UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

Isothiazolinone biocide metabolism in fish liver cells

Name Surname

Catriona Gilland

Degree project for Master of Science (120 hec) with a major in Environmental Science

ES2571, Degree Project Environmental Science, 45 hec

Second cycle

Semester/year: Autumn 2023/ Spring 2024

Supervisor: Malin Celander, Department of Biological and Environmental Sciences

Examiner: Joachim Sturve, Department of Biological and Environmental Sciences

Abstract

Isothiazolinones, widely used preservatives renowned for their antifungal and antimicrobial properties. Despite these benefits, they have long been known to cause skin irritation and sensitization. Slowly more legislation is restricting certain chemicals, but then there are others that are becoming more prominent in consumer products. Benzisothiazolinone (BIT) is one such chemical that has been banned from cosmetics but is still prevalent in day-to-day products. DCOIT, found in antifouling agents, poses environmental risks due to its persistence and toxic effects, yet its mechanisms are not fully understood. This study focuses on these two prominent isothiazolinones BIT and DCOIT. This research aims to investigate the toxic effects of DCOIT and BIT individually and in combination, using the Poeciliopsis lucida hepatocellular carcinoma (PLHC-1) cell line and employing high-performance liquid chromatography (HPLC), ethoxyresorufin-O-deethylase (EROD) assays, and cytotoxicity assays. DCOIT is thought to be more toxic to cells than BIT, with potentially higher induction rates of CYP1A and lower values in cytotoxicity assays. Additionally, we aim to identify potential metabolites of BIT. This study sheds light on the comparative toxicities of these isothiazolinones and their interactions, providing valuable insights into their environmental and health implications.

Acknowledgment

Firstly I would like to thank my supervisor and co-supervisor, Malin Celander and Anne Farbrot for the help and support during this project which I have enjoyed thoroughly. I would also like to thank Anders Blom for all the help with the HPLC machines. This semester would've been a lot more difficult to get through if it was not for Romana Lukášiková, Nele Breitkreutz and Larissa Abfalterer, who were always there to listen and support - Love you girls!

Table of Contents

1.Introduction

1.1 Background

Isothiazolinones are commonly used preservatives from everything to industrial products to cosmetic products (Schwensen & Johansen, 2019). They are used so often due to their antifungal and anti-microbial characteristics. However, since their appearance in the 1970s, they have been known to cause irritation and sensitization (Bruze et al., 1987; Bruze et al., 1988). This family of chemicals is becoming more and more prominent in everyday products, such as washing detergents, which leads to persistence of them in our clothes and increasing the rates of dermatitis (Marrero-Alemán et al., 2020).

1.2 Isothiazolinones

This thesis project will look specifically at the two isothiazolinones. Benzisothiazolinone (BIT) is known to cause skin irritation and dermatitis, and because of this it is banned from cosmetic products (Silva et al., 2020), but it is still becoming more and more common in everyday items. Most commonly, it is used in paints as seen in figure 1.

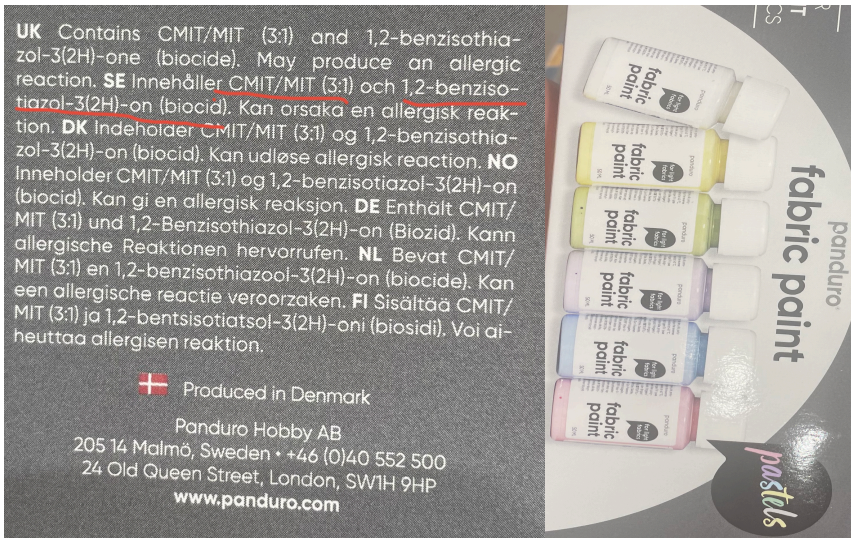

Figure 1: Image of paints and the ingredients, taken at a craft shop (Panduro). Two isothiazolinones can be seen listed in the ingredients and BIT is one of them.

Another isothiazolinone that is very prominent is 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT). This is the main ingredient of Sea-Nine 211®, which is an antifouling agent normally used on boats in order to prevent corrosion. It is known to degrade rapidly and because of this it is found in ecosystems around the world, from marine areas in Europe (Steen et al., 2004) to estuary sediments in Brazil (Abreu et al., 2020).

DCOIT has been around since the 1990s, and over that time some of its toxic effects are known, but the mechanism is not properly understood. It is known to pass easily through the cell membrane (Gabe, Guerreiro and Sandrini, 2021), cause oxidative stress in (Eom et al., 2019) and even disrupt mitochondrial activity as seen in rat livers, according to Bragadin et al. (2005).

Both of these chemicals have hydrocarbon rings, as shown in figure 2. DCOIT also has a small chain, and 2 chlorine molecules which make it more electronegative than BIT.

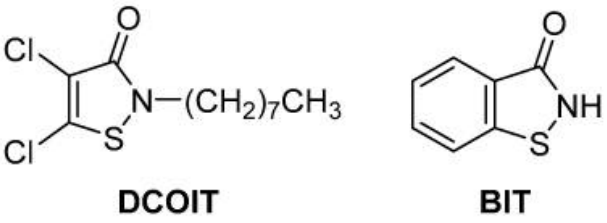

Figure 2: Chemical structure of DCOIT and BIT (Silva et al., 2020).

1.3 PLHC-1 Cells

The *Poeciliopsis lucida hepatocellular carcinoma* (PLHC-1) cell line is fish liver cell line derived from a topminnow and is commonly used for in vitro biomonitoring and cytotoxicity experiments (Huuskonen et al., 1998). This cell line expresses the aryl hydrocarbon receptor (AhR) and thus allows for the induction of the CYP1A enzymes. Measuring the induction of these enzymes is one of the main ways to measure the extent of contamination within an organism. This project uses the cell line, in order to look at the biomarker, which is the induction of the CYP1A pathway, and looking at the cytotoxicity effects on the cells.

1.4 High-performance liquid chromatography

High performance liquid chromatography (HPLC) is an analytical technique evolved from column chromatography. “Like” the classical technique, it is used to separate chemicals, but it also accurately identifies and quantifies them. It is also used to identify impurities and intermediate products, which makes it a valuable technique for pharmaceutical research (Bhardwaj et al., 2015). At its simplest, HPLC analysis begins with the injection of the sample of interest into the column, which contains porous material known as the “stationary phase”. Then various solvents, or “mobile phase”, flow through the column at high pressure. As this happens different molecules in the sample are picked up, and these molecules are separated based on their size, charge, polarity etc. The more affinity with the stationary phase, the slower and shorter distance the molecules will travel (Vidushi & Meenakshi, 2017). The speed of travel of the molecule through the column is the retention time. The separated sample then flows out of the column to the UV detector. The HPLC data is represented as a chromatogram, where the y-axis is the response of the detected signal, and the x-axis is the retention time.

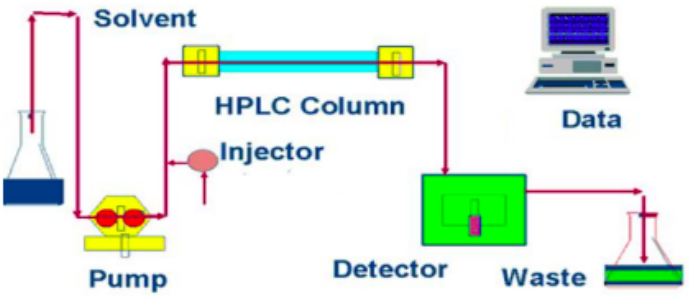

*Figure 3: Diagram of how the HPLC machine works (*Bhardwaj, 2015).

1.5 EROD

The extent of toxic exposure from [environmental] toxicants/contaminants in a given species can be measured by looking at biomarkers. A biomarker is "a toxicant-induced change in gene expression leading to alteration in protein content and enzyme activity that is linked to the amount of contaminant", as defined by Bucheli and Fent (1995).

One of the most commonly studied biomarkers is the induction of the cytochrome P4501A (CYP1A) enzyme, the extent of which is measured by looking at the ethoxyresorufin-*O*-deethylase (EROD) activity. The CYP1A enzyme is known to be induced by chemicals such as polycyclic aromatic hydrocarbons (PAHs), and occurs via receptor-mediated induction.

The detoxification process in fish begins with the binding of a xenobiotic, which is any foreign or toxic substance, attaches to a cytosolic aryl hydrocarbon receptor (AhR) (Whyte et al., 2000). The AhR then goes on to bind to aryl hydrocarbon nuclear transferase (ARNT), before traveling to the nucleus (Bucheli & Fent, 1995). The ARNT binds to a section of the DNA, whereby transcription of the CYP1A gene can occur and subsequently the synthesis of mRNA (Guengerich, 1993).

1.6 Cytotox assay

This project will use 2 cytotoxicity assays using alamar blue (AB) and 5-Carboxy Fluorescein Diacetate-Acetoxymethyl Ester (CFDA-AM). Alamar blue is a marker for mitochondrial activity. The active ingredient is resazurin, is blue, non-fluorescent and permeable through membranes. The mitochondria in cells is able to reduce resazurin to resorufin, which is red and fluorescent, and measurable with a spectrophotometer (Bonnier et al., 2015). The amount of resorufin produced is related to the metabolic activity, whereby damaged cells will have lower absorbance values than healthier cells (Rampersad, 2012).

CFDA-AM is also a non-fluorescent, membrane-permeable dye. It is converted to fluorescent fluorescein by esterases in cell membranes. Only intact membrane would be able to perform this conversion, the amount of fluorescence detected will indicate the integrity of the cell membrane (Bopp & Lettieri, 2008).

1.7 Aim and Hypothesis

As previously mentioned, isothiazolinones such as BIT (and MIT) and their effects are better understood, than DCOIT for example. Given the prominent use of these chemicals in commercial products it is important to know the extent of their effects, and at what concentrations they become toxic. In a real ecosystem, organisms will be exposed to multiple chemicals and concentrations at once, meaning there is possibility of a cocktail effect. When multiple biocides interact, is there any difference in toxicity?

The aim of this thesis is to look at the toxic effects of DCOIT and BIT, both separately and together. The first research question addresses the effects of DCOIT; how it compares and if it acts similarly to BIT in PLHC-1 cells. Following on from this, the second question concerns the cocktail effect of these two chemicals and if there is a difference compared to when they are alone. These two questions will be investigated through HPLC analysis, EROD assays and cytotoxicity assays. Finally, this thesis will also look for potential metabolites of BIT by HPLC analysis.

Research Questions

1. Toxic effects of DCOIT on the PLHC-1 cells
2. Are these chemicals more toxic if they are combined?
3. Look for potential metabolites

The hypothesis is that DCOIT alone is more toxic to the cells than BIT, as shown in Silva et al. (2020). One hypothesis would be that the specific EROD activity would be high in samples treated with DCOIT alone, which would mean higher induction rate of CYP1A. Another hypothesis would be that the values for AB and CFDA-AM in the cytotoxicity assays would be lower in DCOIT only samples.

2. Materials and Methods

2.1 Material and Chemicals

The materials and chemicals were bought, prepared and already available in the lab before this project took place. The isothiazolinones DCOIT and BIT had already been prepared in 100 % dimethyl sulphoxide (DMSO). The chemicals required for the cell culture media, and seeding cells include are Minimum Essential Medium Eagle (MEM) powder, HEPES free acid, fetal bovine serum (FBS), ethylenediaminetetraacetic acid (EDTA) and phosphate buffer saline (PBS). The chemicals required for the EROD analysis are: Bovine Serum Albumin (BSA), 7-ethoxyresorufin (ER, Resorufin ethyl ether), Fluorescamine. The chemicals required to make the cytotoxicity solution are: alamar blue (AB), 5-Carboxy Fluorescein Diacetate-Acetoxymethyl Ester (CFDA-AM), Trizma-base, Trizma-HCL and Sodium hydroxide (NaOH).

2.2 Cell Cultures

As with the media solutions, when seeding and exposing the PLHC-1 cells everything must be kept sterile and all instruments are autoclaved before use. Everything involving the cell cultures is performed under the fume-hood bench. The preparation of cell cultures for each experiment is similar. Both HPLC and EROD analysis used 48-well plates, whereas the cytotoxicity assays use a 96-well plate.

To make the cell culture media, the MEM-powder is dissolved in 850mL of milliQ water. It is continuously mixed, using a magnetic stirrer, for 15 minutes after which it should be a yellow colour. Then add 5.96g of HEPES free acid and 2.2g of $NaHCO_3$, and continue to be stirred for 10 more minutes. The colour should now have changed to red. The pH should be between 7.0 and 7.1, it can be adjusted by adding drops of 10M NaOH. The solution is then made up to 1L, before being filtered with a vacuum suction. The final step is to add 5% FBS to the solution, and then the media culture is kept in the fridge.

2.2.1 Sub-Culturing and seeding cells

The cells were subcultured every week on the first day of analysis. The cell cultures are kept in the incubator at 30ºC in a 75cm^2 cell culture flask. The first step is to remove the media from the flask, using a sterile pipette. Then 5mL of EDTA-PBS solution is added for around 3 minutes, after which it is removed with a sterile pipette. Following on, 1.5mL of trypsin solution is added to the flask for around 1 minute, or until the cells have detached. Then 10mL of the sterile cell culture media is added, and the pipette is used to mix the solution in order to remove clusters of cells. This solution is transferred to a 50mL test tube.

The cell density can be calculated by using a Bürker Chamber. A small sample of the cell suspension is placed on to the chamber, which is then placed under a microscope to count the cells per A-square. The cell density is calculated by the following calculation:

Mean number of cells counted on 3 A-squares x 10,000. Once the cell density is known, the cell suspension can be diluted with more media depending on how much cell suspension is required. For the HPLC and EROD analysis the cell density should be 600,000 cells/mL. For the cytotoxicity assay the cell density should be 500,000 cells/mL.

The sub-culture should be 1:20 dilution of the suspension. This means pipetting 0.5ml of cell suspension into a new 75cm^2 , and adding 9.5ml of cell culture media.

The next step is to add the cell cultures to the well plates. For HPLC and EROD analysis, 500µl of cell culture is added each well. For cytotoxicity assay, 200µL of cell culture is added to each well.

2.3 HPLC analysis

HPLC was used to analyse cell media samples for the presence of isothiazolinones of interest. The PLHC-1 cells were exposed to different concentrations and mixtures of DCOIT and BIT in order to understand how they might interact with the cells.

2.3.1 Machine

The HPLC machine is named SYS-LC-1200 (Agilent series 1200, Agilent 1260 Infinity, Agilent 1290 Infinity, Agilent Technologies Inc, Santa Clara, California) was equipped with a Kromasil 100-3,4-C18 column (3.0 x 100 mm) as well as a UV-detector (1260 DAD VL+). The injection volume was set at 20 µL for each sample. The column temperature was set at 30°C. The UV-detector detected the UV-absorbance at a wavelength of 292 nm for DCOIT and 318 nm for BIT. The mobile phases for HPLC analysis were water and methane solutions of different ratios. Mobile phase A had a ratio of H_2O:MeOH, 95:5, and mobile phase B1 had a ratio of H_2O:MeOH, 25:75. Both mobile phases contained 0.4% acetic acid.

2.3.2 Experimental Set up

The PLHC-1 cells were seeded and prepared as described in 2.4. The day after seeding of the cells, the new media was prepared with the different DCOIT and BIT treatments. For HPLC analysis 500µL of media was prepared for each well. Control samples included media with no exposure and media containing no cells that were exposed to the same treatments. The treatments that the cells were exposed to were; 5µM of DCOIT (DCOIT5), 10µM of DCOIT (DCOIT10) and 10µM of BIT (BIT10). To investigate a potential cocktail effect, the cells were also exposed to mixture treatments; 5µM of DCOIT with 10µM of BIT (DCOIT5BIT10) and 10µM of DCOIT with 10µM of BIT (DCOIT10BIT10). These treatments were then incubated and analysed after 3 hours, 24 hours and 48 hours of exposure.

After the designated exposure time, the cell media was removed and pipetted into eppendorf tubes to be centrifuged at 10,000rpm for 5 minutes. For the final step, the supernatants from each well-treatment were removed and pipetted into 1.5mL HPLC glass vial for HPLC analysis.

Tabel 1: Design of 48-well plate for HPLC analysis. This plate design was repeated for the time points.

Plate	1	2	3	4	5	6	7	8
A	**Media** Cells	**DCOIT10** Cells	**DCOIT5** Cells	**DCOIT10** **BIT10** Cells	**DCOIT5** **BIT10** Cells	**BIT10** Cells	N/A	N/A
B	**Media** Cells	**DCOIT10** Cells	**DCOIT5** Cells	**DCOIT10** **BIT10** Cells	**DCOIT5** **BIT10** Cells	**BIT10** Cells	N/A	N/A
C	**Media** Cells	**DCOIT10** Cells	**DCOIT5** Cells	**DCOIT10** **BIT10** Cells	**DCOIT5** **BIT10** Cells	**BIT10** Cells	N/A	N/A
D	**Media** No Cells	**DCOIT10** No Cells	**DCOIT5** No Cells	**DCOIT10** **BIT10** No Cells	**DCOIT5** **BIT10** No Cells	**BIT10** Cells	N/A	N/A
E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

2.3.3 Analysis

As mentioned in [1.4], molecules and particles separated during HPLC analysis are detected by UV detectors. The injection volume for each sample is 20µL. For this analysis, the samples were detected by 2 wavelengths, 292 nm (A) and 318nm (B), in order to optimally detect DCOIT and BIT, respectively. The resulting chromatograms show 2 wavelengths per sample.

2.4 EROD analysis

2.4.1 Machine

The fluorescence of EROD activity, protein content, resorufin standard and BSA from the EROD analysis were measured using a spectrophotometer, SPECTRA MAX 190 (MOLECULAR DEVICES, United Kingdom). The fluorescence of EROD activity is measured every minute for 10 minutes at excitation wavelength at 530 nm and emission wavelength at 590 nm. The resorufin standard is also measured at excitation wavelength at 530 nm and emission wavelength at 590 nm, but it is only measured one time. The protein content and the BSA standard is measured with excitation wavelength at 405 nm and emission wavelength at 460 nm, and are also only measured one time.

2.4.2 Experimental Set-Up

Following on from 2.4, the day after seeding of the cells, the new media was prepared with the different DCOIT and BIT treatments. The same treatments were chosen for EROD analysis as HPLC, except for the addition of BNF 1µM as a positive control treatment. The cells were exposed to the treatments for 24 hours.

Tabel 2: Design of 48-well plate for EROD analysis.

Plate	1	2	3	4	5	6	7	8
A	**Media** Cells	**BNF** Cells	**DCOIT10** Cells	**DCOIT5** Cells	**DCOIT10 BIT10** Cells	**DCOIT 5 BIT10** Cells	**BIT10** Cells	N/A
B	**Media** Cells	**BNF** Cells	**DCOIT10** Cells	**DCOIT5** Cells	**DCOIT10 BIT10** Cells	**DCOIT 5 BIT10** Cells	**BIT10** Cells	N/A
C	**Media** Cells	**BNF** Cells	**DCOIT10** Cells	**DCOIT5** Cells	**DCOIT10 BIT10** Cells	**DCOIT 5 BIT10** Cells	**BIT10** Cells	N/A
D	**Media** No Cells	**BNF** No Cells	**DCOIT10** No Cells	**DCOIT5** No Cells	**DCOIT10 BIT10** No Cells	**DCOIT 5 BIT10** No Cells	**BIT10** Cells	N/A
E	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
F	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

2.4.3 Analysis

Before starting the EROD analysis, 4 solutions need to be prepared. Firstly, the 7-ethoxyresorufin (ER) work solution needs to be made and the required concentration is 2µm of ER in 50mM sodium-phosphate buffer, with a pH of 8. For a 48-well plate, 15mL of ER work solution is required. A final concentration of 2µM is achieved by diluting the stock solution of ER. To start, 15mL of the sodium phosphate buffer is added

to a 50mL test tube. Then 10µL of ER stock solution is added at a time to the test tube and is mixed well . After each addition, the absorbance of the solution is measured at 482 nm in the spectrophotometer until the absorbance is 0.06.

The next solution is the fluorescamine (FA) solution, which is required to measure the protein content. To make, 3mg FA is dissolved into 10mL of acetonitrile.

Next, two standards are required for EROD analysis, the BSA standard and the resorufin standard. For the BSA standard, a vial of BSA 0.5mg/mL was defrosted. A dilution series of 1:2 is made by using the ER buffer to make 5 standard concentrations, as well as a blank standard (buffer alone). The first standard is the 500µL of stock vial. The second standard is 50µL of the stock solution with 500µL of ER buffer, etc. until standard 5. In a 48-well plate, pipette 200µL in duplicates for each standard.

The resorufin standard is made in a similar way, however 6 standards are made with one “blank” buffer solution.

When the solutions have been prepared, the analysis can begin. The exposed cell plates are removed from the incubator and placed under the fume-hood. The media is vacuumed off. The wells are washed by adding 500µL PBS, then vacuuming off as well. Then, 200µL of ER work solution is added, then place the plate in the fluorometer.

After the EROD analysis, 100µL FA solution was added to the cell treatments and the BSA standard plate, before being incubated at room temperature for 10 minutes. After this both plates can be measured for the protein content.

The resorufin standard plate is then measured in the machine.

2.5 Cytotoxic assays

The extent of cytotoxicity will be measured by using 2 fluorescent dyes, alamar Blue (AB) and CFDA-AM.

2.5.1 Machine

The same spectrophotometer is used for the EROD analysis, see section 2.6.

2.5.2 Experimental Set-Up

Treatments that have been previously tested in HPLC and EROD analysis were also tested for the cytotoxicity assay. Following on from section 2.4, concentrations of DCOIT and BIT are prepared in order to expose the cells. It is important to keep the final column empty as a blank sample. The cells are then exposed for 24 hours. Before the analysis, a cytotoxicity solution is prepared by mixing 10mL of cytotox buffer, 125µL of AB (1,25% v/v) and 10µL of CFDA-AM (4µM). The cytotox buffer had been prepared beforehand, by mixing 1 L milliQ water, 6.61g Trizma-HCL and 0.97g Trizma-base.

The cell plates that have been exposed to the treatments for 24 hours are removed from the incubator. The media is removed and each well is washed twice with 150µL of cytox buffer. Then 100µL of the cytotoxicity-solution is added to each well. The well plate is then covered with aluminium foil and incubated for 45 - 60 minutes at room temperature. The fluorescence is analysed at excitation/emission 530/590 for AB and at 485/530 for CFDA-AM.

Table 3: 96-well plate design for cytotoxicity assay

Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	Media	DCOIT10	DCOIT5	BIT 10	BIT 5	DCOIT 10 BIT10	DCOIT 5 BIT10	DCOIT10 BIT5	DCOIT 5 BIT5	DMSO	N/A	Blank
B	Media	DCOIT10	DCOIT5	BIT 10	BIT 5	DCOIT 10 BIT10	DCOIT 5 BIT10	DCOIT10 BIT5	DCOIT 5 BIT5	DMSO	N/A	Blank
C	Media	DCOIT10	DCOIT5	BIT 10	BIT 5	DCOIT 10 BIT10	DCOIT 5 BIT10	DCOIT10 BIT5	DCOIT 5 BIT5	DMSO	N/A	Blank
D	Media	DCOIT10	DCOIT5	BIT 10	BIT 5	DCOIT 10 BIT10	DCOIT 5 BIT10	DCOIT10 BIT5	DCOIT 5 BIT5	DMSO	N/A	Blank
E	Media	DCOIT10	DCOIT5	BIT 10	BIT 5	DCOIT 10 BIT10	DCOIT 5 BIT10	DCOIT10 BIT5	DCOIT 5 BIT5	DMSO	N/A	Blank
F	Media	DCOIT10	DCOIT5	BIT 10	BIT 5	DCOIT 10 BIT10	DCOIT 5 BIT10	DCOIT10 BIT5	DCOIT 5 BIT5	DMSO	N/A	Blank
G	Media	DCOIT10	DCOIT5	BIT 10	BIT 5	DCOIT 10 BIT10	DCOIT 5 BIT10	DCOIT10 BIT5	DCOIT 5 BIT5	DMSO	N/A	Blank
H	Media	DCOIT10	DCOIT5	BIT 10	BIT 5	DCOIT 10 BIT10	DCOIT 5 BIT10	DCOIT10 BIT5	DCOIT 5 BIT5	DMSO	N/A	Blank

2.6 Ethical Permit

This thesis used PLHC-1 cell lines and was only involved in vitro experiments. There were no animal experiments performed, thus an ethical permit was not required.

2.7 Statistics

Each experimental method used in this project measured replicates of each treatment for statistical value. From these replicates, the averages and standard deviations were calculated and are presented in section 3. Examples of the calculations are presented in appendix_. Both Microsoft Excel 2019 and Numbers 2017 (Apple Inc.) were used to carry out the calculations.

3. Results

This section looks at the results from the HPLC analysis, which includes the chromatograms as well as the retention times of BIT and DCOIT. As well as this, the EROD activity, protein content measurements and cytotoxic analysis. Section 2.3 explains the set up for the chosen experiments and treatments.

3.1 HPLC Analysis

The results from the HPLC analysis are presented in the forms of figures from the chromatograms and tables containing the retention times and response of chemicals of interest. The x-axes are the retention times, measured in minutes, and the y-axes represent the responses, measured in mAU (milli-absorbance unit). As mentioned in section 2.3, each sample is measured at two different wavelengths. The following chromatographs have 2 lines representing an individual sample. Wavelength A is used to look for the peaks of DCOIT and BIT's metabolite, while wavelength B is used to look for the peaks of BIT.

3.1.1 DCOIT

The following figures will focus on the media samples that were treated with DCOIT alone. The different concentration treatments are described in section 2.3.

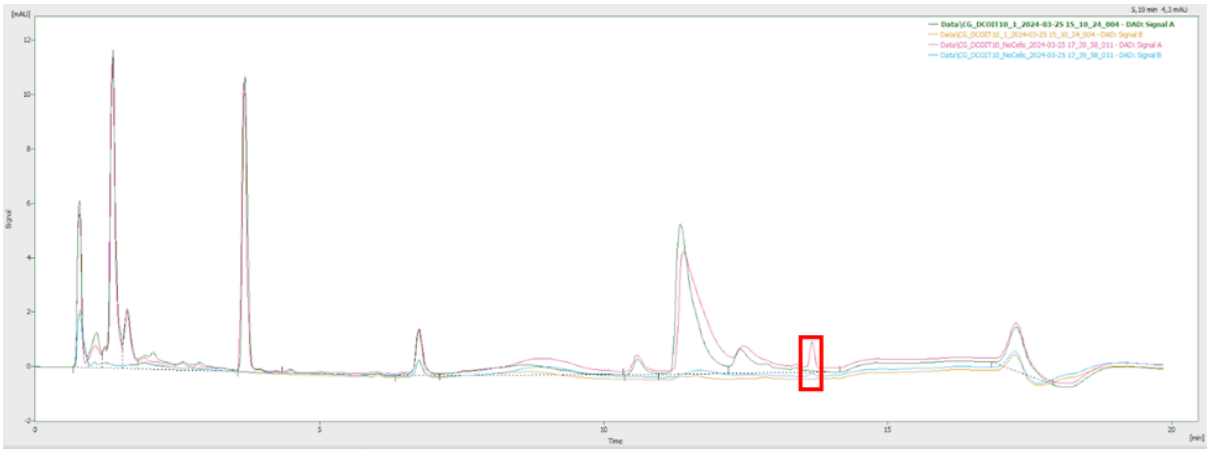

Figure 4: HPLC chromatogram of two samples exposed to a treatment of DCOIT10 for 24 hours, one with cell suspension and one without. Wavelength A is shown in green (sample with cells) and pink (sample without cells). Red box highlights the time and retention where DCOIT is found.

Figures 4 and 5 show the chromatograms of media exposed to DCOIT10, one sample has cell suspension and the other does not. In these figures we are interested in wavelength A since we are looking for DCOIT. The pink peak in fig.5 illustrates the DCOIT that is present in the suspension without cells. From table 4 we can see that the retention time of DCOIT is around 13.6 minutes. The red circle highlights that there is no peak on the green wavelength around this time, meaning that DCOIT is not detectable in the sample containing cells.

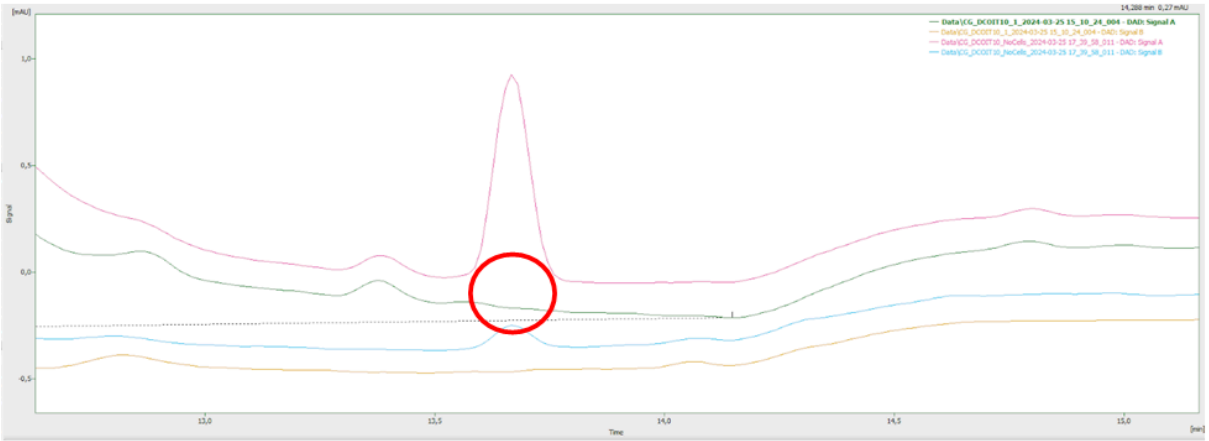

Figure 5: Highlighted peak of figure 4. Wavelength A is represented here in green and pink. The green wavelength represents the DCOIT10 treatment with cell suspension. The pink wavelength represents the DCOIT10 treatment without cell suspension. The red circle highlights discrepancy between samples.

The lack of visible peak is also shown below in table 4, which contains the response values for DCOIT in the corresponding treatments. It shows that DCOIT is not detectable in any samples with cell suspension. In the treatment of DCOIT5BIT10, where there is a lower concentration of DCOIT, we can see that the DCOIT response is also lower.

Table 4: Retention times and responses of DCOIT in the different treatments, with respect to Figures 4,5,8,9,10 and 11.

Treatment	Retention Time (min)	Response
DCOIT10	*Not detected*	*Not detected*
DCOIT10 No Cells	13,667	5,165
DCOIT1OBIT10	*Not detected*	*Not detected*
DCOIT10BIT10 No Cells	13,667	5,348
DCOIT5BIT10	*Not detected*	*Not detected*
DCOIT5BIT10 No Cells	13,667	1,236

3.1.2 BIT

The following figures focus on the media samples that were treated with BIT alone, so the focus will be on wavelength B. Figures 6 and 7 show the chromatogram of samples treated with BIT10,

one with cell suspension and one without. Focusing on wavelength B, the wavelengths of interest are blue and red.

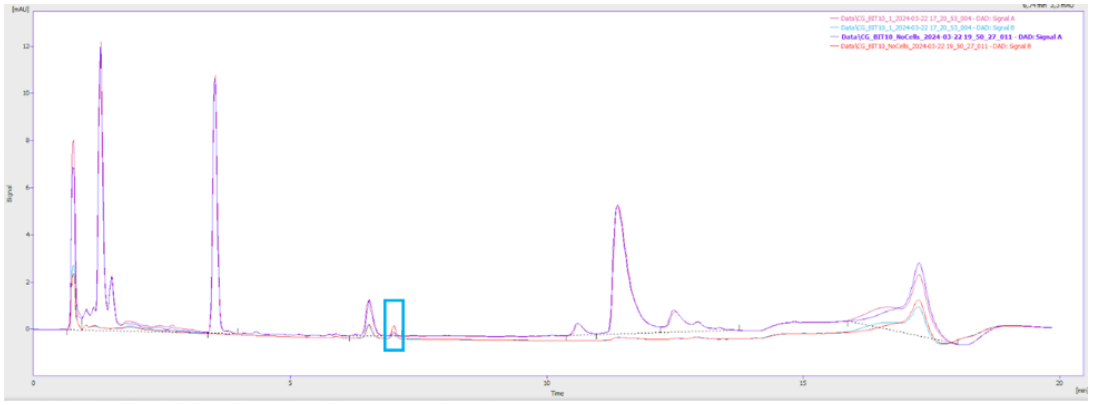

Figure 6: HPLC chromatogram of two samples exposed to a treatment of BIT10 for 24 hours, one with cell suspension and one without. Wavelength B is shown in blue (sample with cells) and red (sample without cells). The blue box highlights the time and retention where BIT is found.

From these figures, and using table 5, the retention time of BIT is around 7 minutes. The data also shows that BIT is detectable in both samples. The response of the sample without cell suspension is almost double the response of the sample with the cells, meaning there is more BIT present when there are no cells.

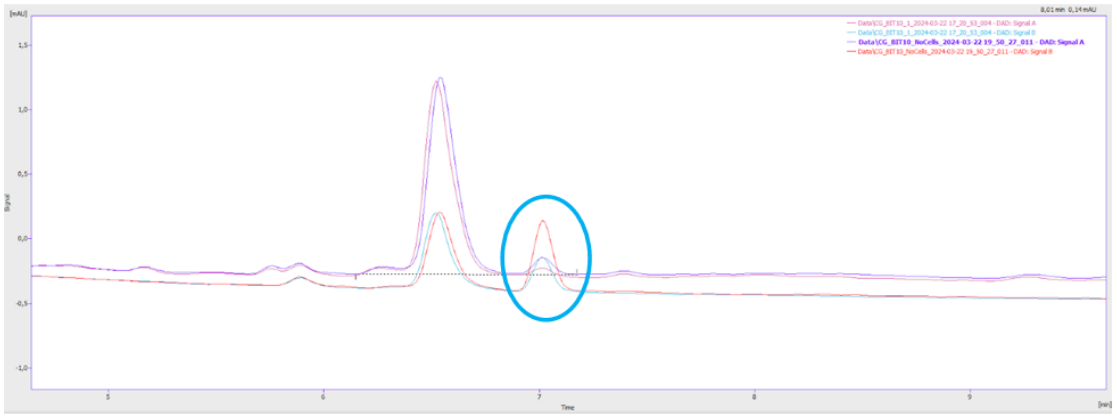

Figure 7: Highlighted peak of figure 6. Wavelength B is represented here in blue and red. The blue wavelength represents the BIT10 treatment with cell suspension. The red wavelength represents the BIT10 treatment without cell suspension. The blue circle highlights discrepancy between samples.

Table 4 also shows that there is a detectable BIT peak when cells are present and when they are not. Focusing on the samples with cell suspension,

Table 5: Retention times and response of BIT in different treatments, with respect to figures 6, 7, 8, 9, 10 and 11.

Treatment	Retention Time	Response
BIT10	7,013	1,606
BIT10 No Cells	7,013	3,364
DCOIT10BIT10	7,0	14,998
DCOIT10BIT10 No Cells	7,013	23,273
DCOIT5BIT10	7,027	12,822
DCOIT5BIT10 No Cells	7,013	29,404

3.1.3 Mixtures

The cell-media samples containing both DCOIT and BIT were prepared as described in section 2.3. Figures 8 and 9 show chromatogram peaks from samples exposed to DCOIT10BIT10. In figure 8 we can see the BIT peak is highlighted in the blue box and the DCOIT peak is highlighted in the red box, and that both of these chemicals are found at their respective retention times.

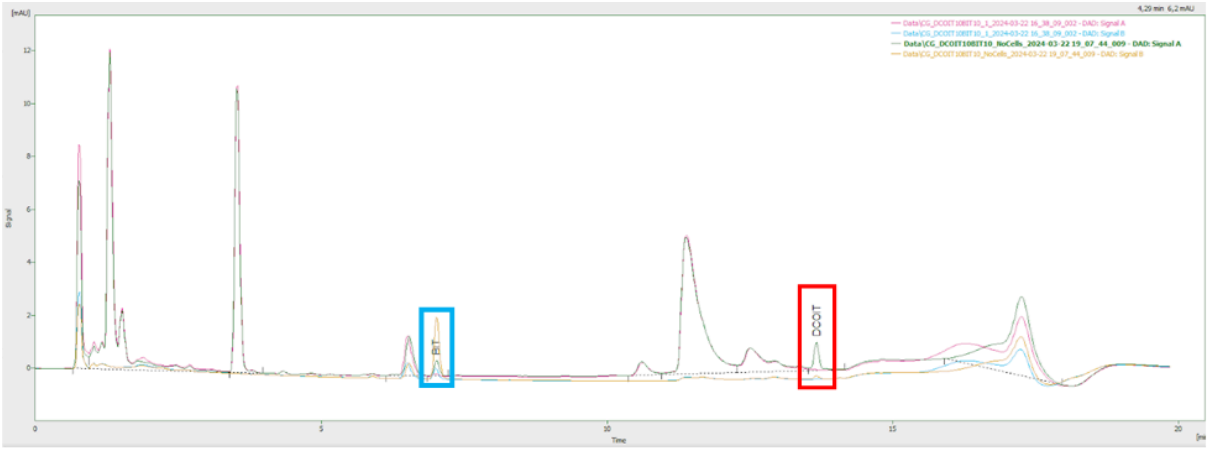

Figure 8: HPLC chromatogram of two samples exposed to a treatment of DCOIT10BIT10 for 24 hours, one with cell suspension and one without. The wavelengths represent the following:
Pink - Wavelength A, with cells
Blue - Wavelength B, with cells
Green - Wavelength A, without cells
Yellow - Wavelength B, without cells

Figure 9 shows a close up of the highlighted peaks of figure 8. Focusing on the BIT peak, we are looking at the yellow (sample without cells) and blue (sample with cells) wavelengths. These

peaks show the same thing as figure 7, whereby there is a higher quantity of BIT when cells are not present. There is also no change in the DCOIT peak; the green wavelength indicates there is DCOIT present in the sample without cells, while the pink wavelength indicates that DCOIT is not detectable in samples with cells.

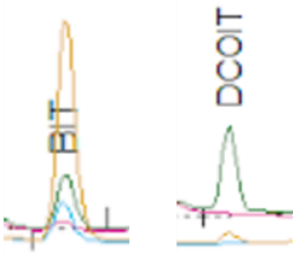

Figure 9: Highlighted BIT and DCOIT peaks from figure 8.

In figure 10 the chromatogram compares the wavelengths of 3 treatments, all containing cells. The blue box highlights the presence of BIT, while the red box highlights that there are no peaks where we would expect to find DCOIT. A close up of the BIT peak is represented by figure 11.

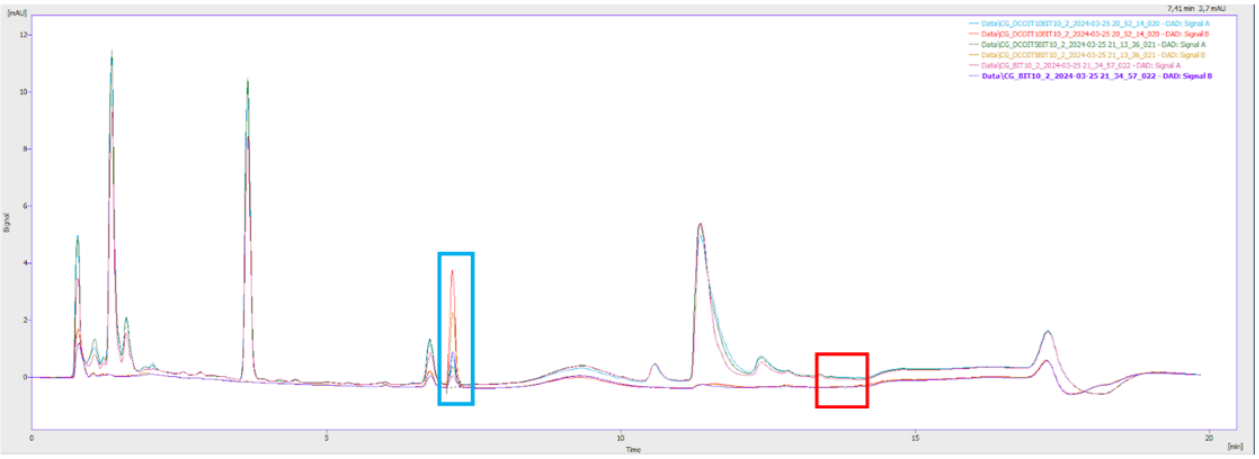

Figure 10: HPLC spectrum of cells exposed for 24 hours to 3 treatments; DCOIT10BIT10, DCOIT5BIT10 AND BIT10. The blue box highlights the BIT peak. The red box highlights where DCOIT is expected to be.

Wavelength B of the 3 different treatments are labeled in the highlighted BIT peak. It shows that the higher the concentration of DCOIT in the samples, the higher the quantity of BIT found in the samples.

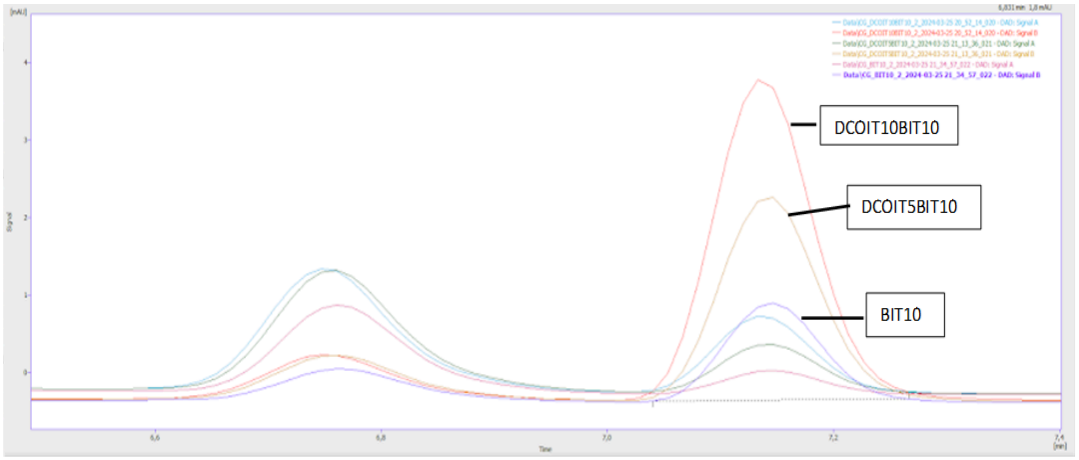

Figure 11: Highlighted BIT peak from figure 10. HPLC chromatogram of 3 samples exposed to treatments DCOIT10BIT10, DCOIT5BIT10 AND BIT10 for 24 hours. All 3 samples have cell suspensions.

3.1.4 BIT Metabolite

The following figures show the potential presence of BIT metabolite. The chromatograms had more of a curve than previous and this may be due to changes in the HPLC phases. However, the following figures show that the BIT peak is found around the same retention time. The BIT peak is highlighted by the light blue box. The dark blue box highlights that there are no peaks.

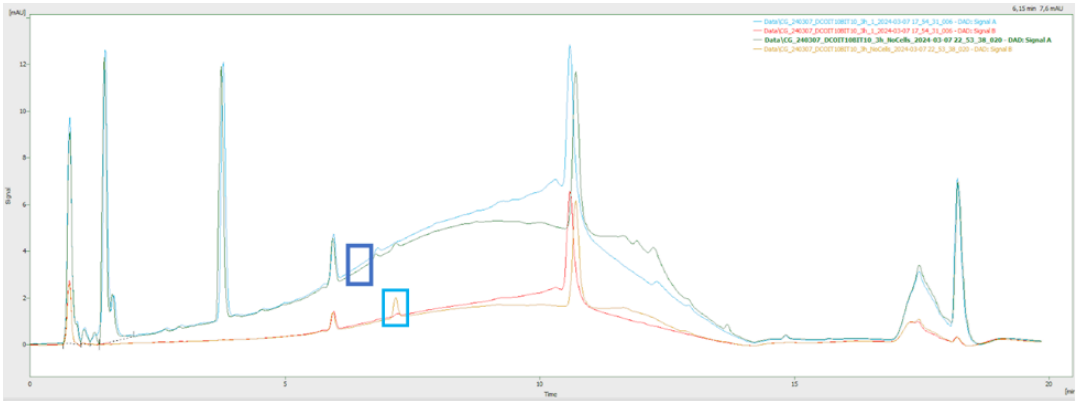

Figure 12: HPLC chromatogram of 2 samples with and without cells, exposed for 3 hours to DCOIT10BIT.

In figure 13, we can see that the blue box highlights the BIT peak, but there is another peak that is found at around the same retention time, and only in the BIT containing samples. This could indicate BIT metabolite. As shown in figure 12, it does not appear in exposure less than 24 hours.

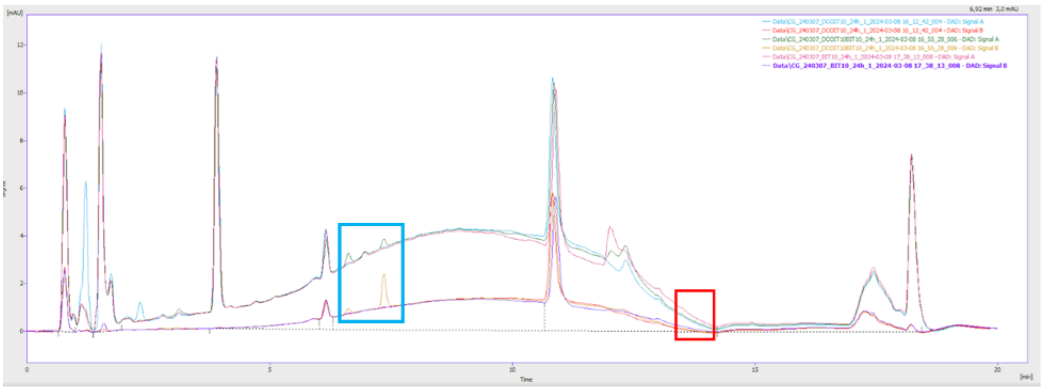

Figure 13: HPLC spectra of cells that were exposed for 24 hours to 3 treatments; DCOIT10BIT10, DCOIT10 and BIT10. The blue box highlights the peaks of BIT and its potential metabolite. The red box highlights where DCOIT peak is expected to show.

This potential metabolite peak is highlighted in figure 14, in the dark blue box. It is found on wavelength A, rather than B like BIT.

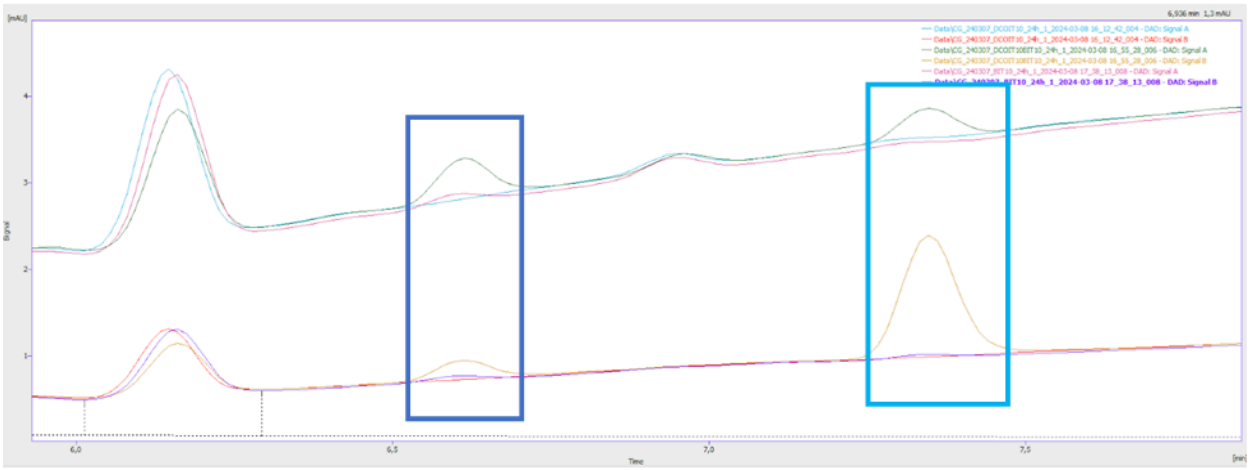

Figure 14: Zoom in on peaks from figure [above]. ***Dark blue box:*** *highlights the potential BIT metabolite (upper-peak), found on the wavelength A (upper). The green peak represents DCOIT10BIT10 sample, signifying a higher concentration of the metabolite. The pink spectrum (wavelength A) is the BIT10 sample, where a small peak can also be seen.*

Light blue box: *highlights the BIT peak, which is found in wavelength B (lower). The yellow peak represents the amount of BIT in the DCOIT10BIT10 sample. The flat spectra under the yellow peak signify that BIT was not detectable in the other samples.*

3.2 EROD Activity

The following figures show the results from the EROD assay. The experimental set up is described in section 2.4. Firstly, figure 15 shows the specific EROD activity of each treatment. The positive control, BNF, indicates induction of CYP1A, however there seems to be a high standard deviation. The sample with cells exposed to DCOIT10 and BIT10 are similar. The samples exposed to DCOIT5 and the mixtures have lower values, meaning a lower rate of

induction. Referring specifically to the DCOITBIT mixtures, the standard deviation looks to be relatively low.

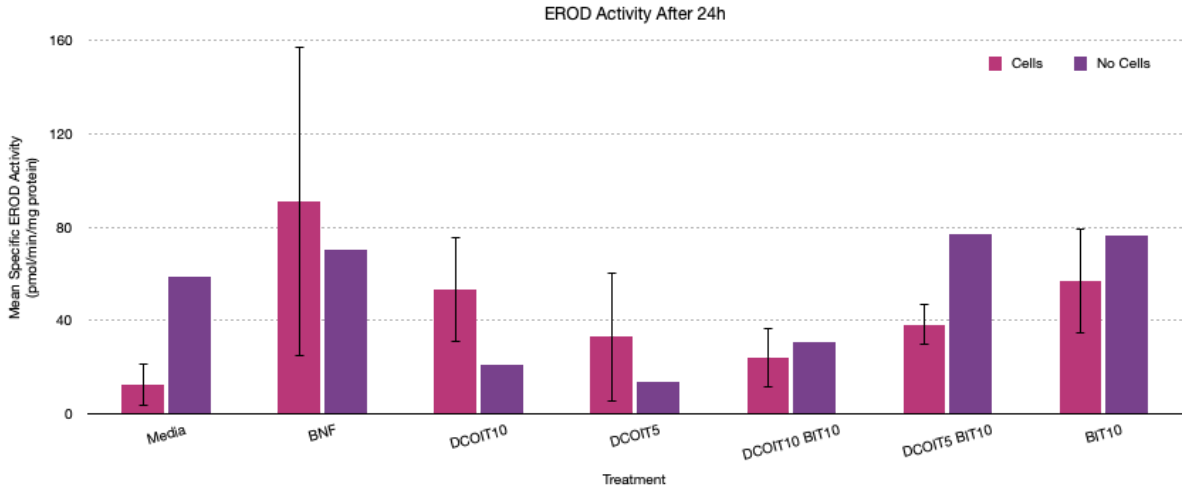

Figure 15: Specific EROD activity samples with and without cell suspension exposed to different treatments for 24 hours. Pink bars represent the values for samples with cells and purple bars represent the samples without cells.

The protein content in each well of the EROD analysis was also measured, as shown in figure 16. The protein content across each treatment seem to be very similar and the results also show relatively high standard deviations. Based on what the figure shows, the DCOIT10 treatment has the lowest protein content, while DCOIT 5 and DCOIT5BIT10 have the highest protein content.

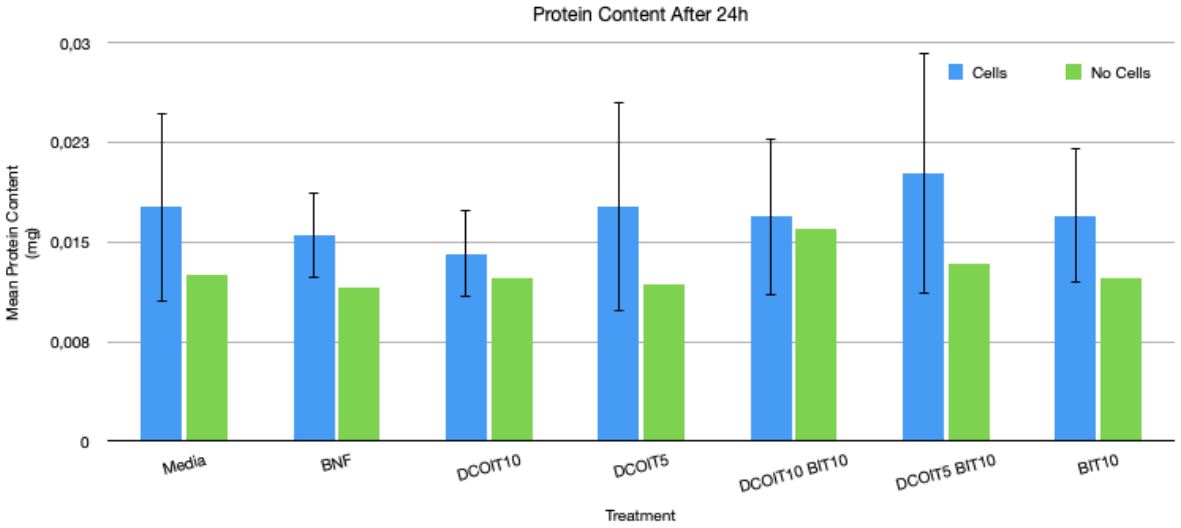

Figure 16: Mean protein content of samples with and without cell suspension, exposed to different treatments for 24 hours.

3.3 Cytotoxicity

The following figures show the results from using the fluorescent dyes AB and CFDA-AM in order to look at the mitochondrial metabolic activity and cell membrane integrity of the cells, respectively. Figure 17 contains the results from the samples only containing media, and it can be seen there is a significant difference compared to samples that have been exposed to either DCOIT or BIT.

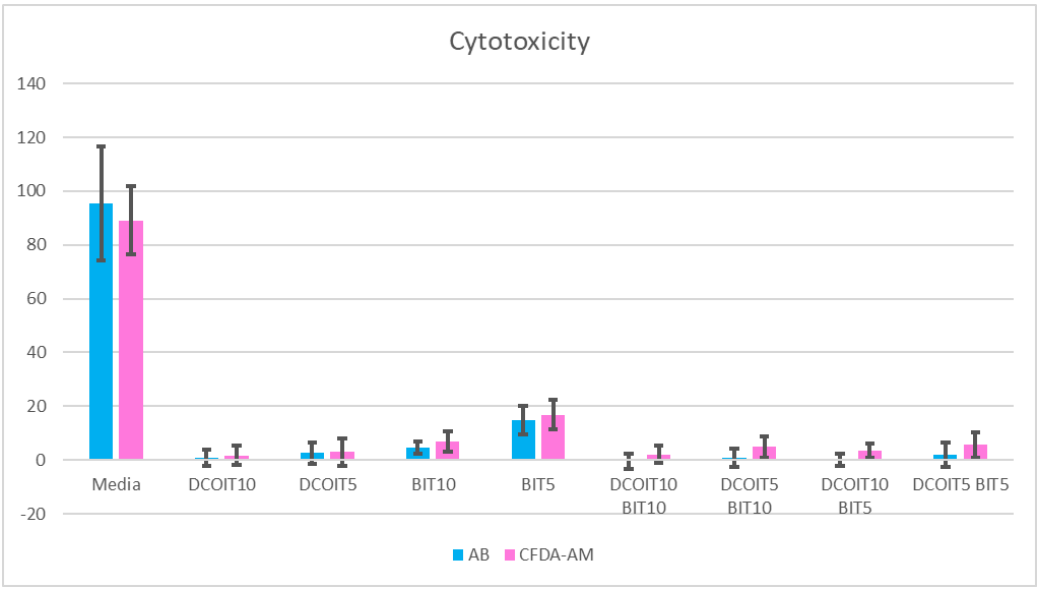

Figure 17: Bar chart comparing the mitochondrial activity (AB) and the cell membrane integrity (CFDA-AM).

In figure 18 we have the same results as figure 17, but a closer look at the treatments of interest. The DCOIT10BIT10 treatment is the only negative value and DCOIT10 also has a low value, which indicates that these treatments have very low metabolic activity. The cells treated with BIT10 seem to have the highest metabolic rate.

The CFDA-AM values are higher, but seem to follow a similar trend. Both DCOIT10 and DCOITBIT10 are the lowest, indicating that these samples have a lower amount of intact cell membranes. The cells treated with BIT10 also have the highest value. Most of the treatments, apart from BIT10, show relatively high standard deviation.

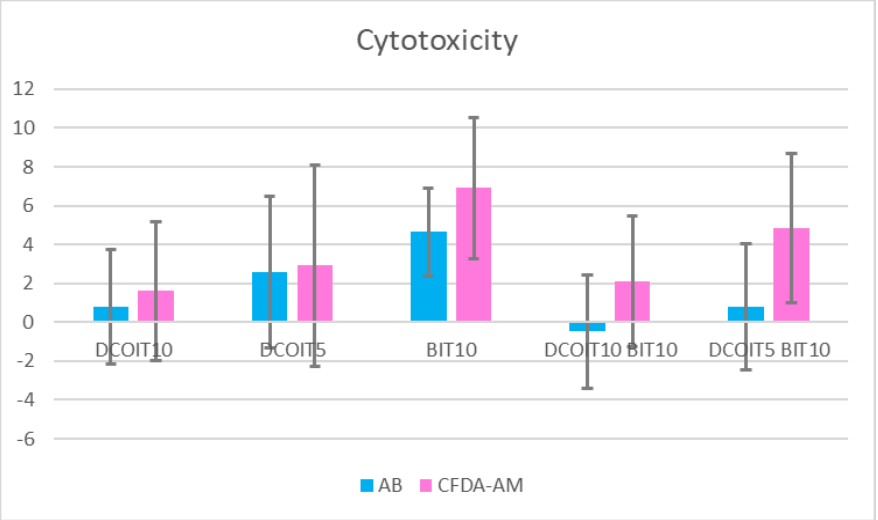

Figure 18: Bar chart comparing the mitochondrial activity (AB) and the cell membrane integrity (CFDA-AM). These are the results from figure 14, without the media alone samples.

4.Discussion

4.1 HPLC

The first results from the HPLC data showed that we were not able to detect DCOIT in samples that had cell suspensions. At first, this was not expected so the experiment technique was questioned, but further analysis showed the same results. A possible explanation for this is that the DCOIT is not present in the cell media due to being inside the cells having been metabolised. The samples that had the same DCOIT treatment, but without cells, have clear peaks that have a retention time we would expect to see for DCOIT, and this shows that the exposure concentrations were made correctly. The BIT only treatments, however, show that BIT is always detectable in the media, whether cells were present or not. There is an observed decrease in the amount of BIT in samples containing cells, this is expected due to the cells metabolising it.

The mixture samples, figures 8 and 10, DCOIT shows the same trend whereby it is not detectable in the sample without cells. From this, a conclusion cannot be drawn about a possible cocktail effect between these biocides. If we then look at the BIT retention peaks we have more information. The concentration of DCOIT seems to affect the amount of BIT in the media, this is highlighted in figure 11. The highest DCOIT treatment, DCOIT10, has a high BIT peak, while when DCOIT is not present the BIT peak is low. Even though DCOIT itself is not detected in the media, it seems to affect the metabolism of BIT. This could mean the cells have “preference” to metabolise DCOIT, for example, if DCOIT more readily enters the cells. It may have something to do with cytotoxicity, if there is some level of cell damage so BIT is less able to be metabolised. This does point to a cocktail effect between these biocides.

In samples that were exposed to treatments of BIT for 24 hours, such as the samples in figure 13, there is a possible metabolite of BIT. The peak is only visible on wavelengths of BIT and is

found around the same retention time. The peak is found on a different wavelength, which could indicate how the BIT molecule is broken down. The peak also has slightly earlier retention time, which might suggest that it is less hydrophobic than BIT itself.

4.2 EROD Activity

The result of BNF in figure 15 represents the induction of CYP1A enzymes. From our hypothesis, we would expect to see the DCOIT only samples to have the higher specific EROD activity. The activity for DCOIT10 is relatively high, but the BIT10 treatment has the highest activity, apart from BNF. There was no expectation about what might happen with the mixture treatments, but they have the lowest activity. The 2 mixture treatments also have the smallest standard deviations, which mean the results are more accurate than the other treatments. Despite DCOIT and BIT inducing the CYP1A enzymes when present alone, there's something about the combination that lowers the ability.

The protein content is harder to draw conclusions from as the results are similar to each other, and most of the samples have high standard deviation. From the data, though we can see that DCOIT5BIT10 and DCOIT5 treatments had the most protein in the well

4.3 Cytotoxicity

Based on our hypothesis that DCOIT is more toxic than BIT, we would expect the DCOIT alone treatments to have the lowest values. The results in figure 18 support our hypothesis about DCOIT being more toxic than BIT, but the lowest values are the mixture treatments. Firstly, looking at the mitochondrial activity, DCOIT10BIT10 has a negative value, then DCOIT5BIT10 has a similar value to DCOIT10. This suggests that DCOIT may target inside the cell and disrupt mitochondrial activity.

The CFDA-AM values follow a similar trend, whereby DCOIT10BIT10 has one of the lowest values of membrane integrity, and BIT10 alone has the highest value, indicating a higher percentage of intact membranes. Although it follows a similar trend, the values (pink bars) are higher, more notably in the samples with DCOIT. If we compare this observation to our data from the HPLC analysis, whereby DCOIT was not found in cell media, instead potentially in the cells then there is a trend. Figure 18 also shows then that BIT alone also has a more toxic effect inside the cell compared to damaging the cell membrane.

Previous studies have shown that DCOIT causes mitochondrial metabolic damage at lower concentrations than BIT (Kim et al., 2021). The results from an in vitro study on the effect of DCOIT on rat livers also showed a negative effect of DCOIT, whereby the production of adenosine 5′-triphosphate that occurs in the liver, was inhibited (Bragadin et al., 2005). Additionally, DCOIT has been shown to disrupt the mitochondrial activity and cause oxidative stress in haemocytes (Cima et al., 2008).

4.4 Answering Aim and Hypothesis

The first research question, looking at the toxic effects of DCOIT and BIT in PLHC-1 cells, can be answered. It would seem that DCOIT is more toxic to the cells when alone, compared to BIT. The toxic effects seem likely to occur within the cells. Looking at the cytotoxicity assays, DCOIT is notably more cytotoxic in any treatment. It's possible the concentration of BIT that was used was not enough to damage the cells. Since BIT was detected in all treatments in the HPLC analysis, maybe it is not as readily taken into the cells, especially when higher concentrations of DCOIT are present.

The second research question was whether or not the mixtures are more toxic. It would seem from our results that the mixture treatments are less toxic than when DCOIT is alone, but more toxic than when BIT is alone. However we did notice that there is more bit detected outside the cells when there is a higher DCOIT concentration, so maybe BIT is less effective when mixed witH DCOIT.

The third research question addresses potential metabolites, which is believed to be found in the HPLC analysis. Since the metabolite is found on the other wavelength and has a slightly earlier retention time, then it would be interesting to analyse further to find out how it occurs. Additionally why it seems to occur after 24 hours of exposure.

There are few studies that look at the toxic mechanisms of these two isothiazolinones, especially concerning the HPLC and measuring EROD activity methods used in this project, there are not many results or studies to compare with. Despite this, the results in this project show similar trends to studies that have been carried out. Specifically concerning DCOIT, where there is evidence that it is involved in some way in the mitochondrial activities as well as being more toxic to the cells than BIT. The hope is that the results of this thesis will prove useful for future studies.

5. Conclusion

The original theory that DCOIT was more toxic than the BIT was proven in our results. Before the HPLC analysis, the DCOIT was thought to have been present in the cell media, but this turned out not to be the case. Additionally we saw that the more DCOIT in the mixture concentration the, the BIT content in the cell media.

The EROD data also showed that DCOIT, more specifically the DCOIT10 treatment to have the stronger effect, by inducing the CYP1A at a higher rate than the other test treatments. Interestingly BIT 10 also showed high activity rates compared to the mixtures. The protein contents of each well were very similar so there were no trends to pick up on from the figure.

The cytotoxicity assays suggest that DCOIT is entering and damaging the cells internally, via metabolism pathways, as was indicated in the HPLC chromatograms. In comparison to DCOIT, BIT alone did not have as strong of cytotoxic effect.

There is still a lot of potential for future research to look at changes in intracellular metabolism and potential synergistic interactions of a wider range of concentrations and mixture treatments. This could also include testing another isothiazolone against one of the ones studied in this project. General questions were able to be answered based on the results that were found, but there is still a lot of complexity with researching these chemicals.

Especially since the concentrations and experimental set-up cannot simulate what a real environment would be like. This project hopefully still highlights the need to further the understanding of isothiazolones and their mechanisms, with the eventual goal to restrict them from being so widely used.

References

Abreu, F.E.L. *et al.* (2020) 'Are antifouling residues a matter of concern in the largest South American port?', *Journal of Hazardous Materials*, 398, p. 122937. doi:10.1016/j.jhazmat.2020.122937.

Bhardwaj, S., Dwivedi, K. and Agarwal, D. (2015) 'A Review: HPLC Method Development and Validation', *International Journal of Analytical and Bioanalytical Chemistry*, 5(4), pp. 76–81.

Bonnier, F. *et al.* (2015) 'Cell viability assessment using the Alamar Blue Assay: A comparison of 2D and 3D cell culture models', *Toxicology in Vitro*, 29(1), pp. 124–131. doi:10.1016/j.tiv.2014.09.014.

Bopp, S.K. and Lettieri, T. (2008) 'Comparison of four different colorimetric and fluorometric cytotoxicity assays in a zebrafish liver cell line', *BMC Pharmacology*, 8(1). doi:10.1186/1471-2210-8-8.

Bragadin, M., Pavoni, B., Scutari, G. and Manente, S. (2005). An in vitro study of the interaction of Sea-Nine® with rat liver mitochondria. *Environmental Toxicology and Chemistry*, 24(5), pp.1074–1078. doi:https://doi.org/10.1897/04-349R.1.

Bruze, M. *et al.* (1987) 'Contact allergy to the active ingredients of Kathon CG in the Guinea pig', *Acta Dermato-Venereologica*, 67(4), pp. 315–320. doi:10.2340/0001555567315320.

Bruze, M., Gruvberger, B. and Agrup, G. (1988) 'Sensitization studies in the guinea pig with the active ingredients of Euxyl® K 400', *Contact Dermatitis*, 18(1), pp. 37–39. doi:10.1111/j.1600-0536.1988.tb05487.x.

Bucheli, T.D. and Fent, K. (1995) 'Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems', *Critical Reviews in Environmental Science and Technology*, 25(3), pp. 201–268. doi:10.1080/10643389509388479.

Cima, F., Bragadin, M. and Ballarin, L. (2008) 'Toxic effects of new antifouling compounds on tunicate haemocytes', *Aquatic Toxicology*, 86(2), pp. 299–312. doi:10.1016/j.aquatox.2007.11.010.

Eom, H.-J., Md. Niamul Haque, Nam, S.-E., Lee, D.-H. and Rhee, J.-S. (2019). Effects of sublethal concentrations of the antifouling biocide Sea-Nine on biochemical parameters of the marine polychaete Perinereis aibuhitensis. *Comparative Biochemistry and Physiology*, 222(222), pp.125–134. doi:https://doi.org/10.1016/j.cbpc.2019.05.001.

Fonseca, V.B., Guerreiro, A. da S., Vargas, M.A. and Sandrini, J.Z. (2020). Effects of DCOIT (4,5-dichloro-2-octyl-4-isothiazolin-3-one) to the haemocytes of mussels Perna perna. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 232. doi:https://doi.org/10.1016/j.cbpc.2020.108737.

Gabe, H.B., Guerreiro, A. da S. and Sandrini, J.Z. (2021). Molecular and biochemical effects of the antifouling DCOIT in the mussel Perna perna. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 239, p.108870. doi:https://doi.org/10.1016/j.cbpc.2020.108870.

Guengerich, F.P. (1993) *American Scientist*, 81(5), pp. 440–447.

Huuskonen, S.E., Hahn, M.E. and Lindström-Seppä, P. (1998) 'A fish hepatoma cell line (PLHC-1) as a tool to study cytotoxicity and CYP1A induction properties of cellulose and wood chip extracts', *Chemosphere*, 36(14), pp. 2921–2932. doi:10.1016/s0045-6535(97)10248-x.

Kim, D., Kim, E.-H. and Bae, O.-N. (2021) 'Comparative study of two isothiazolinone biocides, 1,2-benzisothiazolin-3-one (bit) and 4,5-dichloro-2-n-octyl-isothiazolin-3-one (DCOIT), on barrier function and mitochondrial bioenergetics using murine brain endothelial cell line (bend.3)', *Journal of Toxicology and Environmental Health, Part A*, 84(22), pp. 932–943. doi:10.1080/15287394.2021.1955786.

Marrero-Alemán, G. *et al.* (2020) 'Persistence of isothiazolinones in clothes after machine washing', *Dermatitis*, 32(5), pp. 298–300. doi:10.1097/der.0000000000000603.

Rampersad, S.N. (2012) 'Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays', *Sensors*, 12(9), pp. 12347–12360. doi:10.3390/s120912347.

Schwensen, J.F. and Johansen, J.D. (2019) 'Isothiazolinones', *Kanerva's Occupational Dermatology*, pp. 507–520. doi:10.1007/978-3-319-68617-2_216.

Silva, V., Silva, C., Soares, P., Garrido, E.M., Borges, F. and Garrido, J. (2020). Isothiazolinone Biocides: Chemistry, Biological, and Toxicity Profiles. *Molecules*, 25(4), p.991. doi:https://doi.org/10.3390/molecules25040991.

Steen, R.J.C.A. *et al.* (2004) 'Monitoring and evaluation of the environmental dissipation of the marine antifoulant 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT) in a danish harbor', *Chemosphere*, 57(6), pp. 513–521. doi:10.1016/j.chemosphere.2004.06.043.

Vidushi, Y. and Meenakshi, B. (2017) 'A REVIEW ON HPLC METHOD DEVELOPMENT AND VALIDATION', *Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences*, 2(6), pp. 166–178. doi:10.26479/2017.0206.12.

Whyte, J.J. *et al.* (2000) 'Ethoxyresorufin-*o*-deethylase (EROD) activity in fish as a biomarker of chemical exposure', *Critical Reviews in Toxicology*, 30(4), pp. 347–570. doi:10.1080/10408440091159239.

Appendix A - EROD Calculations

An example of the calculations used to find the specific EROD activity and protein content.

Specific EROD Activity

The method for preparing the analysis is described in section 2.4. The first equation (1) needed is:

EROD activity = Slope of raw data/ Resorufin standard slope

The next equation (2), which calculates the specific EROD activity is:

Specific EROD activity = EROD activity / protein count

EROD Activity

When the plate is read, it measures 10 counts per well and these are the raw data. These 10 counts are plotted, where the x-axis is 1-10 and the y-axis is the counts. Using excel the gradient is found in the line equation function. The slope of the data in table 6 is 0.0979.

Table 6: Raw data example of one DCOIT10 treatment.

Count / Treatment	DCOIT10
1	29,425
2	30,046
3	31,183
4	30,463
5	29,572
6	31,606
7	31,736
8	30,469
9	30,380
10	30,706

Next we need the slope of the resorufin standard plate. This is done the same way as above, except each well has one count. The slope of resorufin is 0.0956. Using equation (1):

EROD Activity = 0.0979 / 0.0956 = 1.024

Table 7: Raw data for resorufin standard

Resorfun st	Concentration	Counts
Standard 1	0pmol	3,211
Standard 1	0pmol	3,77
Standard 2	3,13 pmol	4,067
Standard 2	3,13 pmol	4,246
Standard 3	6,26 pmol	4,572
Standard 3	6,26 pmol	5,063
Standard 4	12,5pmol	6,672
Standard 4	12,5pmol	6,754
Standard 5	25 pmol	7,858
Standard 5	25 pmol	8,011
Standard 6	50 pmol	10,892
Standard 6	50 pmol	12,311
Buffer	100 pmol	14,083
Buffer	100 pmol	11,757

Protein Content

Similarly to above, we need to find the slope of the BSA standard plate, where x-axis is the raw counts and the y-axis is the BSA concentration per in each well. Instead of adding a linear equation, the line equation will be a polynomial: $Y = Ax^2 + Bx - C$

The polynomial equation is:

$Y = -6.785x^2 * 10^{-9} + 6.85x * 10^{-5} + 0{,}0048$

where x is the raw protein counts, and Y is the protein mg/well.

From the raw data, the protein count for DCOIT10 treatment is 120.365. Inserting this value into polynomial equation, we get a Y = 0.013 mg/well

Table 8: Raw data from BSA standard plate

Protein Raw Data	mg/well	Counts
F5	0	60,471
F6	0	57,121
F3	0,01	93,041
F4	0,01	84,617
F1	0,021	160,386

F2	0,021	170,014
E5	0,042	447,928
E6	0,042	451,957
E3	0,083	1346,699
E4	0,083	1403,064
E1	0,167	6297,912
E2	0,167	6325,959

Using equation (2):

Specific EROD activity = 1.024 / 0.013 = 78.77

Each chemical treatment had 3 replicates, and the three values were used to calculate the standard deviation in figure 12.

Appendix B - Example of Cytotoxicity Calculations

The cytotoxic effect is measured in %, and is calculated from the raw absorbance data.

% Cytotoxicity AB

The equation used to calculate:

% AB = [(FU exposed cells - FUblank) / (FUvehicle control cells - FUblank)]* 100

Table 9: Raw data from AB fluorometry

DCOIT 10	DMSO (Vehicle control)	Blank
67.243	197.217	66.027

In this case the % of cytotoxicity of AB is:

% = [(67.243 - 66.027) / (197.217 - 66.027)] * 100

= 0.927 %

% Cytotoxicity CFDA-AM

The equation is the same as above:

% CFDA-AM = [(FU exposed cells - FUblank) / (FUvehicle control cells - FUblank)]* 100

Table 10; Raw data from CFDA-AM fluorometry

DCOIT10	DMSO (Vehicle control)	Blank
107.896	890.1	76.55

Using table 10, the % cytotoxicity of DCOIT10 is:

% = [(107.896 - 76.55) / (890.1 - 76.55)] *100

= 3.85%

Each treatment had 8 replicates, and the values were calculated for each replicate, then used to find the standard deviation in figures 14 and 15.