to wastewater treatment plant effluents. *Environmental Science and Technology*, *44*(13), 4910–4916. https://doi.org/10.1021/es100356f

Plant, P. J., Manolson, M. F., Grinstein, S., & Demaurex, N. (1999). Alternative mechanisms of vacuolar acidification in H+-ATPase-deficient yeast. *Journal of Biological Chemistry*, *274*(52), 37270–37279. https://doi.org/10.1074/jbc.274.52.37270

Pringle, J. R., Preston, R. A., Adams, A. E. M., Stearns, T., Drubin, D. G., Haarer, B. K., & Jones, E. W. (1989). Fluorescence microscopy methods for yeast. *Methods in Cell Biology*, *31*(C), 357–435. https://doi.org/10.1016/S0091-679X(08)61620-9

PubChem Compound Summary for CID 5361250. (2021). National Center for Biotechnology Information. Retrieved November 2, 2021, from https://pubchem.ncbi.nlm.nih.gov/compound/cymoxanil.

Pupo, A., Ku, K. M., & Gallagher, J. E. G. (2019). Effects of MCHM on yeast metabolism. *PLoS ONE*, *14*(10), 1–24. https://doi.org/10.1371/journal.pone.0223909

Quek, N. C. H., Matthews, J. H., Bloor, S. J., Jones, D. A., Bircham, P. W., Heathcott, R. W., & Atkinson, P. H. (2013). The novel equisetin-like compound, TA-289, causes aberrant mitochondrial morphology which is independent of the production of reactive oxygen species in *Saccharomyces cerevisiae*. *Molecular BioSystems*, *9*(8), 2125–2133. https://doi.org/10.1039/c3mb70056a

Rego, A. (2017). *The role of ceramides in the mitochondrial apoptotic pathway induced by acetic acid*.

University of Minho.

Ribeiro, I. C., Veríssimo, I., Moniz, L., Cardoso, H., Sousa, M. J., Soares, A. M. V. M., & Leão, C. (2000). Yeasts as a model for assessing the toxicity of the fungicides penconazol, cymoxanil and dichlofluanid. *Chemosphere*, *41*(10), 1637–1642. https://doi.org/10.1016/S0045-6535(00)00039-4

Rosales, E., Diaz, S., Pazos, M., & Sanromán, M. A. (2018). Comprehensive strategy for the degradation of anti-inflammatory drug diclofenac by different advanced oxidation processes. *Separation and Purification Technology*. https://doi.org/10.1016/j.seppur.2018.04.014

Ryuko, S., Ma, Y., Ma, N., Sakaue, M., & Kuno, T. (2012). Genome-wide screen reveals novel mechanisms for regulating Cobalt uptake and detoxification in fission yeast. *Molecular Genetics and Genomics*, *287*(8), 651–662. https://doi.org/10.1007/s00438-012-0705-9

Sanchez, A., Álvarez, A. M., Benito, M., & Fabregat, I. (1997). Cycloheximide prevents apoptosis, reactive oxygen species production, and glutathione depletion induced by transforming growth factor β in fetal rat hepatocytes in primary culture. *Hepatology*, *26*(4), 935–943. https://doi.org/10.1002/hep.510260420

Schönichen, A., Webb, B. A., Jacobson, M. P., & Barber, D. L. (2013). Considering protonation as a posttranslational modification regulating protein structure and function. *Annual Review of Biophysics*, *42*(1), 289–314. https://doi.org/10.1146/annurev-biophys-050511-102349

Singh, S., Khanna, V. K., & Pant, A. B. (2018). Development of In Vitro Toxicology: A Historic Story. In *In Vitro Toxicology*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-804667-8.00001-8

Skerker, J. M., Leon, D., Price, M. N., Mar, J. S., Tarjan, D. R., Wetmore, K. M., ... Arkin, A. P. (2013). Dissecting a complex chemical stress: Chemogenomic profiling of plant hydrolysates. *Molecular Systems Biology*, *9*(674). https://doi.org/10.1038/msb.2013.30

Smith, A. M., Ammar, R., Nislow, C., & Giaever, G. (2010). A survey of yeast genomic assays for drug and target discovery. *Pharmacology and Therapeutics*, *127*(2), 156–164. https://doi.org/10.1016/j.pharmthera.2010.04.012

Smith, M. R., Boenzli, M. G., Hindagolla, V., Ding, J., Miller, J. M., Hutchison, J. E., ... Bakalinsky, A. T. (2013). Identification of gold nanoparticle-resistant mutants of *Saccharomyces cerevisiae* suggests a role for respiratory metabolism in mediating toxicity. *Applied and Environmental Microbiology*, *79*(2), 728–733. https://doi.org/10.1128/AEM.01737-12

Sousa, M., Duarte, A. M., Fernandes, T. R., Chaves, S. R., Pacheco, A., Leão, C., ... Sousa, M. J. (2013). Genome-wide identification of genes involved in the positive and negative regulation of acetic acid-induced programmed cell death in *Saccharomyces cerevisiae*. *BMC Genomics*, *14*(1). https://doi.org/10.1186/1471-2164-14-838

Svensson, J. P., Quirós Pesudo, L., McRee, S. K., Adeleye, Y., Carmichael, P., & Samson, L. D. (2013). Genomic phenotyping by barcode sequencing broadly distinguishes between alkylating agents, oxidizing agents, and non-genotoxic agents, and reveals a role for aromatic amino acids in cellular recovery after quinone exposure. *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0073736

Terra-Matos, J., Teixeira, M. O., Santos-Pereira, C., Noronha, H., Domingues, L., Sieiro, C., ... Côrte-Real, M. (2022). *Saccharomyces cerevisiae* cells lacking the zinc vacuolar transporter Zrt3 Display improved ethanol productivity in lignocellulosic hydrolysates. *Journal of Fungi*, *8*(1). https://doi.org/10.3390/jof8010078

Thumm, M. (2000). Structure and function of the yeast vacuole and its role in autophagy. *Microscopy Research and Technique*, *51*(6), 563–572. https://doi.org/10.1002/1097-0029(20001215)51:6<563::AID-JEMT6>3.0.CO;2-8

Toffolatti, S. L., Venturini, G., Campia, P., Cirio, L., Bellotto, D., & Vercesi, A. (2015). Sensitivity to cymoxanil in Italian populations of *Plasmopara viticola* oospores. *Pest Management Science*, *71*(8), 1182–1188. https://doi.org/10.1002/ps.3906

Torres, N. P., Lee, A. Y., Giaever, G., Nislow, C., & Brown, G. W. (2013). A high-throughput yeast assay identifies synergistic drug combinations. *Assay and Drug Development Technologies*, *11*(5), 299–307. https://doi.org/10.1089/adt.2012.503

Troppens, D. M., Dmitriev, R. I., Papkovsky, D. B., O'Gara, F., & Morrissey, J. P. (2013). Genome-wide investigation of cellular targets and mode of action of the antifungal bacterial metabolite 2,4-diacetylphloroglucinol in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, *13*(3), 322–334. https://doi.org/10.1111/1567-1364.12037

Tun, N. M., O'Doherty, P. J., Perrone, G. G., Bailey, T. D., Kersaitis, C., & Wu, M. J. (2013). Disulfide stress-induced aluminium toxicity: Molecular insights through genome-wide screening of *Saccharomyces cerevisiae*. *Metallomics*, *5*(8), 1068–1075. https://doi.org/10.1039/c3mt00083d

Ueda, K. (2009). Effect of environmental chemicals on genes and the expression. *Yakugaku Zasshi*,

129(12), 1501–1506. https://doi.org/10.1248/yakushi.129.1501

Vachon, J., Pagé-Lariviè re, F., Sirard, M. A., Rodriguez, M. J., Levallois, P., & Campagna, C. (2018). Availability, quality, and relevance of toxicogenomics data for human health risk assessment: A scoping review of the literature on trihalomethanes. *Toxicological Sciences*, *163*(2), 364–373. https://doi.org/10.1093/toxsci/kfy050

Vatakuti, S. (2016a). Toxicogenomics of precision-cut liver slices for prediction of human liver toxicity. *PhD Thesis*, 141.

Vatakuti, S. (2016b). *Toxicogenomics of precision-cut liver slices for prediction of human liver toxicity.* University of Groningen.

Wang, T., Wei, J. J., Sabatini, D. M., & Lander, E. S. (2014). Genetic screens in human cells using the CRISPR-Cas9 system. *Science*, *343*(6166), 80–84. https://doi.org/10.1126/science.1246981

Wang, X., Lee, J. H., Li, R., Liao, Y. P., Kang, J., Chang, C. H., … Nel, A. E. (2018). Toxicological profiling of highly purified single-walled carbon nanotubes with different lengths in the rodent lung and *Escherichia coli*. *Small*, *14*(23), 1–11. https://doi.org/10.1002/smll.201703915

Webster, J., Bollen, P., Grimm, H., & Jennings, M. (2010). Ethical implications of using the minipig in regulatory toxicology studies. *Journal of Pharmacological and Toxicological Methods*, *62*(3), 160–166. https://doi.org/10.1016/j.vascn.2010.05.002

Welihinda, A. A., Beavis, A. D., & Trumbly, R. J. (1994). Mutations in LIS1 (ERG6) gene confer increased sodium and lithium uptake in *Saccharomyces cerevisiae*. *BBA - Biomembranes*, *1193*(1), 107–117. https://doi.org/10.1016/0005-2736(94)90339-5

Yakes, F. M., & Van Houten, B. (1997). Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(2), 514–519. https://doi.org/10.1073/pnas.94.2.514

Yeast Deletion Project. (n.d.). *Sacchairomyces* genome deletion project. Retrieved May 7, 2020, from http://www-sequence.stanford.edu/

Youle, R. J., & Van Der Bliek, A. M. (2012). Mitochondrial fission, fusion, and stress. *Science*, *337*(6098), 1062–1065. https://doi.org/10.1126/science.1219855

Zhang, J., Chang, V. W. C., Giannis, A., & Wang, J. Y. (2013). Removal of cytostatic drugs from aquatic

environment: A review. *Science of the Total Environment*, *445–446*, 281–298. https://doi.org/10.1016/j.scitotenv.2012.12.061

Zhang, L., Liu, N., Ma, X., & Jiang, L. (2013). The transcriptional control machinery as well as the cell wall integrity and its regulation are involved in the detoxification of the organic solvent dimethyl sulfoxide in *Saccharomyces cerevisiae*. *FEMS Yeast Research*, *13*(2), 200–218. https://doi.org/10.1111/1567-1364.12022

Zhou, X., Ma, Y., Fang, Y., gerile, W., Jaiseng, W., Yamada, Y., & Kuno, T. (2013). A genome-wide screening of potential target genes to enhance the antifungal activity of micafungin in *Schizosaccharomyces pombe*. *PLoS ONE*, *8*(5). https://doi.org/10.1371/journal.pone.0065904

Zimmermann, R., Eyrisch, S., Ahmad, M., & Helms, V. (2011). Protein translocation across the ER membrane. *Biochimica et Biophysica Acta - Biomembranes*, *1808*(3), 912–924. https://doi.org/10.1016/j.bbamem.2010.06.015

Ziogas, B. N., & Davidse, L. C. (1987). Studies on the mechanism of action of cymoxanil in *Phytophthora infestans*. *Pesticide Biochemistry and Physiology*, *29*(2), 89–96. https://doi.org/10.1016/0048-3575(87)90066-6

Zounkova, R., Kovalova, L., Blaha, L., & Dott, W. (2010). Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites. *Chemosphere*, *81*(2), 253–260. https://doi.org/10.1016/j.chemosphere.2010.06.029

7. Annexes

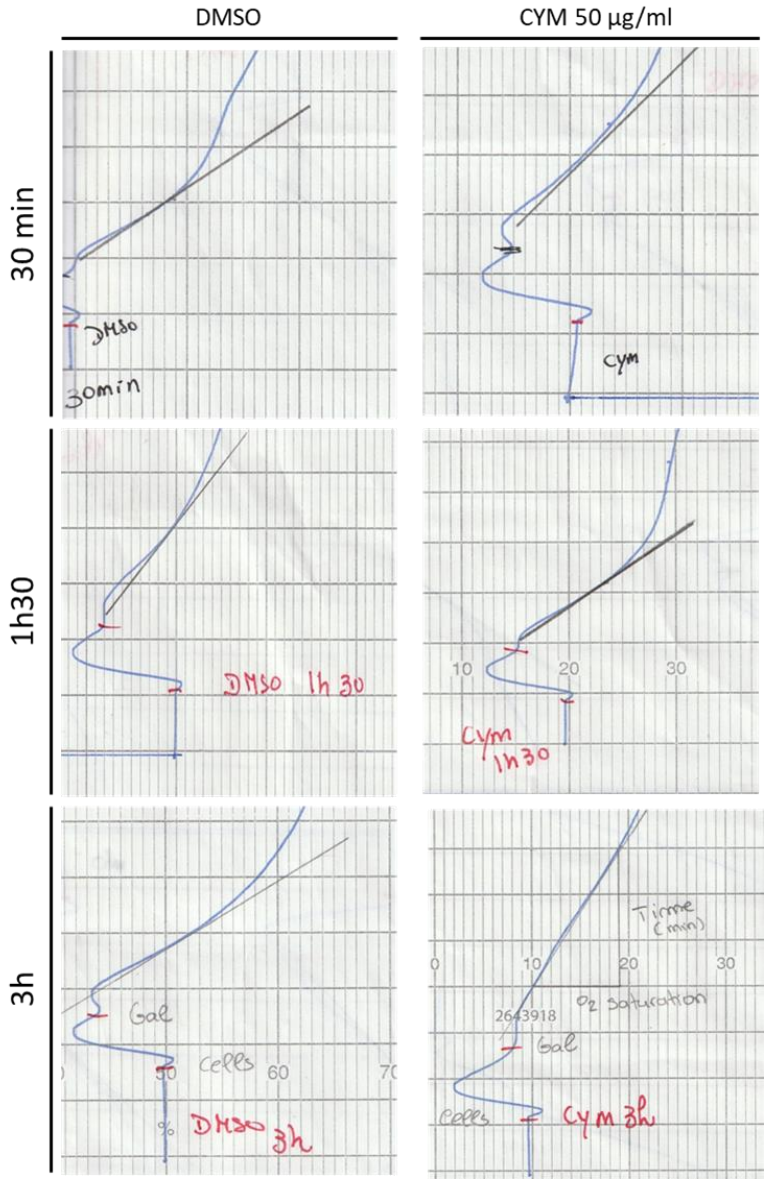

Figure A1 – Oxygen consumption in the presence or absence of 50 µg/ml of CYM. Cells were diluted to an OD_{640nm} = 0.1, exposed to 50 µg/ml cymoxanil for 24 hours in SC-Gal media. O_2 consumption measured with Clark electrode. The inhibition is not a process that occurs immediately. In our conditions, the respiration inhibition occurs when cells are incubated with the compound for 3 hours, as we can see when we compare the straight slope (straight gray line) of control whit the treatment.