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Project Summary

An environmental risk assessment was conducted to characterise and compare the risk of seven shortlisted partitioning inter-well tracer candidates. The candidates were: Pyridine, 2-3-Dimethylpyrazine, 2,6-Dimethylpyrazine, 4-Methoxybenzyl alcohol, 3,4-Dimethoxybenzyl alcohol, 4-Chlorobenzyl alcohol, and 2,6-Dichlorobenzyl alcohol. A literature review for each candidate was done along with a determination of the assessments required for the environmental risk assessment. Once determined, the assessments were conducted to find three properties of each partitioning inter-well tracer candidate. These properties were: the bioaccumulation potential, the biodegradation potential over 28 days, and the toxicity characteristics of the candidates on algae (*Skeletonema costatum*) and on fish cells (Rainbow Trout gill cells).

The bioaccumulation potential was calculated based on results from work done before this thesis in shortlisting the candidates. Based on these calculations, none of the candidates were found to be effectively bioaccumulating in organisms to a large extent. The biodegradation potential of 2,3-Dimethylpyrazine, 4-Chlorobenzyl alcohol, and 2,6-Dichlorobenzyl alcohol were found to be near the threshold that characterises a compound as persistent while 4-Methoxybenzyl alcohol and Pyridine were the only candidates readily biodegradable. The toxicity characteristics of all the compounds were found to be relatively low and of no substantial concern for the partitioning inter-well tracer applications. The two most toxic compounds of the candidates were 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol. The property of most concern for PITT applications of the candidates was found to be the biodegradation potential.

Discharge data for each candidate and its properties were individually added to a real produced water profile and their discharge scenario simulated over a 30-day period using the dynamic risk and effects assessment model. The model determined the average contribution to the environmental impact factor, a method of characterising risk contribution of compounds. All candidates had an environmental impact factor less than commonly used threshold values and therefore the risk for all seven candidates was characterised as “acceptable”. Regarding contribution to risk comparison of the candidates based on the environmental impact factor it was concluded that the compound with the least risk contribution was 2,3-Dimethylpyrazine, followed by 2,6-Dimethylpyrazine, 3,4-Dimethoxybenzyl alcohol, 4-Methoxybenzyl alcohol, Pyridine, 2,6-Dichlorobenzyl alcohol, and the highest, 4-Chlorobenzyl alcohol. However,

based on all assessment conducted, 4-Methoxybenzyl alcohol and Pyridine were determined to be the “best” environmental candidates, as they had acceptable results for all aspects assessed. The other candidates may have better tracer properties and be preferred for applications, in these situations the outcomes of this ERA are designed to form the basis for risk-informed decisions.

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A special thanks must go to my family and friends who have been immense throughout my studies and more so during this thesis. I am eternally grateful for the support, motivation, and much needed encouragement from my mother, Siv.Ing. Inger-Johanne Gamlem Njau, my siblings Maria and Jonas, and my partner Courtenay. Last, but not least, I would like to thank my late father Dr. Ing. Gregory Joseph Njau who continues to be my inspiration and who I wish to emulate.

Foreword

This thesis has been conducted as an Environmental Risk Assessment (ERA). The ERA has not been an individual effort due to the size of the task and the time constraint. The work, particularly in the laboratories, was done together with PhD candidate Mehul Arun Vora as part of his ongoing research, of which an article will be published accordingly. The establishing and modifications of the methods used was a joint effort. The literature review conducted and included in this thesis was an individual effort. The Dynamic Risk and Effects Assessment Model (DREAM) simulations included in this thesis were carried out by PhD candidate Mehul Arun Vora. Upon consultation with my supervisor, Associate Professor Steinar Sanni, we decided to have the thesis written based on the entire work done.

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List of Abbreviations

BCF	Bioconcentration Factor
BOD	Biological Oxygen Demand
DMSO	Dimethyl sulfoxide
DREAM	Dynamic Risk and Effects Assessment Model
EC_{50}	Concentration where 50% of the organisms are affected
EIF	Environmental Impact Factor
EOR	Enhanced Oil Recovery
ERA	Environmental Risk Assessment
FBS	Fetal Bovine Serum
FSU	Fluorescence Units
HEPB	Hill Equation with Prediction Band
HOCNF	Harmonised Offshore Chemical Notification Format
IFE	Norwegian Institution for Energy technology (Institutt For Energiteknikk)
IOR	Improved Oil Recovery
K_{OW}	Octanol/water Partitioning Coefficient
NCS	Norwegian Continental Shelf
NIVA	Norwegian Institute for Water Research
NOEC	No Observable Effect Concentrations
NORCE	Norwegian Research Centre AS
OECD	Organisation for Economic Co-operation and Development
OSPAR	Oslo and Paris Commission
PBS	Phosphate Buffered Saline
PEC	Predicted Environmental Concentration
PITT	Partitioning Inter-well Tracer Tests
PNEC	Predicted No-Effect Concentration
RF	Recovery Factor
RTgill-W1	Rainbow Trout Gill Cells
SWCTT	Single-Well Chemical Tracer Tests
ThOD	Theoretical Oxygen Demand
UiS	University of Stavanger

Chapter 1: Introduction

The efficiency of production in the oil and gas industry has, and continues to, significantly improve as new methods of both Improved Oil Recovery (IOR) and Enhanced Oil Recovery (EOR) are being developed. These methods are of increasing importance as the rate of new oil and gas field discoveries are no longer as high as they once were and is in fact falling rapidly. This has led to more emphasis on improving the recovery factor (RF) in current fields (1). The progress of inter-well tracers in aiding the improvement of the RF, among other EOR methods, have led to optimism within the industry (2,3). Partitioning inter-well tracer tests (PITT), in particular, have witnessed a growing interest and are being considered as the way forward for the oil and gas industry because of their ability to provide an enhanced understanding of mature reservoirs. Research conducted by the Norwegian Institution for Energy technology (IFE), the Norwegian Research Centre AS (NORCE) and the University of Stavanger (UiS), based on offshore tracer stability assessments has provided a shortlist of seven PITT candidates. These are: Pyridine, 2,3-Dimethylpyrazine, 2,6-Dimethylpyrazine, 4-Methoxybenzyl alcohol, 3,4-Dimethoxybenzyl alcohol, 4-Chlorobenzyl alcohol, and 2,6-Dichlorobenzyl alcohol (3–5).

1.1 Why an Environmental Risk Assessment?

An environmental risk assessment (ERA) is yet to be conducted on these shortlisted PITT candidates. Therefore, the risk related to the potential fate and effect on the environmental surroundings from the eventual point of discharge are not known. The environmental impact for any chemical used in the offshore oil and gas industry is of extreme significance. This is the reason for implemented measures of an ERA before discharge, monitoring during discharge, and analysing impact after discharge (6,7).

A characteristic of all shortlisted PITT candidates is their stability, meaning they are of persistent nature. Any chemical compound that shows signs of persistency is a cause for concern for the environment it is released into. Also known is the bioaccumulation potential of the PITT candidates, otherwise known as the octanol/water partitioning coefficient (K_{OW}) (3). This is another chemical property that could prove negatively impactful to the environment, as K_{OW} values determine the path of the chemicals within organisms (8). However, other ERA parameters, namely the biodegradation potential and toxicity of these chemicals in offshore environments, are unknown and need to be determined experimentally in order to portray the PITT candidate's environmental impact and overall risk during application.

1.2 Objective and Scope of Thesis

The goal of this thesis is to establish an understanding of the potential environmental impact and characterize as well as compare the risk of each shortlisted PITT candidate when discharged. This is achieved by conducting an ERA following the strict guidelines set by the Oslo and Paris Commission (OSPAR), known as the Harmonised Offshore Chemical Notification Format (HOCNF). According to the HOCNF guidelines, it is essential to conduct both an exposure and an effect assessment of the chemical. The exposure assessments take into account the biodegradation and bioaccumulation potential of the chemical, among other factors, and determines their environmental concentrations upon a simulated discharge; while the effect assessments determine the toxicity of the chemicals on standardized organisms (9). The results of these assessments are to be used to characterize the risk and the environmental impact by applying the Dynamic Risk and Effects Assessment Model (DREAM) (10). Thereafter, the PITT candidates with the least environmental impact and contribution to risk are to be highlighted to allow for environmental risk-informed decisions (11).

The report from herein has the following sections: theory, materials and methods, results and discussion, and conclusion. The theory section consists of a literature review conducted on PITT's and the candidates shortlisted, as well as a general overview of ERA's and how it can be applied to the PITT candidates. The materials and methods section details the international standards that were followed strictly or modified, and the equipment used in all experiments. The results and discussion section shows the findings of each experiment and explains the significance of its individual result; thereafter the findings are collectively discussed, and the environmental impact of each PITT candidate is understood via DREAM simulations. A conclusion is then drawn and stated in the last section of this thesis. The appendix contains the raw data of the experiments conducted in the laboratories.

Chapter 2: Theory

Before conducting an ERA, it is important to have an overview and understanding of the substances being assessed. Therefore, this section gives a detailed theoretical review of the shortlisted PITT candidates before entailing what an ERA is and specifically how it will be conducted.

2.1 Partitioning Inter-well Tracer Tests

The application and technique of PITT has been well utilised in hydrogeology when analysing contamination of non-aqueous liquids in the subsoil (12–14). PITT works by introducing an oil/water partitioning tracer, with a known partition coefficient ($K_{OW} = \frac{Conc.\ Tracer\ in\ Oil}{Conc.\ Tracer\ in\ water}$), together with a passive tracer, into an entrance well and effectively timing the lag difference between the two tracers at the exit well. The lag between the tracers is a result of the passive tracer remaining in the aqueous phase and not interacting with the oil in the reservoir whilst the partitioning tracer interacts with both phases. The results are analysed using the reversible, yet constant, equilibrium distribution of the partitioning tracer between the aqueous and hydrocarbon phases of the reservoir (15,16).

This is nowadays being applied in the offshore industry where it provides data that can be used to understand both the distribution and concentration or oil saturation (S_o) of the hydrocarbon phase of the reservoir (17,18). The tracers improve the production and overall RF by giving a better understanding of the given reservoir (3). It should be mentioned that as of now, S_o is most commonly calculated in the oil industry using single-well chemical tracer tests (SWCTT) however, as inter-wells continue to increase with mature oil fields, then PITT is the better solution (2,19).

The conditions the PITT is exposed to offshore are remarkably different to those experienced in contamination analysis of non-aqueous liquids in subsoil hydrogeology. Therefore, although the principle is the same, not all tracers known to be successful in hydrogeology can be applied offshore (3). This is the reason for the lack of tested tracers for offshore use, resulting in rare application of PITT. However, research within the field points toward the potential of several tracers being applicable in extreme offshore conditions. Industrial interest has already led to the commercialisation of tracers for PITT through patents (20). The criteria for these tracers is that they: do not pre-exist in the reservoir, be environmentally accepted, have good partitioning characteristics, shown to be stable both chemically and biologically, and have negligible

sorption on rock surfaces (21). Although environmentally accepted, not enough knowledge regarding the environmental impact of tracers, including PITT tracers, has been acquired; specifically within the context of being applied offshore where they may be released with other harmful toxins in produced water discharges (6,22).

2.2 The PITT Candidates

Research on the stability in offshore conditions of potential PITT tracer candidates has been conducted by IFE in collaboration with NORCE and UiS. The tracer candidates tested were varying chemicals that could be divided into 4 groups: linear diols, pyrazines, pyridines, and benzyl alcohols. IFE concluded that the most stable candidates did not include linear diols and were the chemicals: Pyridine, 2,3-Dimethylpyrazine, 2,6-Dimethylpyrazine, 4-Methoxybenzyl alcohol, 3,4-Dimethoxybenzyl alcohol, 4-Chlorobenzyl alcohol, and 2,6-Dichlorobenzyl alcohol (3,4). The characteristics of these shortlisted PITT tracer candidates will be discussed in the following subsections.

2.2.1 Pyridine

Pyridines are naturally occurring chemicals that can be found in natural products, pharmaceuticals and materials (23). All Pyridines have a structure very similar to benzene, with the difference being the substitution of a carbon atom with a nitrogen atom as can be seen in Figure 1.

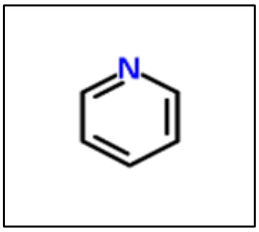

Figure 1: Chemical structure of Pyridine.

Pyridines are synthesised for use in varying industries, with the most reputable use being within medicine. They are receiving growing interest due to their optical and physical properties, such as those seen in Table 1, and because they are regarded as safe to use (24,25).

Table 1: Known properties of the shortlisted Pyridine PITT tracers (3).

Compound	Melting point ˚C	Boiling point ˚C	K_{OW}	pK_a
Pyridine	-42	115	4.4	5.25

The properties seen in Table 1 can be viewed as good PITT properties, as the temperature range is within many reservoirs, the K_{OW} value indicates good partitioning, and the pK_a value does not indicate incompatibility with the reservoirs. IFE performed stability tests on other forms of pyridines, these were 3-Hydroxypyridine, 4-Methoxypyridine, and 2-Hydroxy-6-methylpyridine. None of these were as stable as Pyridine, which was the only compound that was not significantly broken down by the offshore conditions (3).

2.2.2 Pyrazine

Pyrazines are a family of chemicals that can be found in nature but also synthesized and commonly used as food additives, where they are applied for aroma and flavouring. Other uses of pyrazines include the pharmaceutical industry. They are monocyclic aromatic compounds, that have two nitrogen atoms instead of carbon in the para positions and have their highest production yield at a basic pH, with relatively high temperatures between 120 and 150˚C (26). The specific structure of the shortlisted PITT pyrazines can be seen below in Figure 2 and 3, these are 2,3-Dimethylpyrazine and 2,6-Dimethylpyrazine.

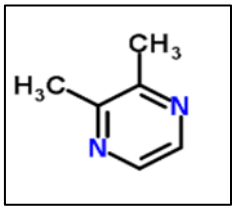

Figure 2: Chemical structure of 2,3-Dimethylpyrazine.

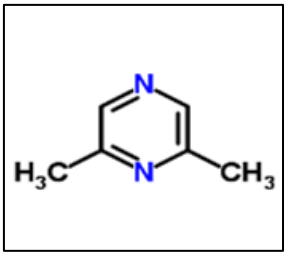

Figure 3: Chemical structure of 2,6-Dimethylpyrazine.

As can be seen in the figures, these aromatic compounds have features that allow them to partition well in both the water and hydrocarbon phases of the reservoir. The physicochemical properties known for the two pyrazines can be seen in Table 2.

Table 2: Known properties of the shortlisted pyrazine PITT tracers (4).

Compound	Melting point ˚C	Boiling point ˚C	K_{OW}	pK_a
2,3-Dimethylpyrazine	-12	156	3.47	1.62
2,6-Dimethylpyrazine	37	154	3.54	1.55

The pyrazines shortlisted display the characteristics needed for a PITT tracer candidate, as they are water soluble, have a good K_{OW} value, do not pre-exist in the reservoirs and have a suitable temperature and acidity range that have proven to befit offshore conditions through a stability assessment test (4). Additionally, they are deemed safe to use in their current applications (27).

2.2.3 Benzyl alcohols

The majority of shortlisted PITT tracer candidates are benzyl alcohols. Benzyl alcohols are a group of naturally occurring aromatic hydrocarbons found in plants, that are often used as reaction intermediates in chemical industries (28). The structures of the shortlisted tracer candidates are seen below in Figures 4-7, these are 4-methoxybenzyl alcohol, 3,4-Dimethoxybenzyl alcohol, 4-Chlorobenzyl alcohol, and 2,6-Dichlorobenzyl alcohol.

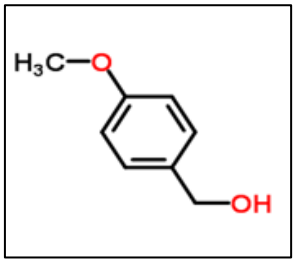

Figure 4: Chemical structure of 4-methoxybenzyl alcohol.

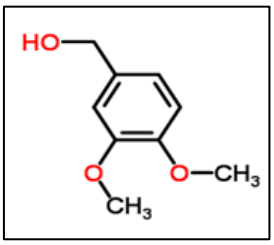

Figure 5: Chemical structure of 3,4-dimethoxybenzyl alcohol.

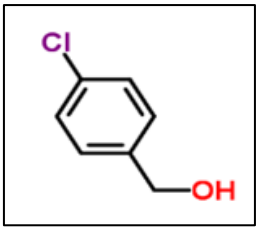

Figure 6: Chemical structure of 4-chlorobenzyl alcohol.

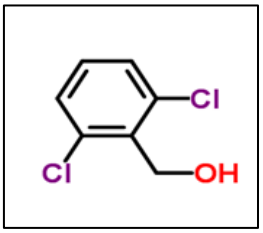

Figure 7: Chemical structure of 2,6-dichlorobenzyl alcohol.

The chemical features of the above figures show that these candidates are water soluble and can partition into the hydrocarbon phase, they also show that these compounds are basic. Table 3 below contains proof of these characteristics, they all have good K_{OW} values, relatively high pK_a values, and a decent temperature range regarding offshore conditions.

Table 3: Known properties of the shortlisted benzyl alcohol tracers (5).

Compound	Melting point ˚C	Boiling point ˚C	K_{OW}	pK_a
4-Methoxybenzyl alcohol	23	259	5.1	13.6
3,4-Dimethoxybenzyl alcohol	22	297	4.2	13.3
4-Chlorobenzyl alcohol	71	232	4.2	13.9
2,6-Dichlorobenzyl alcohol	98	268	7.0	13.5

IFE had initially shortlisted a hydroxybenzyl alcohol as well, but it was the methoxy and chlorinated benzyl alcohols that proved to be most stable in offshore conditions. The main reason was the temperature range, hydroxybenzyl alcohol was not stable beyond 50˚C which is impractical in offshore applications. During the stability assessment both 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol were found to be stable in offshore conditions for 12 weeks at 150˚C, whilst both 4-Methoxybenzyl alcohol and 3,4-Dimethoxybenzyl alcohol remained stable for 12 weeks at 125˚C but would degrade after one week at 150˚C. 4-

Methoxybenzyl alcohol and 3,4-Dimethylbenzyl alcohol still remain potential PITT candidates as there are many reservoirs that do not exceed temperatures of 125˚C (5).

2.3 Environmental Risk Assessment of the PITT Candidates

An Environmental Risk Assessment (ERA) is a method of understanding the potential consequences of the chemical compounds and picturing the risk of their use. The framework of a standard ERA, seen in Figure 8, begins with identification of a potential problem, followed by both an exposure and effect assessment, which then provides a risk characterization (29,30).

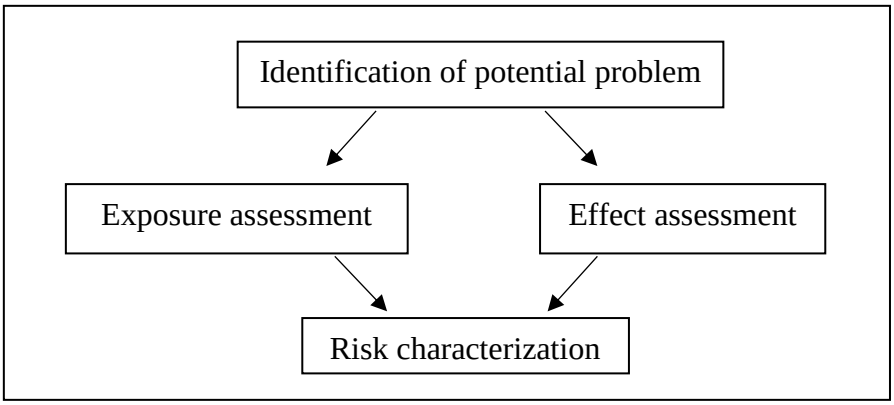

Figure 8: Standard ERA framework.

In the following subsections the potential problems focused on in this ERA will be identified, and the exposure and effects assessments chosen highlighted, as well as how the risk will be characterized.

2.3.1 Identification of Potential Problem

A good ERA begins with a well formulated identification of a potential problem that allows to narrow the focus of the ERA in the following assessments and consider the nature of the effects. The Oslo and Paris Commission (OSPAR) provides thorough guidelines that can be applied to IOR and EOR solutions (31). These guidelines are known as the Harmonised Offshore Chemical Notification Format (HOCNF). The HOCNF guidelines highlight three key properties that are to be known of each chemical in order to determine their environmental impact, these are bioaccumulation (partitioning coefficient), biodegradation, and aquatic toxicity (9). The overarching problem in this ERA is therefore identified as the potential environmental impact of the PITT candidates. This can be divided into the following areas of concern: the potential bioaccumulation in organisms; the fate of the chemicals in the

environment and how they biodegrade; and the potential toxicity of these PITT candidates to the exposed organisms (9).

2.3.2 Exposure Assessment

The goal of the exposure assessment is to understand where the potential contaminant will spread to throughout the environment where it is introduced (29). Once understood, the exposure assessment produces a predicted environmental concentration (PEC) value that will be used for risk characterization. PEC is estimated by combining the chemical's fate factors such as ocean currents, dilution, sedimentation among other measured or modelled values, with the biodegradation and bioaccumulation potential of the compounds (32). The biodegradation and bioaccumulation potential are the focus parameters of this exposure assessment. The reason being that these are compound specific values that will contribute to the fate and reach of the chemicals. This eventually leads to the calculated exposure scenarios of the risk assessment (9).

2.3.2.1 Biodegradation Potential

There are well practiced procedures for determining the biodegradation potential of chemicals. The OSPAR commissions guidelines, HOCNF, recommend chemicals to be analysed using the Organisation for Economic Co-operation and Development (OECD) standard test number 306, "Biodegradability in Seawater – Closed Bottle Method". This method has therefore been chosen to be conducted for all shortlisted PITT candidates. The principle of the method is to dissolve a known amount of the test substance in seawater and monitor the degradation by measuring the oxygen levels in intervals during a period of 28 days (9,33).

The biodegradation potential, also known as biological oxygen demand (BOD), is calculated using the theoretical oxygen demand (ThOD). The ThOD can be found using the compounds formula and its molecular weight (33). The first step is to find the amount of Oxygen needed to breakdown the chemical. This is done by balancing the chemical reaction of the compound using its formula, an example of this can be seen with Pyridine (C_5H_5N) in equation (1) below:

$$C_5H_5N + aO_2 \rightarrow bCO_2 + cNO_3^- + dH^+ + eH_2O \qquad (1)$$

equation (1) is then balanced to find the values of a-e. The balanced equation for Pyridine can be seen in equation (2):

$$C_5H_5N + 7.5O_2 \rightarrow 5CO_2 + NO_3^- + H^+ + 2H_2O \qquad (2)$$

The amount of Oxygen in moles needed to breakdown Pyridine is found to be 7.5 $\frac{mole\ O_2}{mole\ Pyridine}$. The second step is using this value to find the ThOD, which is expressed as $\frac{mg\ O_2}{mg\ Substance}$. This can be found by combining the expression in moles with the molecular weights of Oxygen and Pyridine, as seen in formula (3).

$$ThOD = 7.5\ \frac{mmol\ O_2}{mmol\ Pyridine} \times \frac{32\ \frac{mg\ O_2}{mmol\ O_2}}{79.09\ \frac{mg\ Pyridine}{mmol\ Pyridine}} = 3.04\ \frac{mg\ O_2}{mg\ Pyridine} \qquad (3)$$

These calculations are repeated for the remaining 6 PITT candidates and their ThOD can be seen in Table 4.

Table 4: ThOD calculated for each PITT candidate.

Compound	Formula	Molecular Weight (mg/mmol)	ThOD (mg O_2/mg compound)
Pyridine	C_5H_5N	79.09	3.04
2,3-Dimethylpyrazine	$C_6H_8N_2$	108.12	3.11
2,6-Dimethylpyrazine	$C_6H_8N_2$	108.12	3.11
4-Methoxybenzyl alcohol	$C_8H_{10}O_2$	138.16	2.20
3,4-Dimethoxybenzyl alcohol	$C_9H_{12}O_3$	168.19	2.00
4-Chlorobenzyl alcohol	C_7H_7OCl	126.63	1.79
2,6-Dichlorobenzyl alcohol	$C_7H_6OCl_2$	177.02	2.36

The biodegradation potential after 28 days is found by comparing the consumed oxygen values in the seawater to the theoretical consumption, ThOD, calculated in Table 4. The equation to be used, (4), is seen below:

$$\%\ biodegradation\ (BOD\ 28) = \frac{mg\ O_2^c/mg\ Compound}{mg\ ThOD/mg\ Compound} \times 100 \qquad (4)$$

where O_2^c is the difference between the Oxygen consumed with the test compound and the amount consumed by the blank seawater. The compound is regarded as persistent (red) if the biodegradation potential is less than 20% after 28 days, and biodegradable (green) if it is above 60%. The compounds are listed as yellow when the biodegradation is in between 20 and 60% (9,33,34).

2.3.2.2 Bioaccumulation Potential

Bioaccumulation potential is acquired using the octanol-water partitioning coefficient mentioned earlier, K_{OW}. It is the logarithm of this value that provides information on the potential bioaccumulation in organisms and is used in models together with biodegradation to determine the PEC value. When $log\ K_{OW}$ is equal to or larger than 3, the HOCNF guidelines deem the compound to be actively bioaccumulating in the organisms and therefore is seen as hazardous to life in that environment and must undergo further assessments to determine the bioconcentration factor (BCF) (9). The K_{OW} of each PITT candidate has already been determined through the assessments and experiments conducted when shortlisting them (3–5). The $log\ K_{OW}$ of each chemical is therefore easily determined and can be seen in Table 5.

Table 5: Bioaccumulation potential of shortlisted PITT candidates.

Compound	Bioaccumulation potential log (K_{OW})
Pyridine	0.643
2,3-Dimethylpyrazine	0.540
2,6-Dimethylpyrazine	0.549
4-Methoxybenzyl alcohol	0.708
3,4-Dimethoxybenzyl alcohol	0.623
4-Chlorobenzyl alcohol	0.623
2,6-Dichlorobenzyl alcohol	0.845

All shortlisted PITT candidates have a log (K_{OW}) less than 3. Therefore, according to HOCNF, the compounds are not actively bioaccumulating in organisms. Thus, they are not required to undergo further assessments on their persistence within specific organisms as part of this ERA.

2.3.3 Effect Assessment

During the effect assessment, the goal is to monitor the actual impact on surrounding organisms by the exposure. A chemical specific value known as the predicted no-effect concentration (PNEC) will be determined for use in this assessment in order to characterize risk. PNEC is the predicted concentration of the exposure where no effect is detected on the different organisms under consideration. PNEC is a threshold value for the ecological community. It can be based on different no observable effect concentrations (NOEC) determined in tests on varying organisms. Alternatively, it can be derived from another significant value that can be found in

similar tests, known as EC_{50}. This is the concentration of the compound where 50% of the organisms are affected (35,36). Both NOEC and EC_{50} can be determined from toxicity tests (29).

The HOCNF guidelines state that toxicity tests are mandatory for all new chemicals being introduced offshore (9). There are various toxicity tests that can be implemented. Generally, they consist of observing the effect of the compound on the organism or its tissue at different concentrations of the chemical. The effect measured is often the death or growth inhibition of the organism, but there is a steady rise in less harmful methods that do not require whole organisms, such as cell testing (37).

The toxic effects of the PITT candidates, like other chemicals, need to be determined on varying species. According to the HOCNF guidelines, toxicity tests should be conducted on three surrogate species: an alga, a crustacean, and a representative juvenile fish. Algae, crustaceans and fish allow for observation of the different levels of potential contamination (9). As the PITT candidates will be released with the produced water, the organisms primarily effected will be the surrounding algae and fish species. Additionally, toxicity results of algae species are often like those of the crustacean species. Therefore, the model species to be tested during this ERA will be an alga representative and a fish representative.

2.3.3.1 Toxicity Test on Algae

The algae to be used is *Skeletonema costatum*, a common and representative marine alga. The international standard for toxicity tests on *Skeletonema costatum* is the ISO10253:2016(E). The test is also known as a water quality test and works by determining the extent the growth of the algae is inhibited by a foreign compound dissolved in seawater. The algae are first cultured to a point of exponential growth before being inoculated and exposed to varying concentrations of the test substance for 72 hours. The cell density is measured every 24 hours and the average specific growth rate is determined using equation (5) below:

$$\mu = \frac{\ln(N_L) - \ln(N_0)}{t_L - t_0} \qquad (5)$$

where μ is the average specific growth rate; N_L is the measured cell density after a specified time, t_L; and N_0 is the initial cell density at start time, t_0.

Thereafter the growth inhibition is observed as the difference between the specific growth rate of the control cultures growing under identical conditions and the cultures under exposure. This is found using equation (6) below:

$$I_{\mu i} = \frac{\mu_c - \mu_i}{\mu_c} \times 100 \qquad (6)$$

where $I_{\mu i}$ is the percentage growth inhibition attributed to the compound; μ_c is the average growth rate for the control cultures; and μ_i is the growth rate of the algae exposed to a concentration of the test compound.

The inhibitory effects of the concentrations can then be plotted and visually observed on a dose-response curve, where the EC_{50} concentration can also be determined (38). There are several methods and models that can be utilised to plot a dose-response curve. The most common in biological practices is the use of a four-parameter regression model, known as Hills equation. This is because dose-response curves tend to have a sigmoidal shape when plotted and the Hills equation is known to produce a well-fitting and representative sigmoidal regression curve (36). The modified equation adopted in biological practices can be seen in equation (7) below:

$$\hat{Y} = a + \frac{(b - a)}{(1 + (\frac{c}{X})^d} \qquad (7)$$

where $\hat{Y}$ is the approximate response at dose X; a is the approximate response with no dosage; b is the maximum and stable response above a certain dosage; c is the dosage where 50% show a response, or EC_{50}; and d is the gradient at the steepest part of the curve, known as the Hills slope. There are computational tools that can be used to estimate the four parameters in equation (7). The computational tool chosen to be used in this ERA is the Hill Equation with Prediction Band (HEPB) program as it is freely available online and user-friendly (36).

2.3.3.2 Toxicity Test on Fish

Over the years, focus has been on reducing the number of whole fish organisms being used for toxicity testing by rather exposing fish cells through cell line assays. In fact, testing on the cellular level may well be more efficient or conservative as the effects are said to be seen much earlier and at significantly lower concentrations (39). Two types of fish cells have shown to be useful in toxicity tests, these are gill and liver cells. Fish gill cell line assays have recently been internationally approved and thus a standard, OECD Test 171, has been issued for this form of

toxicity testing using the fish species *Oncorhynchus mykiss*, also known as Rainbow Trout (40). The OECD Test 171 standard has been selected for this ERA.

The principle of the OECD Test 171 method is to plate Rainbow Trout gill cells, RTgill-W1, on 24, 48 or 96-well culture plates and expose them to varying concentrations of the chemicals for 48 hours. Thereafter the cell viability of the gill cells is assessed by adding indicator dyes that can be picked up spectrophotometrically or fluorometrically. The OECD Test 171 suggests one basic protocol and two alternate protocols that can be used to determine cell viability. The protocols differ in the assessment of cell viability (39). The basic protocol assesses the metabolic activity of the cells using the Resazurin (Alamar Blue) indicator dye (41). The two alternate protocols assess the membrane integrity and the lysosomal activity of the cells using substrate 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) for membrane integrity and Neutral Red dye for lysosomal activity (42,43). The basic protocol is the only protocol chosen for the cell line assays due to it being favoured in the standard and the time constraint of this thesis.

Once the Alamar Blue dye has been added, the well culture plate is read on a fluorimeter (or spectrophotometer). The percentage cell viability can then be found using equation (8):

$$\%CV = \frac{FS_{\text{exp.}\,cells} - FS_{\text{exp.}\,no\ cells}}{FS_{cont.cells} - FS_{cont.no\ cells}} \times 100 \qquad (8)$$

where $\%CV$ is the percentage of viable cells, compared to the control, after exposure to the chemical; $FS_{\text{exp.}\,cells}$ is the fluorescence (or spectrophotometric) unit measured for the wells with cells exposed at a particular concentration of the chemical; $FS_{\text{exp.}\,no\ cells}$ is the fluorescence unit measured for the wells containing no cells at the same concentration of chemical; $FS_{cont.cells}$ is the fluorescence unit measured for the control wells with cells and no exposure; $FS_{cont.no\ cells}$ is the fluorescence unit measured for the control wells without cells and without exposure, only media used to cultivate the cells (39).

The percentage cell viability can be plotted against the concentration of the chemical, producing a dose-response curve that highlights the EC_{50} and its corresponding PNEC. The dose-response curve can be found using the same method and tools described under the previous subsection: Toxicity Tests for Algae (40).

2.3.4 Risk Characterization

The characterization of risk allows for risk-informed decisions to be made (11). The environmental risk related to operational discharges of produced water offshore is commonly characterised by taking into account the relation between PEC and PNEC (29,44). This is also valid for the discharge of the potential PITT candidates in this study, as they are released into the sea (31). The value generated from the ratio between PEC and PNEC is used to determine the risk of the discharge in terms of the environmental impact factor (EIF). An EIF unit of one is defined as the specific volume of water, 100m x 100m x10m in depth, where the value of $\frac{PEC}{PNEC}$ is greater than one. As Figure 9 shows, the risk within this cubic volume corresponds to a probability of environmental impact greater than 5%, the recognised threshold. The risk is therefore characterised as significant and substantial (44).

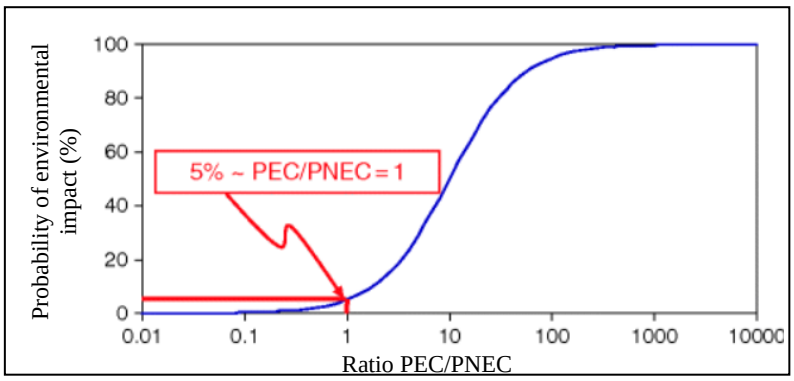

Figure 9: PEC/PNEC ratio versus probability of environmental impact (44,45).

When the value of $\frac{PEC}{PNEC}$ is less than 1, the risk in the cubic volume is considered acceptable as the probability of environmental impact is less than 5% (46). The EIF in this case has a corresponding unit of zero (35).

The risk related to the overall discharge is the sum of all the specific cubic volumes where the EIF has a unit of one (10). Various models have been established that aim to portray and assess the overall risk in the recipient environment using the combined EIF values of all the specific cubic volumes. The model with the best reputation and most frequently used to analyse produced water and drilling discharges in the aquatic environments of Norway, and indeed Europe, is the Dynamic Risk and Effects Assessment Model (DREAM) (47).

DREAM is a complex model that combines the characteristics of the surrounding environment with the cocktail of chemicals released, in order to simulate the potential risk over a period of time often 30-days. The model uses the unique properties of each chemical including the

biodegradation and bioaccumulation potential as well as the toxicity of each compound. It also has extensive information on the ocean currents and other physical factors that would influence the risk picture.

DREAM can provide the total EIF value in specified grids within the area of interest giving an understanding of the impact at the source of pollution as well as its surroundings over the period simulated. An example of this can be seen in Figure 10, with a DREAM simulation producing a horizontal risk field and its vertical profile. The DREAM simulation therefore provides the entire "worst-case" risk picture, using the accumulated EIF values of each chemical compound in the produced water (44).

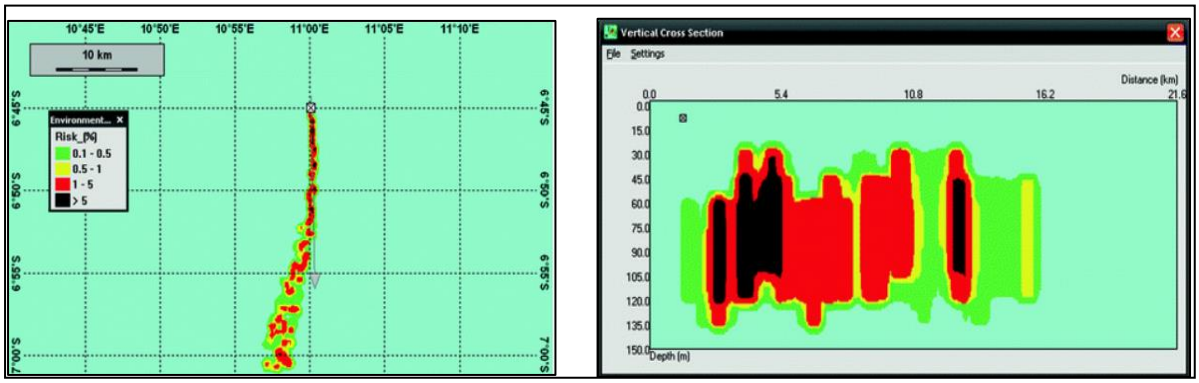

Figure 10: Risk field (left) and vertical profile (right) produced by DREAM simulation (44).

The example from DREAM shown in Figure 10 also easily indicates the value of $\frac{PEC}{PNEC}$ in colour codes, represented here by green and yellow for when $\frac{PEC}{PNEC}$ is less than one and red and black for when it is larger than one.

DREAM can also specify the contribution of each included chemical to the overall EIF or total risk, allowing for observations of the individual chemicals impact on the total EIF value. This is often expressed as either percentages, pie charts, or both as seen in the example in Figure 11. Alternatively, they are presented as time averaged EIF contributions, which is the average EIF contribution of the chemicals in a specific volume of water (1,000,000 m^3) over the simulated period (35,44).

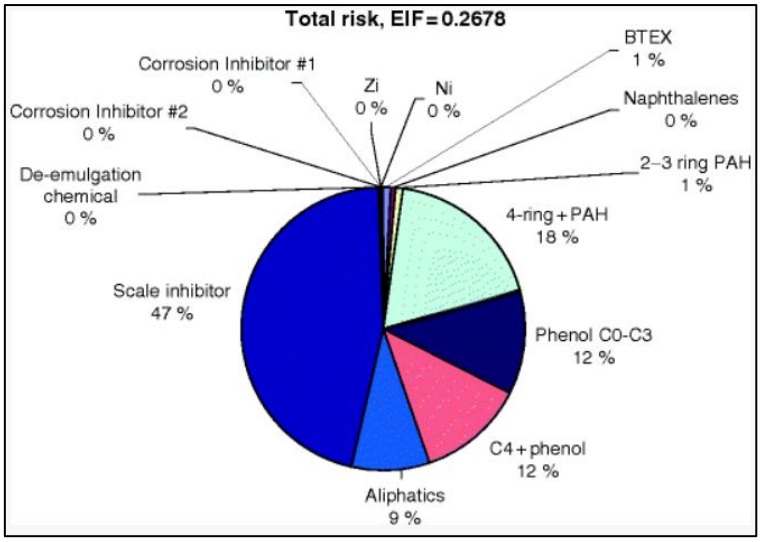

Figure 11: Total risk expressed in a pie-chart format (44).

The example in Figure 11 has a very low total risk, nevertheless the contribution to the total risk of each compound can be easily evaluated when looking at the percentages. It is also common to see no or very little contribution from compounds. Compounds that show high contributions to the total risk are flagged, often rejected, sometimes replaced or if possible removed from the produced water via treatments before discharge (35).

Assessing and characterising the risk offshore from produced water, and the individual substances, were made much more practical with the use of DREAM (10). The shortlisted PITT candidates will therefore be added to a 30-day DREAM simulation once the exposure and effect assessments are conducted. Currently there are no real cases where these PITT candidates are planned to be used on the Norwegian Continental Shelf (NCS). However, a pragmatic approach with a real produced water profile will be used with the goal of obtaining a realistic impression of the PITT candidate's risk contribution relative to other chemicals released in the produced water. The individual PITT candidate's EIF contribution and thus contribution to risk will be highlighted and compared to the other shortlisted in order to allow for risk-informed decisions.

Chapter 3: Materials and Methods

This section introduces the different materials used and the subsequent methodologies adopted to determine the biodegradation potential and toxicity characteristics of the PITT candidates as well as their potential environmental impact and risk on the marine environment. The section also highlights the modifications and practical adaptations that were made during this thesis. First the materials of each experiment will be presented followed by the methods of each experiment. The last sub-section is dedicated to the statistical analysis adopted.

3.1 Materials

Table 6 is an origin of chemicals table showing the seven shortlisted PITT candidates and the suppliers they were acquired from.

Table 6: Origin of shortlisted PITT candidates.

Compound	CAS	Supplier, Origin
Pyridine	110-86-1	Alfa Aeser, Germany
2,3-Dimethylpyrazine	5910-89-4	TCI, Japan
2,6-Dimethylpyrazine	108-50-9	TCI, Japan
4-Methoxybenzyl alcohol	105-13-5	Acros Organics, India
3,4-Dimethoxybenzyl alcohol	93-03-8	Acros Organics, India
4-Chlorobenzyl alcohol	873-76-7	Alfa Aeser, Germany
2,6-Dichlorobenzyl alcohol	15258-73-8	TCI, Japan

3.1.1 Biodegradation Potential

Table 7 on the next page shows all compounds used for the biodegradation potential experiments and Table 8 shows the other materials and instruments used for the same experiment.

Table 7: Chemicals and compounds used during biodegradability tests.

Compound	CAS	Supplier, Origin
Aniline	62-53-3	Merck, Germany
Sodium acetate	127-09-3	Riedel-de Haën, Germany
Potassium dihydrogen orthophosphate	7778-77-0	Merck, Germany
Dipotassium hydrogen orthophosphate	7758-11-4	Merck, Germany
Disodium hydrogen phosphate anhydrous	7558-79-4	AppliChem, Germany
Ammonium chloride	12125-02-9	VWR, Belgium
Calcium chloride dihydrate	10035-04-8	Merck, Germany
Magnesium sulphate heptahydrate	10034-99-8	Merck, Germany
Iron (III) chloride hexahydrate	10025-77-1	Merck, Germany
Distilled water	-	UiS, Norway
Seawater	-	NORCE, Norway

Table 8: Equipment and instruments used during biodegradability tests.

Equipment/Instrument/ Software	Model	Purpose
Standard laboratory equipment	-	Diverse usage
275 mL BOD bottles	-	Test vessels used for all samples
Glass/Rubber stoppers	-	Used to keep BOD bottles air-tight
0.45 µm pore filter	Pall ULTIPOR	Used to filter seawater
Incubator	Liebherr Lovibond TC 445	Used to store bottles at 20˚C and no light
Dissolved Oxygen measuring probe	VWR Multi-parameter meter (MU 6100 L)	Used to measure dissolved Oxygen
Microsoft Excel	Excel 2016	Used to tabulate and process results

Figure 12 shows a couple of pictures containing some of the equipment and instruments stated in Table 8.

Figure 12: Measuring oxygen levels (left) and test samples stored in incubator (right).

The equipment and instruments seen in Figure 12 are: the BOD bottles with the stoppers, the dissolved Oxygen measuring probe, and the incubator set at steady temperature 20˚C and no light.

3.1.2 Toxicity Tests on Algae

Table 9 shows the test organism used for this experiment, the strain number and where it was acquired from. Table 10 shows the compounds that were used for the toxicity test on algae.

Table 9: Information of test organism used for toxicity tests on algae.

Test organism	Strain number	Supplier, origin
Skeletonema costatum	NIVA-BAC 1	NIVA, Norway

Table 10: Compounds used during toxicity tests on algae.

Compound	Supplier, Origin
Z8 Growth media kit	NIVA, Norway
Carbon dioxide (CO_2)	Nippon Gases, Norway
Dimethyl sulfoxide (DMSO)	AppliChem GmbH, Germany
Distilled water	UiS, Norway
Seawater	NORCE, Norway

The Z8 growth media kit supplied by Norwegian Institute for Water Research (NIVA) contained four stock solutions: stock solution I, stock solution II, stock solution III, and stock solution IV. These stock solutions can be seen in Table 11 and were used to make a 20% Z8 growth media in seawater.

Table 11: Contents in the Z8 growth media kit received from NIVA.

Stock solution I	Stock solution II	Stock solution III	Stock solution IV
$NaNO_3$ (46.7 g) $MgSO_4 \cdot 7H_2O$ (2.5 g) $Ca(NO_3)_2 \cdot 4H_2O$ (5.9 g) Dissolved in distilled water to 100 ml	K_2HPO_4 (3.1 g) Na_2CO_3 (2.1 g) Dissolved in distilled water to 1000 ml	a) $FeCl_3 \cdot 6H_2O$ (2.8 g) in 100 ml 0.1 N HCl b) $Na_2EDTA \cdot 2H_2O$ (3.9 g) in 100ml 0.1 N NaOH 10 ml solution a) and 9.5 ml solution b) in distilled water to 1000 ml	c) $Na_2WO_4 \cdot 2H_2O$ (0.330 g/l) d) $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.880 g/l) e) KBr (1.20 g/l) f) KI (0.830 g/l) g) $ZnSO_4 \cdot 7H_2O$ (2.87 g/l) h) $Cd(NO_3)_2 \cdot 4H2O$ (1.54 g/l) i) $Co(NO_3)_2 \cdot 6H_2O$ (1.46 g/l) j) $CuSO_4 \cdot 5H_2O$ (1.25 g/l) k) $NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$ (1.98 g/l) l) $Cr(NO_3)_3 \cdot 9H_2O$ (0.41 g/l) m) V_2O_5 (0.089 g/l) n) $Al_2(SO_4)_3K_2SO_4 \cdot 24H_2O$ (4.74 g/l) o) H_3BO_3 (3.10 g) and $MnSO_4 \cdot H_2O$ (1.69 g) in 1000 ml distilled water 10 ml of solutions c-n) and 100 ml solution o) were dissolved to 1000 ml with distilled water

The equipment, instruments and software used for the toxicity tests on algae experiment can be seen in Table 12 on the next page.

Table 12: Equipment and instruments used during toxicity tests on algae.

Equipment/Instrument/ Software	Model	Purpose
Standard laboratory equipment	-	Diverse usage.
250 mL Erlenmeyer flasks	-	Test vessels used for all samples.
Aluminium foil	Standard aluminium foil	Used to protect flasks from particles.
Temperature controlled room	-	Used this 20˚C space for exposure conditions.
Lights	Osram*	To expose algae with 24 hours of light.
Incubator	Termaks AS KBP 6395 FL	Used to grow algae at 15˚C with 12h light.
pH probe	VWR Multi-parameter meter (MU 6100 L)	Used to measure pH.
Fluorimeter	SpectraMax Paradigm Multi-Mode Microplate Reader	Used to measure cell density of the algae.
24-well microplate	-	To collect samples to be measured in fluorimeter
Microscope	-	Used to observe and count algae cells.
Bürker cell counting chamber	0.0025mm^2 Neubauer from Marienfeld	Placed on microscope to count algae cells.
Autoclave	-	Used to sterilize equipment.
Microsoft Excel	Excel 2016	Used to tabulate, process and plot results.
HEPB program	HEPB – Hill Equation with Prediction Band	Computational tool to determine parameters in Hills equation.

Figure 13 shows the algae, *Skeletonema costatum*, in the Erlenmeyer flasks being cultivated in an incubator instilled with a 12-hour light cycle at 15˚C for slow and steady growth.

Figure 13: *Skeletonema costatum* growing steadily at 15˚C with a 12-hour light cycle.

Figure 14 shows the set-up for the toxicity tests on *Skeletonema costatum*. This was set up in a temperature control room set at 20˚C with continuous light.

Figure 14: Exposed *Skeletonema costatum* in the temperature control room set at 20˚C.

Figure 15 shows the fluorimeter, SpectraMax Paradigm Multi-Mode Microplate Reader, used for both toxicity tests on algae and on fish cells.

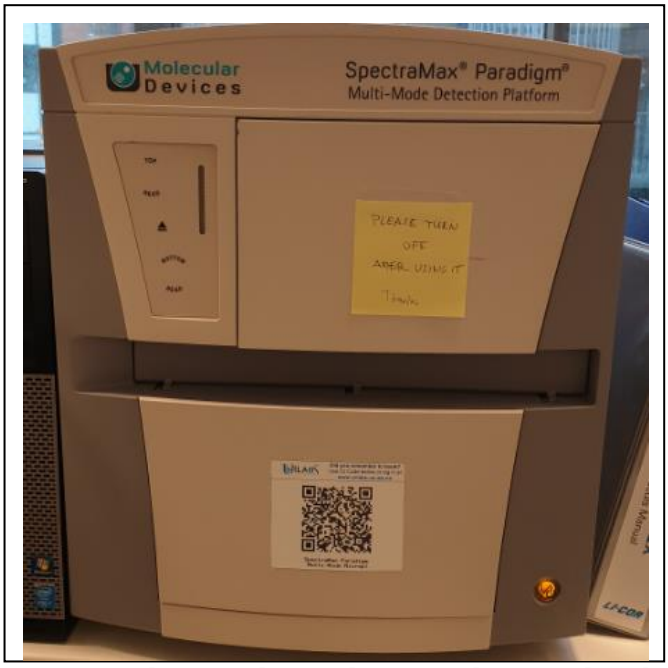

Figure 15: SpectraMax Paradigm Multi-Mode Microplate Reader, the fluorimeter used.

3.1.3 Toxicity Tests on Fish Cells

Table 13 details the fish species used for the experiment as well as the particular cells that were obtained for the toxicity tests.

Table 13: Information of test organism used for toxicity tests on fish cells.

Test organism	Cell line	Specific batch	Supplier, origin
Oncorhynchus mykiss (Rainbow Trout)	RTgill-W1	RT gill P_4(3)	UiS, Norway

Table 14 shows all the compounds used for the toxicity tests on fish cells and Table 15 shows all the equipment, instruments and software used.

Table 14: Compounds used during experiment on fish cells.

Compound	Catalogue number/CAS	Supplier, Origin
Gibco™ Leibovitz's L-15 Medium	11415064	Thermo Fisher Scientific, Sweden
Fetal bovine serum (FBS)	-	Biowest, France
Penicillin streptomycin	15140122	Life Technologies AS, Norway
Phosphate buffered saline (PBS)	-	Life Technologies, Netherlands
Trypsin–EDTA	59417C	Merck, Norway
Dimethyl sulfoxide (DMSO)	67-68-5	AppliChem GmbH, Germany
Resazurin (Alamar Blue)	62758-13-8	Alfa Aeser, Germany
Hydrogen Peroxide (H_2O_2)	7722-84-1	Merck, Norway
100% ethanol solution	64-17-5	Fisher Scientific AS, Norway
MUSE® Count and viability kit	MCH600103	Luminex Corporation, USA
Instrument Cleaning Fluid (ICF)	-	Luminex Corporation, USA
Distilled (Milli-Q) water	-	UiS, Norway

Table 15: Equipment, instruments and software used during toxicity tests on fish cells.

Equipment/Instrument/ Software	Model	Purpose
Standard biological laboratory equipment	-	Diverse usage.
75 cm^2 tissue culture flask	-	To grow the fish cells in
Biological Safety Cabinet	Class II	For a sterile working environment
Inverted phase-contrast microscope	Olympus CKX41	Used to observe cells in flask
Vacuum aspirator	-	To remove growth media
Incubator	-	Used to grow cells at 18˚C.
Normal and multichannel Pipettor	-	Used to pipette solutions.
Autoclave	-	Used to sterilize equipment.
Fluorimeter	SpectraMax Paradigm Multi-Mode Microplate Reader	Used to measure fluorescence of the stained cells.
96-well tissue-culture treated microwell plate	-	To expose cells in and thereafter measure fluorescence
SoftMax Pro	-	Software used when reading plates in SpectraMax
Microsoft Excel	Excel 2016	Used to tabulate, process and plot results.
HEPB program	HEPB – Hill Equation with Prediction Band	Computational tool to determine parameters in Hills equation.

Seen in Figure 16 is the microscope used to observe cells in the 75 cm^2 tissue culture flask and the same kind of flask under sterile conditions in a biosafety cabinet during a transfer of cells.

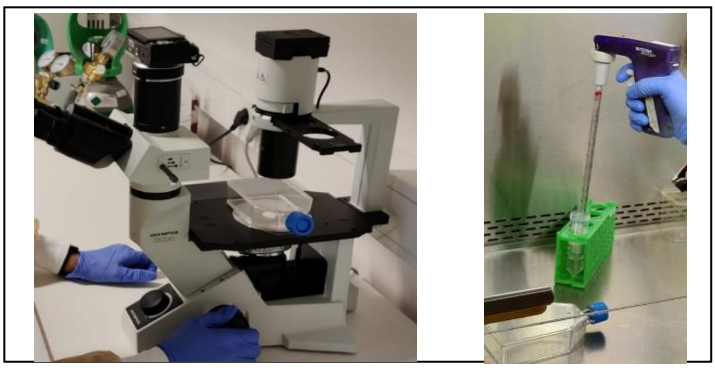

Figure 16: Observation of cells under microscope (left) and cell transfer in cabinet (right).

Figure 17 displays one of the 96-well tissue-culture treated microwell plate used.

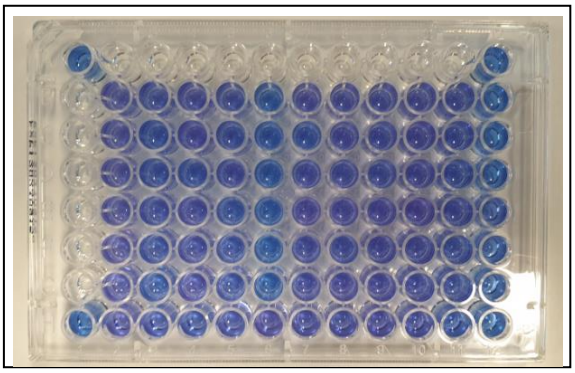

Figure 17: A 96-well tissue-culture treated microwell plate with stained cells.

The plate in Figure 17 contains wells with Alamar Blue stained cells as well as blank wells for reference. The difference in colour was picked up fluorometrically using the fluorimeter in Figure 15.

3.1.4 DREAM Simulations

Table 16 gives information on the software and produced water release profile used for the DREAM simulations. Table 17 shows the other significant input parameters for the simulations.

Table 16: Software and produced water profile used for DREAM simulations.

Software	Produced water profile	Data supplied by
Dynamic Risk and Effects Assessments Model (DREAM)	Brage field, 2019	Wintershall DEA

Table 17: Input parameters used in DREAM simulations.

Fate factors/Model parameters	Input data
Longitude/Latitude	3˚2.0’E/60˚32’N
Volume produced water	15571.5 m^3/d
Depth of release	17 m below sea surface
Concentration grid	100 m × 100 m × 10 m
Habitat grid	50 km × 50 km
Duration of release/simulation	30 days
Time step of simulation	10 minutes
Output interval	6 hours
Ocean currents	90may.dir
Winds	gullfaks.wnd

3.2 Methods

The methodology for the biodegradation potential experiments will be detailed first followed by the toxicity test on algae, then the toxicity test on fish cells and finally the DREAM simulation. A safe-job analysis was conducted prior to any experiments.

3.2.1 Biodegradation Potential

The OECD 306 closed bottle standard was followed as best as possible when determining the biodegradation potential (9). The procedure for determining the biodegradation potential is detailed step-by-step below, where modifications from the standard are highlighted:

1) The theoretical oxygen demand (ThOD) of the test substances was determined as seen in section 2.3.2.1 of this thesis
2) Two reference compounds, Aniline and Sodium acetate, were selected to certify microbial activity in the seawater and validate the results. These are two of the three recommended by the OECD 306 standard.
3) Seawater was collected from NORCE's Mekjarvik laboratories. This seawater originates from the deep waters of the fjord nearby. Due to the low organic content of the water, the seawater was aged only for a couple of days.
4) Four stock solutions of mineral nutrients were prepared:
    a) Potassium dihydrogen orthophosphate (8.50g)
       Dipotassium hydrogen orthophosphate (21.75g)
       Disodium hydrogen phosphate anhydrous* (33.3g)
       Ammonium chloride (0.50g)
       Dissolved and topped up to one litre with distilled water.
       *The OECD 306 closed bottle method recommends the use of Disodium hydrogen orthophosphate dihydrate. Due to a lack of availability, Disodium hydrogen phosphate anhydrous was chosen.
    b) Calcium chloride dihydrate* (27.50g)
       Dissolved and topped up to one litre with distilled water.
       *The OECD 306 closed bottle method recommends the use of Calcium chloride. Calcium chloride dihydrate was chosen due to availability.
    c) Magnesium sulphate heptahydrate (22.50g)
       Dissolved and topped up to one litre with distilled water.
    d) Iron (III) chloride hexahydrate (0.25g)

Dissolved and topped up to one litre with distilled water.

5) The test medium was prepared by adding 1 mL of each nutrient stock solution per litre seawater. The dissolved oxygen of the test medium was determined using a dissolved Oxygen measuring probe. The dissolved Oxygen of the test medium measured in this experiment ranged between 6.3 and 7.0 mg/L.

   *The OECD 306 standard suggests aerating the test medium with clean compressed air to saturate the medium with air. However, the seawater collected was already well aerated and the dissolved oxygen measured after the addition of nutrients was very close to saturated levels. Therefore, the test medium was not subjected to saturation with air.

6) Duplicate BOD bottles for each measurement (day 0, 5, 15, and 28) of test samples, reference compounds, and blanks were collected and prepared by ensuring cleanliness of each bottle.

7) Amount of test substance and reference compounds to be added in the 275 mL BOD bottles was calculated based on their ThOD's. They were also calculated based on the criteria that the amount of chemical added should not theoretically consume more than 50% of the dissolved Oxygen available in the test medium. This criterion means that the test substances and reference compounds should not consume more than 50% of 6.3 mg/L, or 1.7325 mg O_2/275 mL bottle.

   The OECD 306 closed bottle method gives two suggestions on how to add the substances, either by direct addition of substances to the bottle or by a stock solution method to all bottles in that test series. The initial experiment was conducted using the direct addition method, but due to a higher level of uncertainty in weighing and inconsistencies in duplicate measurements of dissolved Oxygen, the method was altered to the stock solution method. This improved the consistency between duplicates and reduced the uncertainty when weighing the compounds, these results were chosen. The following was calculated based on ThOD and the above criterion for both direct addition of compounds to bottles and the stock solution method:

   a) Pyridine:
      - 0.2398 mg directly in a 275 mL BOD bottle.
      - 2.0940 mg in 2400 mL nutrified seawater, 275 mL poured in each bottle.

   b) 2,3-Dimethylpyrazine
      - 0.2650 mg directly in a 275 mL BOD bottle.

- 2.3120 mg in 2400 mL nutrified seawater, 275 mL poured in each bottle.

c) 2,6-Dimethylpyrazine
   - 0.2650 mg directly in a 275 mL BOD bottle.
   - 2.3120 mg in 2400 mL nutrified seawater, 275 mL poured in each bottle. *Complications arose with this compound as it is air-sensitive, the pure substance solidifies when exposed to air. The solution was to dissolve the easily soluble compound in distilled water and serially dilute it to the desired concentration

d) 4-Methoxybenzyl alcohol
   - 0.3750 mg directly in a 275 mL BOD bottle.
   - 8.270 mg in 2400 mL nutrified seawater, 275 mL poured in each bottle.

e) 3,4-Dimethoxybenzyl
   - 0.4125 mg directly in a 275 mL BOD bottle.
   - 3.60 mg in 2400 mL nutrified seawater, 275 mL poured in each bottle.

f) 4-Chlorobenzyl alcohol
   - 0.4583 mg directly in a 275 mL BOD bottle.
   - 3.9997 mg in 2400 mL nutrified seawater, 275 mL poured in each bottle. *There were difficulties in dissolving this compound. Instead a 250 mg/L stock solution was made. 16 mL of this solution was added to 2384 mL nutrified seawater.

g) 2,6-Dichlorobenzyl alcohol
   - 0.3496 mg directly in a 275 mL BOD bottle.
   - 3.0511 mg in 2400 mL nutrified seawater, 275 mL poured in each bottle. *There were difficulties in dissolving this compound. Instead a 250 mg/L stock solution was made. 12.20 mL of this solution was added to 2387.8 mL nutrified seawater.

h) Aniline
   - 0.2670 mg directly in a 275 mL BOD bottle.
   - 2.330 mg in 2400 mL nutrified seawater, 275 mL poured in each bottle.

i) Sodium acetate
   - 1.0580 mg directly in a 275 mL BOD bottle.
   - 9.230 mg in 2400 mL nutrified seawater, 275 mL poured in each bottle.

8) A blank series containing only the nutrified seawater is made by pouring the seawater into eight BOD bottles (duplicates for each day measurements).

9) Once samples were prepared, the day 0 BOD bottles were measured for dissolved Oxygen whilst the remaining bottles were stored in an incubator set with no light at 20˚C, this was visualised in Figure 12. The measured values of day 0 were reported for all test substances, reference compounds, and blanks.
10) The dissolved Oxygen was measured and recorded for all test substances, reference compounds and blanks on days 5, 15, and 28.
11) Some experiments that were run observed a significant reduction in the blank samples. These were nullified as per the validity criteria of the OECD 306 standard. The experiments were repeated from the start by collecting new fresh seawater and using different bottles.
12) The Oxygen consumption of the compounds was determined based on the difference in dissolved Oxygen between the blank samples and the test samples on day 28 compared to the measurements on day 0. Thereafter, equation (4) in section 2.3.2.1 was applied to find the amount of the compound that had been degraded, the biodegradation potential, over the period of 28 days.
13) A validity criterion in OECD 306 is that the reference compounds are comparable to previous results obtained that have been approved. A couple of experiments that were run did not satisfy this criterion and were consequently nullified and repeated.
14) Only the results that satisfied all the validity criteria in the OECD 306 standard were accepted and will be shared in the following chapter.

3.2.2 Toxicity Test on Algae

The international standard followed during the toxicity test on algae was the ISO10253:2016(E) (38). The standard was followed as best as possible using the algae *Skeletonema costatum*. The way this was practically conducted during this thesis is explained step-by-step below:

Growing the algae

1) All glassware equipment to be used was autoclaved and the working environment sterilized with ethanol.
2) Seawater was collected from the NORCE Mekjarvik laboratory.
3) The Z8 growth media kit, containing stock solutions I-IV seen in Table 11, was acquired from NIVA together with two 10 mL *Skeletonema costatum* stock cultures.

4) A 100% Z8 growth media solution was made by dissolving 10 mL of stock solutions I-III, and 1 mL stock solution IV in distilled water. The solution was aerated with CO_2 for 20 minutes as recommended by the supplier. The solution was topped up to 1 L with distilled water to make a 100% Z8 solution.
5) The algae thrive in a 20% Z8 in seawater solution, thus the 1 L solution of 100% Z8 was diluted with 4 L seawater to make a 5 L 20% Z8 growth media solution. Steps 3) and 4) were repeated when the growth medium was nearing depletion, to keep the medium as fresh as possible.

   *The ISO10253:2016(E) standard suggests a similar recipe for the nutrient stock solutions that can be prepared in the laboratory using various chemicals. However, the supplier of the algae provided a growth medium that was economically feasible and ready-to-use.
6) The *Skeletonema costatum* cultures were cultivated in five 40 mL solutions containing 36 mL of 20% Z8 growth media and 4 mL of the algae stock cultures. These were placed in an incubator instilled with a 12-hour light cycle at 15˚C for steady growth, seen earlier in Figure 13, as recommended by the supplier. The flasks were given a gentle shake by hand once a day. The shake was to resuspend the algae and allow for movement of air within the flasks.
7) The algae were inoculated into new Erlenmeyer flasks when the cell density appeared as a dense brown colour or the algae had been growing in the same flask for more than a week.

<u>Preparing algae for exposure</u>

8) The ISO10253:2016(E) standard requires an approved method for the determination of cell density (cells/mL). Three potential methods are suggested in the standard: a microscope equipped with a cell counting chamber, a fluorimeter calibrated with the cell counting chamber, or a spectrophotometer calibrated with the cell counting chamber. The method chosen was the use of a fluorimeter calibrated with the cell counting chamber. The reason for this choice was the availability of a reference article on this method and its perceived reliability (48).
9) Calibrating the fluorimeter:
   a) First, a pre-inoculum was started by transferring approximately 5 mL from a stock culture growing steadily in the incubator to an Erlenmeyer flask containing 95 mL growth medium. This was allowed to grow for 4 days,

ensuring that the algae are at exponential growth when they are to be inoculated for calibration purposes.

b) After 4 days, a sample of the solution is placed on the microscope with the cell counting chamber. The cell density is determined by counting the cells observed in the chamber. The cell density was found to be roughly 500,000 cells/mL.

c) The number of cells to be inoculated for calibration purposes was determined based on the exposure conditions stated in the ISO10253:2016(E). The standard states that the cell density after inoculation should be between 2,000 and 10,000 cells/mL. The cell density opted for during inoculation was 5,000 cells/mL. Thus, 1 mL of the pre-inoculum was added to 99 mL growth medium in an Erlenmeyer flask to start the inoculum for calibration purposes and placed in the exposure conditions visualised in Figure 14.

d) After a good swirl of the flask, 1 mL of the solution containing the inoculum is transferred to the microscope and another 1 mL to a 24-well microplate. The remaining wells are filled with growth media for the blank reading. The plate is placed in the SpectraMax Paradigm Multi-Mode Microplate Reader and the fluorescence units (FSU) are measured with wavelengths between 430 and 671 nm. The FSU is then compared to the cell density determined on the microscope.

e) The same procedure in d) is repeated after 24, 48, and 72 hours. The same as the criteria in the ISO10253:2016(E) standard. However, for the sake of a reliable correlation between cell density and FSU, further measurements were made. These were done at 96, 120, 144, and 168 hours after inoculation

f) The results were then analysed by plotting the calibration curve, Figure 18, that portrays the linear relation between FSU and algae cell density.

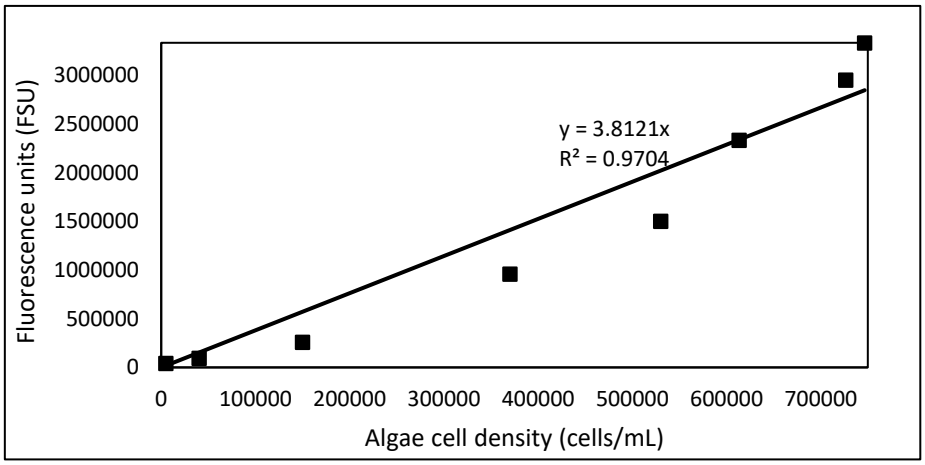

Figure 18: Calibration curve of the relation between fluorescence units and algae cell density

10) Once the fluorimeter was calibrated, a pre-inoculum was started for the toxicity tests. This was started by inoculating roughly 5,000 cells/mL for a period of 4 days. After 4 days, the cell density was determined by measuring the FSU. The cell density found was used to calculate the required inoculum volume for the exposing of the algae. A new batch of pre-inoculum was started prior to each exposure.

Exposing the algae

11) The first set of toxicity tests conducted was the concentration range-finding tests, as suggested in the ISO10253:2016(E) standard. A fixed concentration range was established for the PITT candidates. The range-finding concentrations were 100, 1,000, and 10,000 mg/L, with the main interests being whether or not the toxicity would be found above or below 100 mg/L. Blanks were also readied and consisted of only growth media and inoculum.
    a) The highest concentration, 10,000 mg/L, was made twice by dissolving the chemicals in 10 mL distilled water. One was directly used for exposure while the other was diluted further in distilled water for the remaining concentrations. For reliable readings, duplicates were made for each concentration and the blanks.
    b) After 24, 48, and 72 hours, 1 mL is siphoned from each sample and placed in the 24-well plate. The FSU is measured, and the cell density determined.
    c) Using equation (5), the specific growth rates were found using the differences in cell densities and their respective growth inhibitions found using equation (6).
    d) The concentration ranges were found to be below 100 mg/L for 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol. The rest had toxicity concentration ranging above or around 100 mg/L.
12) Results from the toxicity test on fish cells were also used as an inspiration for the concentration ranges. Once the concentration range was established for each PITT candidate, a geometric series of concentrations to be tested was individually selected for the PITT candidates. The ISO10253:2016(E) standard requires that the geometric series of the concentrations has a ratio of less than 3.2; the ratio was kept well below the threshold for each PITT candidate. The geometric series of the concentrations to be tested for the PITT candidates were:

a) Pyridine in mg/L (ratio 1.67) – 100, 167, 278.89, 465.74, 777.79, 1298.91, 2169.19, 3622.55, 6049.67, and 10102.95
b) 2,3-Dimethylpyrazine in mg/L (ratio 1.43) – 300, 429, 613.46, 877.26, 1254, 1793.91, 2565.29, 3668.37, 5245.77, 7501.45
c) 2,6-Dimethylpyrazine in mg/L (ratio 1.65) – 75, 123.75, 204.19, 336.9, 555.9, 917.23, 1513.4, 2497.2, 4120.3, 6798.6
d) 4-Methoxybenzyl alcohol in mg/L (ratio 2.0) – 10, 20, 40, 80, 160, 320, 640, 1280, 2560, and 5120
e) 3,4-Dimethoxybenzyl alcohol in mg/L (ratio 1.50) – 200, 300, 450, 675, 1012.5, 1518.75, 2278.13, 3417.19, 5125.78, and 7688.67
f) 4-Chlorobenzyl alcohol mg/L (ratio 1.84) – 0.1, 0.184, 0.339, 0.623, 1.146, 2.109, 3.881, 7.14, 13.14, 24.17, 44.48, 81.84, 150.59, 277.09, 509.85
g) 2,6-Dichlorobenzyl alcohol in mg/L (ratio 1.63) – 0.5, 0.815, 1.32, 2.16, 3.52, 5.75, 9.37, 15.28, 24.91, 40.61, 66.19, 107.9, 175.88, 286.68, 467.3

13) The concentrations (duplicates*) were made by dissolving the required amount of chemical in the growth media. Two PITT candidates, 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol, have lower solubility. These were dissolved in Dimethyl sulfoxide (DMSO) and then dissolved in the growth media. The total volume of the solution containing growth media and the chemical was topped up to 99 mL with growth media.
14) Controls (duplicates*) were prepared with only growth media (99 mL) for the compounds that did not require DMSO. For the compounds that required DMSO to dissolve them, a control was prepared by adding the same volume of only DMSO to the growth media and topped up to 99 mL.

    *The ISO10253:2016(E) standard recommends the use of triplicates for each test compound concentration and control. However, duplicates were used during this thesis due to the time constraint and availability of equipment.
15) An inoculum that had been prepared four days prior was used per compound. 1 mL of this algae stock culture was added to each Erlenmeyer flask. The flasks were given a swirl. 1 mL was pipetted out of each flask, including the controls, and transferred to the 24-well microplate. The microplate was read straight away, and the FSU obtained noted. The pH at the start of the exposure was also noted.

16) An aluminium foil with holes was used to cover the flasks and avoid airborne contamination. The flasks were stored under the exposure conditions stated in the standard; continuous light at 20˚C. This was visualised in Figure 14.
17) The flasks were given a shake after 24 hours. 1 mL was withdrawn from each flask and transferred to a 24-well microplate. The microplate was read, and the FSU was used to determine the cell density. The specific growth rate, μ, from equation (5) and the percentage growth inhibition, $I_{\mu i}$, from equation (6) were calculated.
18) Step 17) was repeated at 48, and 72 hours. The presence of cells was also confirmed via the use of a microscope.

Analysis of the results of the exposure

19) The measurements made of the control at 72 hours showed a decline in growth. The ISO10253:2016(E) standard suggests using the measurements taken at 48 hours when this occurs. This was therefore applied to all compounds.
20) A check was done on the validity criteria of the ISO10253:2016(E) standard:
    a) The pH was measured after 72 hours to ensure that a pH shift greater than one had not occurred. This was the case for all compounds.
    b) The cell density of the control should have also increased by a factor of 16 after 72 hours. This was the case for all controls including those containing DMSO.
    c) The specific growth rate of the controls should not exceed a variation coefficient of more than 7%. This was the case for all controls.
21) The HEPB program was used to plot the percentage growth inhibition of the algae *Skeletonema costatum* against the compound's concentration. The program returns a regression curve with a 95% confidence interval. It also determines the estimated EC_{50} value with its upper and lower bounds.

3.2.3 Toxicity Test on Fish Cells

The standard adopted for the toxicity test on fish cells was the OECD test number 171 (OECD 171). The cells used in this thesis were those stated in the standard, the Rainbow Trout Gill Cells (RTgill-W1) (40). A few modifications were made based on the availability of instruments and the common practices in the allocated cell lab for this thesis. The procedure for this experiment was as follows:

Culturing the fish cells

1) All equipment and benches were sterilized with ethanol. All work was done in a biological safety cabinet when possible.
2) Growth medium for the cells was prepared by mixing 500 mL Gibco^{TM} Leibovitz's L-15 Medium, 10% Fetal bovine serum (FBS), and 1% Penicillin streptomycin.
   *The standard states that the growth medium should be Leibovitz's complete medium with added FBS. However, Penicillin streptomycin was added based on the common practice in the allocated lab.
3) Two vials containing RTgill-W1 cells were collected from a cryotank and thawed in a water bath. The growth medium was also added to the water bath.
4) A pipette gun was used to draw 9 mL growth medium. 4.5 mL was transferred to a 15 mL tube and the remaining 4.5 mL was transferred into a 75 cm^2 tissue culture flask. This step was repeated for a second flask.
5) The vials containing RTgill-W1 cells were gently homogenised by rotating the vial several times. Thereafter, an automatic pipette was used to withdraw the cells and transfer them into the media in the 15 mL tubes.
6) The cell containing solution in the 15 mL tubes was then pipetted up and down. This was done to homogenise the solution. The cells in media were then transferred into the 75 cm^2 tissue culture flask. Care was taken by slowly transferring the solution to ensure the cells remain intact. The flask is tilted carefully back and forth to disperse the cells.
7) The flasks were placed on an inverted phase-contrast microscope and the cells were observed. The presence of RTgill-W1 cells was confirmed. The two flasks were placed in an incubator set at 20˚C with no light.

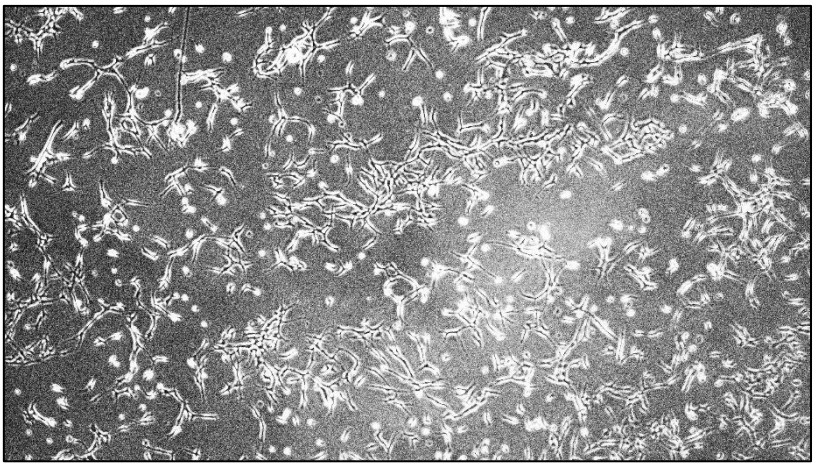

Figure 19: Healthy RTgill-W1 cells attached to bottom of flask.

8) After 24 hours, the flasks were observed on the microscope. A healthy number of RTgill-W1 cells were attached to the bottom of both flasks, some were floating in the media. This is visualised in Figure 19. Floating cells are dead cells and can be seen in the figure as individual dots.
9) The growth media was changed after 24 hours. This is common protocol when starting a fish cell culture. The growth media was changed for both flasks using the following steps:
    a) The growth media present in the flask was carefully removed via aspiration.
    b) Approximately 10 mL of fresh growth medium was calmly pipetted into the flask.
    c) The flask is tilted back and forth before being placed on the microscope for inspection.
    d) The flask was placed back into the incubator.
10) The cells were kept in the incubator for 48 hours before being viewed again on the microscope and their growth media changed using the same steps in step 9).
11) Step 10) was repeated for both flasks until each flask was roughly 90% confluent with cells. In other words, around 90% covered in cells. An example of such a confluent flask is seen in Figure 20.

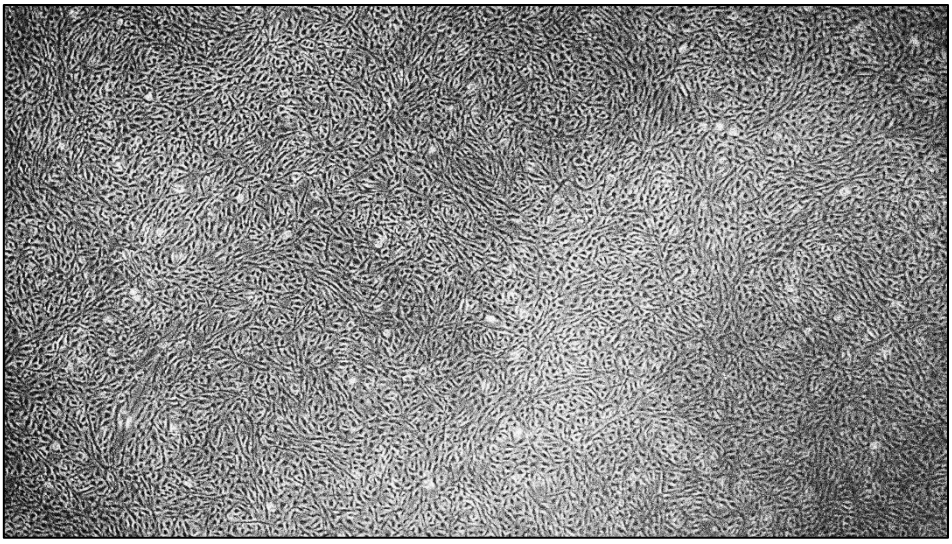

Figure 20: An example of a flask that is at least 90% confluent with RTgill-W1 cells.

12) After the flask had reached the confluency seen in Figure 20, a process known as cell passaging was started. Cell passaging is the splitting of cells from one flask into two new flasks. The steps taken during cell passaging were:
    a) The growth media present in the flask with cells was carefully aspirated.

b) The cell layer was briefly “washed” using 3 mL Phosphate buffered saline (PBS). This step removes dead cells that may not have been aspirated. PBS is then aspirated.

c) 2 mL of Trypsin–EDTA solution is added to the cell containing flask and evenly dispersed. The flask is tilted back and forth before being kept at room temperature for approximately three minutes. The addition of Trypsin–EDTA detaches the cells from the bottom of the flask without damaging them. A microscope is used to observe the cells and ensure that they are detached.

d) 3 mL of growth media was added to the cell containing flask. The 5 mL solution in the cell containing flask was pipetted up and down and throughout the flask to ensure that all cells are evenly dispersed within the solution. This was then transferred to a 10 mL tube.

e) 7.5 mL of growth media was added to the two new flasks.

f) The cell containing solution in the 10 mL tube was homogenised by pipetting gently up and down several times. 2.5 mL of the solution was then added to each flask. Care was taken to add the cell containing solution into the medium in the new flasks. The flasks were tilted back and forth to distribute the cells evenly.

g) Confirmation of cell presence was made using the microscope and the flasks were placed in the incubator.

h) The flasks were observed on the microscope after 24 hours to confirm the attachment of cells to the flasks. The growth media was changed, as in step 9), to remove the Trypsin–EDTA. Thereafter step 10) was followed.

13) Steps 10-12) were repeated throughout the experiment. The exposing of the fish cells did not start until enough confluent flasks were readied.

14) The cells were continuously observed for abnormalities. Flasks containing poor growing cells or abnormal cells were discarded.

<u>Preparing fish cells for exposure</u>

15) A confluent flask was chosen for each exposure. The flask was keenly observed under the microscope to ensure no contaminants were present.

16) The growth media present in the flask was aspirated off.

17) 3 mL PBS was added to “wash” the cells in the flask. The standard recommends a compound called Versene. PBS was used based on common practice in the cell lab and availability.
18) 2 mL Trypsin–EDTA solution was added to detach the cells. The flask was tilted several times and viewed on microscope after 3 minutes to confirm detachment.
19) 3 mL growth media was added to the flask. The mixture was homogenised thoroughly by pipetting it up and down before transferring it to a 10 mL tube. Approximately 100 µL of this mixture was sampled into an Eppendorf tube for a cell count.
20) The MUSE® Count and viability kit was used to determine the cell density of the sample. The standard recommends using the Hemocytometer method to determine cell density. This was done with the first sample and the results were comparable with the MUSE® Count and viability kit. The latter method was chosen based on the automated simplicity and reliability compared to the Hemocytometer, which is based on counting cells using the microscope. The steps followed to determine cell density were:
    a) 25 µL of the sample was added to 225 µL of the MUSE® Count and viability reagent and stored in the dark for 5 minutes.
    b) The solution in a) is then placed in the MUSE® Count and viability instrument and the cell density is returned.
    c) Steps a) and b) were repeated with a fresh sample to confirm the cell density. A confluent flask was confirmed to have between 10^5 and 10^6 cells/mL.
21) Two 96-well plates were collected. A mapping of the plates was conducted beforehand to know where the cells should be seeded.
22) The OECD 171 standard requires at least 30,000 cells seeded per well. The cell density found in step 20) was used to calculate the volume of the cell mixture in step 19) that was to be added to a certain volume of growth media to ensure the concentration of cells per well was indeed at least 30,000. The volume of a well in 96-well plate is 200 µL.
23) The volume of cell mixture and growth media calculated in step 22) was added to a small reservoir. A multi-pipette was used to seed the cells in the designated wells mapped out in step 21). Several wells were not seeded with cells, these were wells left for only growth media and only PBS.
24) The 96-well plate was put under the microscope for confirmation of the presence of cells and no contamination. This is seen to the left in Figure 21.

25) The plates were placed in the incubator until a monolayer of cells had formed at the bottom of the wells. This often took two days, sometimes three, and can be seen to the right in Figure 21.

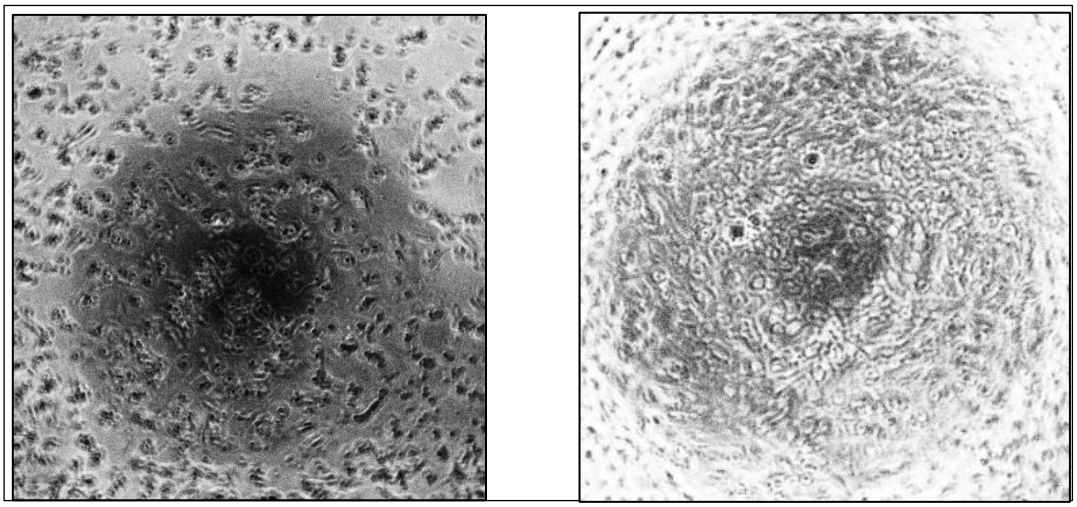

Figure 21: Confirmation of: cells in the well (left); formation of monolayer (right).

Exposing the fish cells

26) Once a monolayer was formed the plates were regarded as ready for exposure.

27) Two plates were used in the first round of cell line assays to determine the concentration range of the toxicity of the PITT candidates. The concentration range-finding test was done in the same way as with the main exposure assessment, seen later in step 29). The concentrations to be tested for the range-finding tests of all compounds was determined to be 0.1, 1, 10, 100, and 1,000 mg/L. The concentration ranges for each PITT candidate were found to be:

    a) Less than 100 mg/L: 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol.
    b) More than 100 mg/L: Pyridine*, 2,3-Dimethylpyrazine*, 2,6-Dimethylpyrazine, 4-Methoxybenzyl alcohol, and 3,4-Dimethoxybenzyl alcohol*.

       *The concentration range for Pyridine, 2,3-Dimethylpyrazine and 3,4-Dimethoxybenzyl alcohol were found to be much larger than 100 mg/L during the range-finding tests. Particularly 2,3-Dimethylpyrazine and 3,4-Dimethoxybenzyl alcohol.
    c) A second screening test for the toxicity concentration range was applied in the range 0.05-8367 mg/L for Pyridine, 2,3-Dimethylpyrazine, 4-Methoxybenzyl alcohol, and 3,4-Dimethoxybenzyl alcohol; and in the range 0.002-5156.59 mg/L for 2,6-Dimethylpyrazine.

28) Once the concentration ranges of toxicity were found for each compound, a specific geometric series of concentrations was selected for the main exposure assessment. A 96-well plate was either used for one compound or two, depending on the number of concentrations of the compounds to be added. The concentrations to be tested for the PITT candidates were:
    a) Pyridine in mg/L – 100, 145, 210.25, 304.86, 442.05, 640.97, 929.41, 1347.64, 1954.08, 2833.42, 4108.46, 5957.28, 8638.05, and 12525.1.
    h) 2,3-Dimethylpyrazine in mg/L – 100, 500, 1000, 1230, 1512.89, 1860.86, 2288.86, 2815.30, 3462.82, 4259.27, 5238.90, 6443.85, 7925.94, and 9748.91.
    i) 2,6-Dimethylpyrazine in mg/L – same as Pyridine.
    j) 4-Methoxybenzyl alcohol in mg/L – 26.56, 44.81, 75.61, 127.55, 215.18, 363.01, 612.4, 1033.13, 1742.89, 2940.26, 4960.23, 8367.9.
    k) 3,4-Dimethoxybenzyl alcohol in mg/L – 100, 500, 1230, 1512.89, 1860.86, 2288.86, 2815.30, 3462.82, 4259.27, 5238.90, 6443.85, 7925.94, and 11991.
    l) 4-Chlorobenzyl alcohol mg/L – 0.002, 0.0038, 0.0072, 0.0137, 0.026, 0.049, 0.094, 0.178, 0.339, 0.645, 1.226, 2.329, 4.426, 8.41, 15.98, 30.36, 57.68, 109.60, 208.25, 395.68, 751.79, 1428.41*, 2713.99*, 5156.59*.
    m) 2,6-Dichlorobenzyl alcohol in mg/L – same as 4-Chlorobenzyl alcohol

    *Both compounds in l) and m) precipitated out of solution above the concentration 751.79. The readings from these concentrations were therefore invalidated.
29) The procedure followed for exposing the fish cells to the concentrations of the PITT candidate compounds was as follows:
    a) For soluble compounds, the concentrations were made by dissolving the highest concentration required in growth media and thereafter diluted to the lower concentrations. The total volume of each concentration was made to be 1000 µL. This was to allow for triplicate samples as each well holds 200 µL and for dilution purposes.
    b) The compounds which had lower solubility, 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol, were first dissolved in DMSO at a concentration 200 times higher than the highest concentration and then diluted down. The concentration must be 200 times higher because compounds dissolved in DMSO will be added dropwise to each well.

c) A 100 μM solution of H_2O_2 in growth media was made for positive control or reference purposes.
d) The growth media in the 96-well plates was aspirated off. Care was taken to not disturb the cells attached at the bottom.
e) For the soluble PITT candidates: 200 μL of each concentration was added to three separate cell-containing wells.
f) For PITT candidates with lower solubility: the three wells were first filled with 199 μL of growth media followed by 1 μL of the respective concentrations. This was added as a drop on the surface as suggested by the standard.
g) The 100 μM solution of H_2O_2 was added to three wells and the remaining wells were the control wells that had only growth media.
h) The plates were closed tightly and placed on the microscope to be observed for potential cell damage during the procedure.
i) The plates were placed in the incubator for the exposure time of 48 hours.

Assessment of exposure effect on fish cells

30) The Alamar Blue dye to be used in staining the cells was readied during the exposure by dissolving it in PBS to make a 484 μmol solution. This was wrapped in aluminium foil and placed in the fridge.
31) After the 48 hours of exposure, the contents of the plate were gently aspirated.
32) The solution in step 30) was added to growth media with a ratio of 1:10. This was done so that when 200 μL was added to each well there would be 20 μL of the solution in step 30) and 180 μL of growth media.
33) 200 μL of the mixture in step 32) was added to all wells. The plate is wrapped in Aluminium foil and placed in the incubator for 2-3 hours.
34) The 96-well plate is then placed in the SpectraMax microplate reader and the fluorescence is measured at wavelengths between 530 and 590 nm. An example of how the plate looked like at that point was visualised in Figure 17.

Analysing the results of the exposure on the fish cells

35) The FSU measured is directly inserted into equation (8) to give the percentage cell viability after exposure compared to the control.
36) The HEPB program was then used to plot the percentage cell viability of the RTgill-W1 cells against the concentration of the PITT candidates. The program determined the

EC_{50} values based on the regression curve calculated using the percentage cell viability and gave a 95% confidence interval. The regression curve was the dose-response curve.

3.2.4 DREAM Simulations

The DREAM simulations were conducted once all results of the three experiments were established. Prior to obtaining the results, the various input parameters for the simulations were acquired. Model parameters for the fate factors of the chemicals were used during the simulations. Additionally, a real produced water release profile was used from the "Brage field" in 2019. These parameters and produced water release profile were shown in Table 16 and 17.

An initial simulation, consisting of only the model parameters and the produced water release profile, was run. The results of the total EIF from the simulation were compared to certified results of the "Brage field". This was done to validate the parameters and procedure adopted for this ERA. The parameters and procedure were validated, as the results were similar.

Once all the experiments on biodegradability and toxicity of the PITT candidates were completed, the following characteristics of each chemical was prepared for the DREAM simulation: the bioaccumulation potential, $\log(K_{OW})$; the percentage biodegradation over 28 days, BOD 28; and the lowest EC_{50} concentration found between the two toxicity tests. An assessment factor of 1000 was applied to the EC_{50} values, as stated and accepted by the European Union's technical guidance document (49).

The expected concentration of PITT tracers in produced water is around 0.003 ppm (21). Therefore, the starting concentration for all PITT candidates in the simulation was chosen to be 0.003 ppm. The outcome of this simulation determined the following concentrations to be simulated. Each chemical and its properties were therefore simulated at varying concentrations together with the model parameters and the produced water release profile. The results of the simulations were presented as the time averaged EIF contribution of each chemical, or the compounds average EIF over the simulated period of 30 days. The time averaged EIF contribution was then used to characterize and compare the risk of each PITT candidate.

3.3 Statistical Analysis

All results were statistically analysed using version 26 of the SPSS software. They were specifically analysed using Dunnett's Post Hoc test (ANOVA) on the software, which compared the PITT candidate results to the control. The null hypothesis significance test was applied here, where P-values less than 0.05 were to be determined statistically significant (50).

Chapter 4: Results and Discussion

The aim of this thesis was to characterize and compare the risk of each PITT candidate, thus this chapter contains the results and discussion relevant to the ERA. Raw data from the experiments can be seen in the Appendix. The significance of the results in each experiment is first discussed in their dedicated subsections before being summed up with the candidates' risk characterised and compared as a result of the DREAM simulations. The last subsection is dedicated to an overall discussion about the results of the assessments and simulations.

4.1 Biodegradability Potential of PITT candidates

The OECD 306 closed bottle standard was followed as best as possible to gain accepted results (33). The results of the biodegradability potential of the PITT candidates over a period of 28 days, expressed in percentages, can be seen in Table 18. Raw data for this experiment can be found in Appendix A. As mentioned in Chapter 2, a compound is regarded as persistent or in the red zone when it has biodegradation potential of less than 20% and is regarded as biodegradable or in the green zone when it is over 60%. Compounds with biodegradation potential between 20 and 60% are in the yellow zone (9,34).

Table 18: Percentage of biodegradation of each PITT candidate over 28 days.

Compound	BOD 28 (%)
Pyridine	91
2,3-Dimethylpyrazine	22
2,6-Dimethylpyrazine	49
4-Methoxybenzyl alcohol	100
3,4-Dimethoxybenzyl alcohol	45
4-Chlorobenzyl alcohol	25
2,6-Dichlorobenzyl alcohol	32

The results obtained in Table 18 show that the biodegradation potential of most of the compounds is in the yellow zone. This view is strengthened by the plot visualised in Figure 22. The error bars in Figure 22 represent the standard deviation between the duplicate samples.

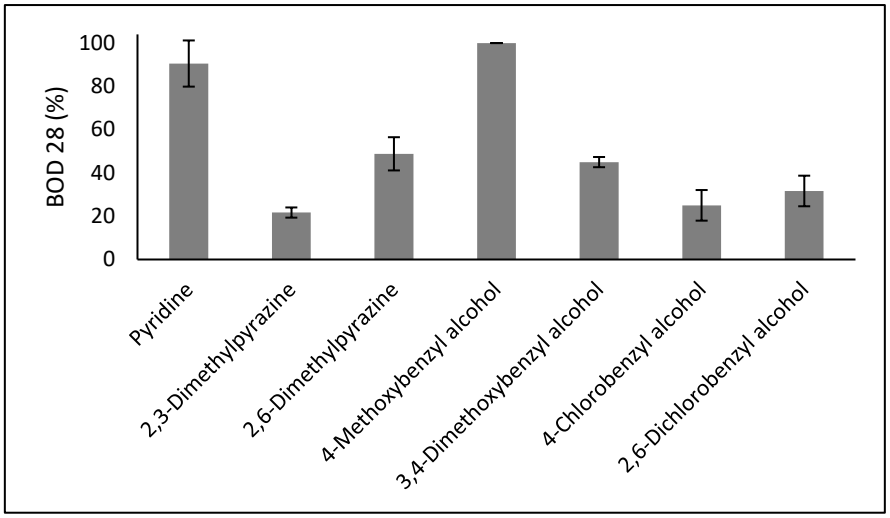

Figure 22: A comparison of the biodegradation potential of each PITT candidate.

Two compounds can be regarded as biodegradable and in the green zone, Pyridine and 4-Methoxybenzyl alcohol. The least biodegradable compound is 2,3-Dimethylpyrazine, which is very close to the red zone threshold. 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol were also close to the red zone threshold. 2,6-Dimethylpyrazine was not too far from the green zone threshold of 60% (34).

An observation during the experiment was made on the low levels of biological activity in all the samples involved. This was attributed to the seawater collected and must be mentioned as the percentage of biodegradation could be higher with seawater collected from areas with higher biological activity (33). However, the seawater collected was representative of the seawater found on the NCS and therefore the results are a good indication of the compounds persistence in Norwegian waters.

Initial results were invalidated due to inconsistent duplicates. This was attributed to the presence of leftover surfactants from the soap used when cleaning the BOD bottles. Surfactants are known to be readily biodegradable (51). The BOD bottles were consequently flushed with very little acetone and thoroughly rinsed with distilled water. Due to concern over the potential biodegradation influence of acetone, the BOD bottles were heavily rinsed using distilled water (52). This proved beneficial as duplicate readings were more consistent. This improvement also confirmed the reliability of the oxygen probe instrument being used to measure dissolved oxygen.

4.2 Toxicity of PITT Candidates on Algae

The ISO10253:2016(E) standard was carefully followed to achieve recognition of the results (38). The toxicity effects measured during this experiment was the growth inhibition of the algae *Skeletonema costatum.* The toxicity dose-response curves of all PITT candidates plotted using the HEPB program and Microsoft Excel are presented in Figure 23 on the next page. Table 19 shows the EC_{50} of all the PITT candidates data determined from these experiments, that is based on the regression curves plotted in Figure 23. Both Table 19 and Figure 23 are presented with a 95% confidence interval provided by the HEPB program (36). The relevant values measured in the laboratory as well as the regression data from HEPB can be seen in Appendix B.

Table 19: The EC_{50} concentration of all PITT candidates for their growth inhibition of algae.

Compound	EC_{50} (min-max) mg/L
Pyridine	347 (282-420)
2,3-Dimethylpyrazine	1106 (953-1278)
2,6-Dimethylpyrazine	792 (578-1036)
4-Methoxybenzyl alcohol	317 (283-353)
3,4-Dimethoxybenzyl alcohol	540 (415-696)
4-Chlorobenzyl alcohol	71 (59-84)
2,6-Dichlorobenzyl alcohol	67 (48-142)

As noted earlier, the expected concentration of PITT tracers in produced water discharge is 0.003 ppm (or mg/L)(21). Comparing the concentrations typically used for PITT applications with the EC_{50} results in Table 19, a conclusion can be drawn that there are no or very low levels of toxicity for all compounds in PITT applications. This is confirmed in Figure 23 as none of the compounds are toxic to algae at such a low concentration of 0.003 mg/L. The regression curves in Figure 23 were put together in one plot so as to compare all the candidates, this is shown in Figure 24.

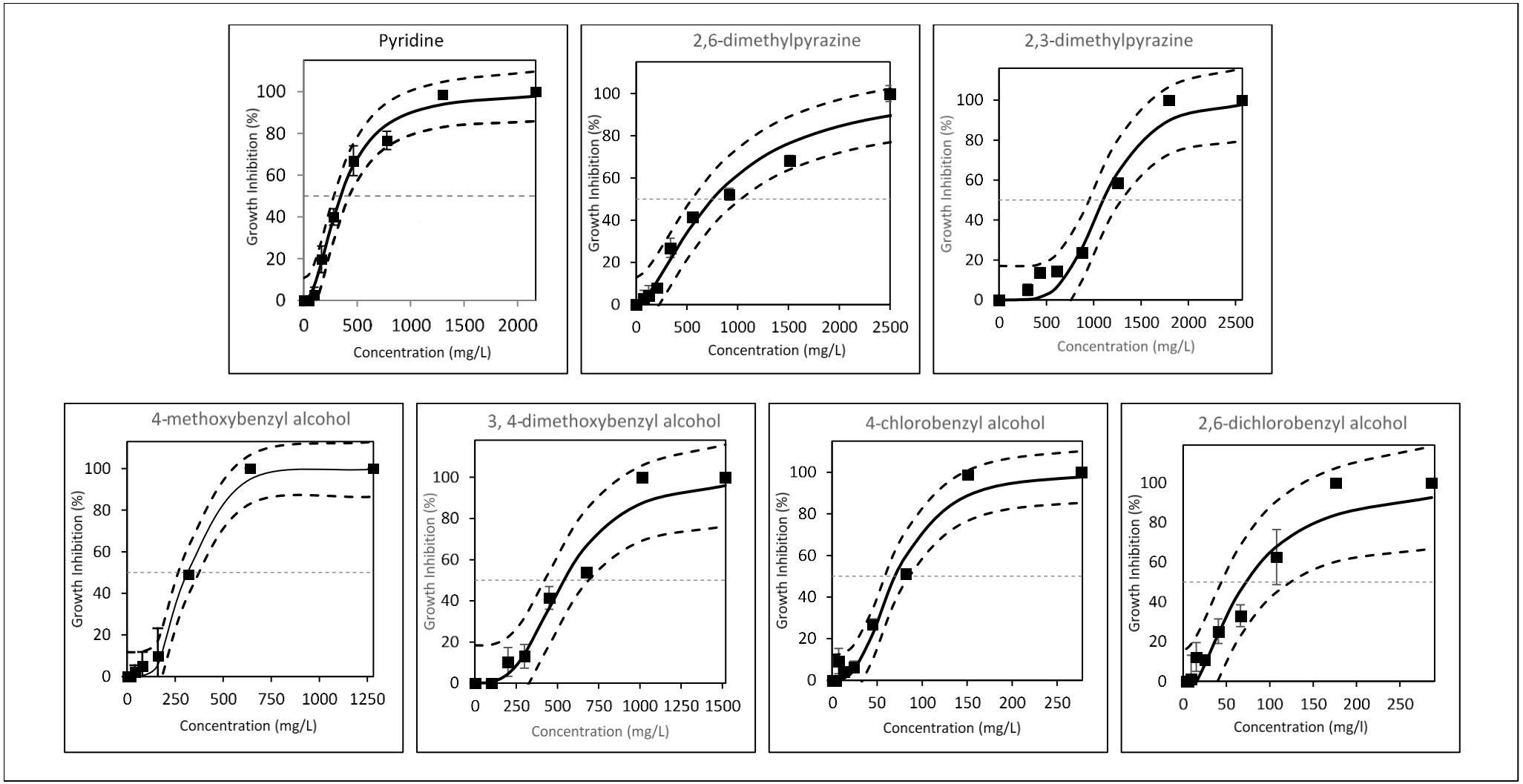

Figure 23: Dose-response curves with 95% confidence interval (dotted lines) based on growth inhibition of *Skeletonema costatum* for all PITT candidates.

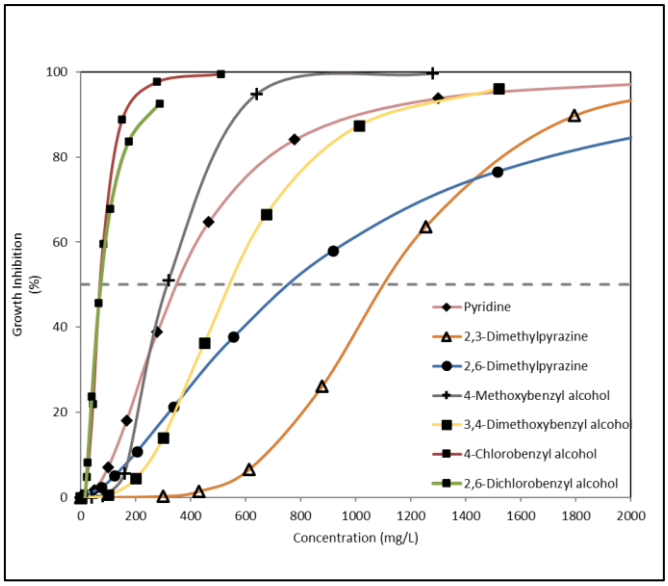

Figure 24: Comparison of all the PITT candidates' toxic effects on *Skeletonema costatum*.

The most toxic compound when it comes to inhibiting the growth of *Skeletonema costatum* was 2,6-Dichlorobenzyl alcohol, 67 mg/L. 2,6-Dichlorobenzyl alcohol and 4-Chlorobenzyl alcohol are the most toxic perhaps due to the presence of chlorine in these candidates (53). The other PITT candidates were a lot less toxic, with the least toxic being 2,3-Dimethylpyrazine.

The results obtained using the ISO10253:2016(E) procedure were consistent throughout the experiment and the application of it in the laboratory was practical. The main modification that was applied was that of using the supplier's growth media instead of synthesising a batch based on the recipe in the standard. As a result of this modification there was no doubt on the growth capability of the algae, in fact the algae had the specific growth rate stated in the standard (38).

An observation was made on the specific growth rate of the control, this was mentioned in chapter 3 of this thesis. The algae had a lag phase of 24 hours and grew exponentially between 24 hours and 48 hours. Thereafter the growth rate of the control decreased slightly. This gave a false indication of growth inhibition for the samples containing the PITT candidates. The standard mentions that this phenomenon can occur and recommends using the last readings from when the algae was growing exponentially (38). Although some control samples were still in the exponential phase at 72 hours, all measurements used to find the growth inhibition concentrations were taken at 48 hours.

An observation from the results was the stimulating effect of certain PITT candidates at lower concentrations. The algae grew better with small dosages of some of the candidates than with the control. This was however not of interest when determining the toxicity concentrations.

4.3 Toxicity of PITT Candidates on Fish Cells

OECD 171 test on RTgill-W1 cells was followed to determine the toxicity of PITT candidates on fish cells. The test looked at the number of Rainbow Trout gill cells that remained viable at increasing concentrations (39). The data for the dose-response curves were found using the HEPB program and plotted in Microsoft Excel, this is seen in Figure 25. For raw data, see Appendix C. The program also provided the EC_{50} values, Table 20. Both Table 20 and Figure 25 show the determined EC_{50} values with a 95% confidence interval. The dose-response curves in Figure 25 indicate the variety in relation between the candidate's dosage and cell viability. The curve is particularly steep for 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol meaning that a small spike in dosage can lead to a large drop in cell viability (54).

Table 20: The EC_{50} concentration of all PITT candidates for their toxicity on fish cells.

Compound	EC_{50} (min-max) mg/L
Pyridine	1796 (1173-2953)
2,3-Dimethylpyrazine	1743 (1095-2710)
2,6-Dimethylpyrazine	755 (569-994)
4-Methoxybenzyl alcohol	734 (462-1148)
3,4-Dimethoxybenzyl alcohol	1939 (1431-2655)
4-Chlorobenzyl alcohol	43 (37-51)
2,6-Dichlorobenzyl alcohol	50 (35-71)

When comparing the two forms of toxicity testing, the EC_{50} values vary inconsistently. For example, the value of Pyridine in Table 19 is lower than the value in Table 20 while the opposite is true for both 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol. Nevertheless, the toxicity of all PITT candidates on fish cells is at much higher concentrations (mg/L or ppm range) than that expected to be released in produced water discharges (21). Therefore, from the EC_{50} obtained in this toxicity experiment the same conclusion can be drawn as that for the algae. The compounds are not significantly toxic for PITT applications.

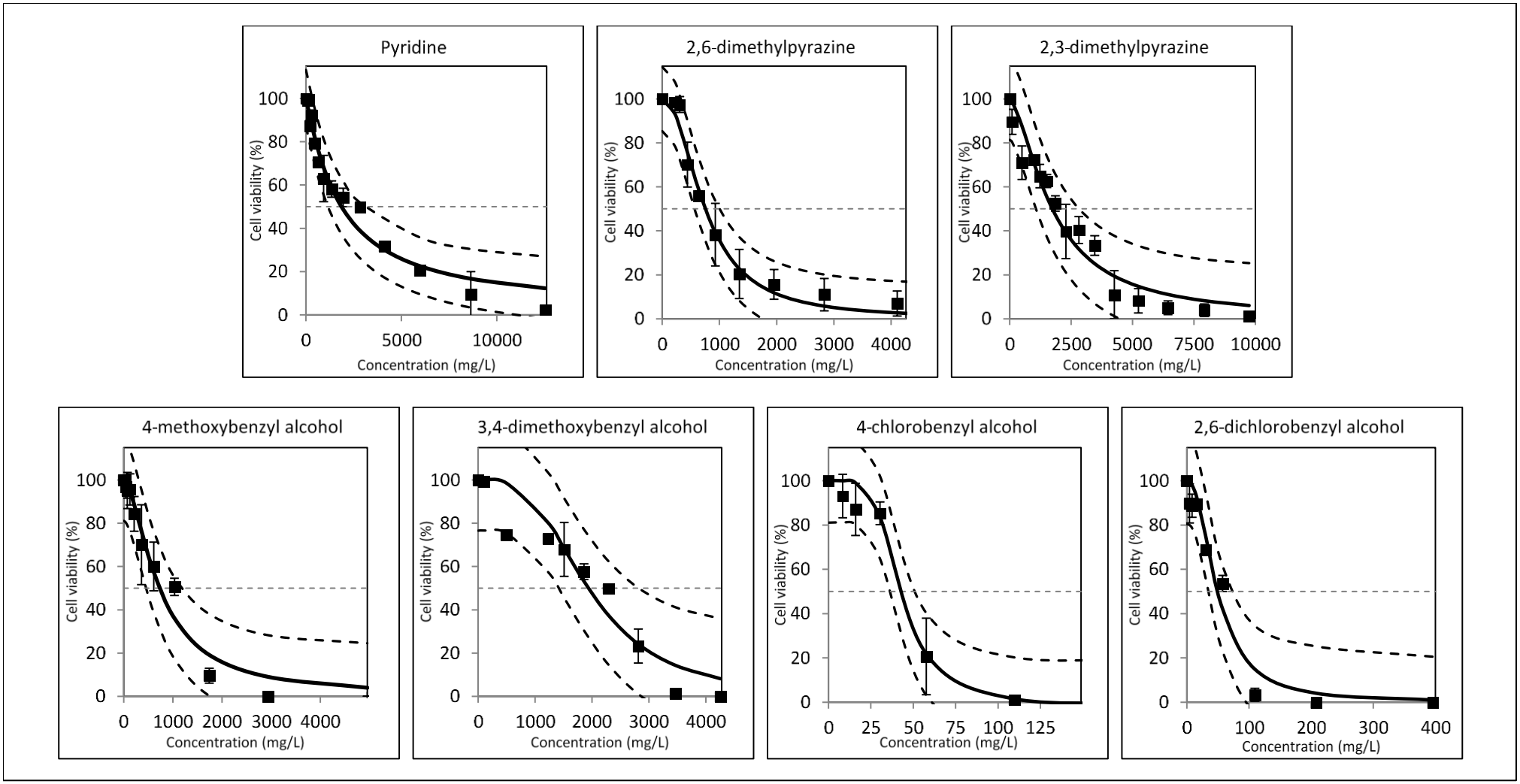

Figure 25: Dose-response curves with 95% confidence interval (dotted lines) based on the cell viability of RTgill-W1 cells for all PITT candidates.

The regression curves in Figure 25 were compiled into a plot, in Figure 26, to compare the PITT candidates.

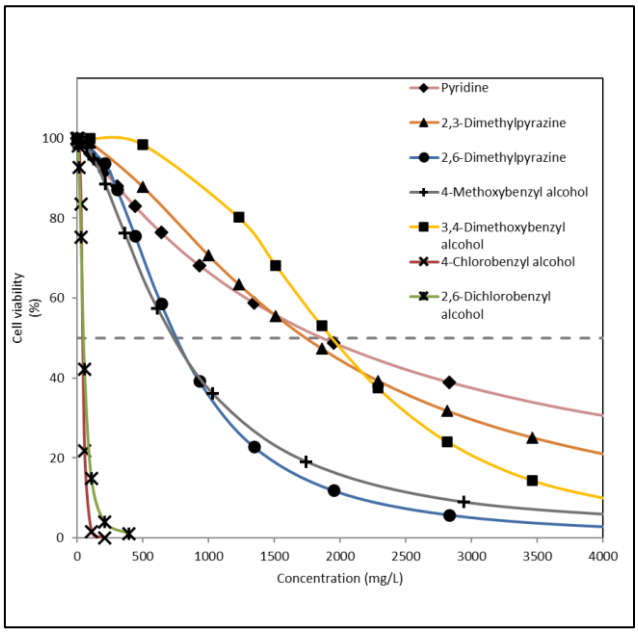

Figure 26: Comparison of all the PITT candidates' toxic effects on the fish cell RTgill-W1.

The most toxic compounds were 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol. These were followed by 2,6-Dimethylpyrazine and 4-Methoxybenzyl alcohol, which had very similar profiles. The remaining three PITT candidates had varying profiles but were the least toxic to the fish cells.

Because of the sensitivity of the fish cells, high level of care had to be practiced in the laboratory during this experiment to maintain cell viability before and after exposure. Slight carelessness during the experiment could have had a significant effect on the result (39). Biological differences between samples was observed, with some cells growing better or worse than others at the same concentration. This is a phenomenon that occurs regularly when working with the same cells as they are often genetically different and thus grow slightly differently (55). However, the deviation between triplicate samples accounts for this and gives an impression of the results' reliability (39). Additionally, the uncertainty that comes with working with very small measurements, in this case volumes in the microlitre (µL) range, could have also implicated end values (56).

4.4 DREAM Simulations and Risk Characterization

Data collected from the effect and exposure assessments for each candidate together with the fate factor parameters and the produced water release profile from the "Brage field", were added to a DREAM profile. A 30-day simulation was run using the model, and the results are seen in Table 21 and visualised in Figure 27 on the next page. The distribution and concentration of the different chemicals' in the environment based on the discharge scenario simulated, PEC, are not visualised here. However, they were calculated within the simulations and used by DREAM to compare with the threshold PNEC values obtained from the toxicity tests to determine the average EIF contributions of each candidate. Table 21 shows the various discharge concentrations of the PITT candidates that were simulated and their average EIF contribution over the 30-day period. The time averaged EIF contribution on the y-axis of Figure 27 is the average EIF contribution of the candidates over the 30-day simulation, this was plotted against the various discharge concentrations of each PITT candidate seen in Table 21.

Table 21: Simulated EIF contributions of PITT candidates at various discharge concentrations.

	Average EIF contribution over 30 days of each candidate						
Conc. (mg/L)	Pyridine	2,3-Dimethyl pyrazine	2,6-Dimethyl pyrazine	4-Methoxy benzyl alcohol	3,4-Dimethoxy benzyl alcohol	4-Chloroben zyl alcohol	2,6-Dichloro benzyl alcohol
0.003	0	0	0	0	0	0	0
0.03	0	0	0	0	0	0.003	0.003
0.3	0.003	0	0.003	0.003	0.003	0.068	0.055
3	0.072	0.021	0.033	0.054	0.049	0.916	0.766
30	0.982	0.238	0.460	0.737	0.690	11.1	9.11

As mentioned in section 2.3.4 of this thesis, and illustrated in Figure 9, the risk is considered "substantial" when the probability of environmental impact is greater than 5%; this corresponds to an EIF value of one in a given cubic volume of 10^5 m^3 (44,45). The average EIF contribution portrays the chemicals contributions in the cubic volumes where the value of EIF is one. The risk can be considered "acceptable" when the value of the contribution to EIF is zero (46). The first discharge concentration applied in the simulation for each candidate was 0.003 mg/L (or ppm), the highest concentration reported in similar produced water profiles (21). None of the

candidates showed any contribution to EIF at this concentration, therefore simulations were conducted with concentrations increasing progressively by factors of 10 for comparing between the different PITT candidates. Based on the simulations at the concentration 0.003 mg/L, the risk of all candidates can be characterised as "acceptable" for PITT applications (44–46).

It should be noted that an assessment factor of 1000 was applied to the toxicity values of EC_{50} during the simulations in case the relevant concentration was lower than that found during the toxicity tests. The assessment factor is also meant to account for other uncertainties related to the differences between laboratory exposure and field exposure, such as acute toxicity versus chronic toxicity (49). The output data, contribution to EIF, provided by DREAM is strongly dependent on the tested input data for a whole discharge composition. Therefore, the contribution to EIF values could vary from the values of actual fields (44). However, the consistency in low contribution values of EIF found even at much higher concentrations proves that the candidates' relevant contribution to EIF of different field discharges should not deviate very much from those calculated in Table 21.

4.5 Comparison and Overall Discussion

The PITT candidates can be compared by observing the results of their EIF contributions at the higher concentrations, although these concentrations are not likely in real PITT applications (21). The time averaged EIF contribution, seen in Figure 27, remains below the value of one even at 30 mg/L for all but two candidates, 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol. At this unrealistically high concentration, the risk of these two candidates is therefore characterized as "substantial" (44,45). They did have an average EIF contribution value around one at 3 mg/L, also a concentration higher than expected in a real produced water discharge.

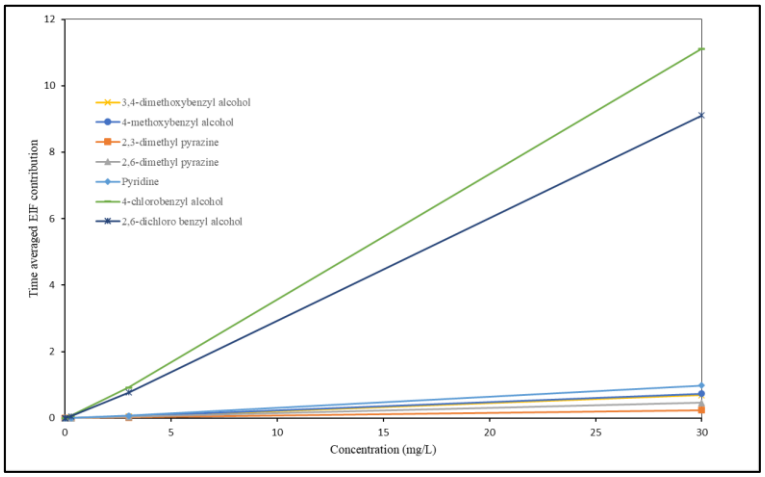

Figure 27: EIF contribution of each PITT candidate plotted against their concentration.

A full comparison of the candidates for all assessments conducted in this ERA can be seen in Table 22 where they have been ranked from 1-7) to indicate the "best" environmental candidate. The ranking of the candidates for all the assessments and EIF contribution gives basis for risk-informed decisions regarding PITT applications (11).

Table 22: Rank of best PITT candidate based on assessments determined during this ERA.

Rank	Biodegradation Potential	Bioaccumulation Potential	Toxicity on algae	Toxicity on fish cells	EIF contribution
1)	4-Methoxybenzyl alcohol	2,3-Dimethyl pyrazine	2,3-Dimethyl pyrazine	3,4-Dimetheoxy benzyl alcohol	2,3-Dimethyl pyrazine
2)	Pyridine	2,6-Dimethyl pyrazine	2,6-Dimethyl pyrazine	Pyridine	2,6-Dimethyl pyrazine
3)	2,6-Dimethyl pyrazine	3,4-Dimetheoxy benzyl alcohol	3,4-Dimetheoxy benzyl alcohol	2,3-Dimethyl pyrazine	3,4-Dimetheoxy benzyl alcohol
4)	3,4-Dimetheoxy benzyl alcohol	4-Chloro benzyl alcohol	Pyridine	2,6-Dimethyl pyrazine	4-Methoxy benzyl alcohol
5)	2,6-Dichlorobenzyl alcohol	Pyridine	4-Methoxy benzyl alcohol	4-Methoxy benzyl alcohol	Pyridine
6)	4-Chlorobenzyl alcohol	4-Methoxy benzyl alcohol	4-Chlorobenzyl alcohol	2,6-Dichloro benzyl alcohol	2,6-Dichloro benzyl alcohol
7)	2,3-Dimethyl pyrazine	2,6-Dichloro benzyl alcohol	2,6-Dichloro benzyl alcohol	4-Chlorobenzyl alcohol	4-Chlorobenzyl alcohol

The PITT candidate with the lowest contribution to EIF (last column) at all concentrations, thus the compound with the lowest contribution to risk, was 2,3-Dimethylpyrazine (35). This was followed by 2,6-Dimethylpyrazine, 3,4-Dimethoxybenzyl alcohol, 4-Methoxybenzyl alcohol, Pyridine, 2,6-Dichlorobenzyl alcohol, and finally, 4-Chlorobenzyl alcohol.

Although the candidates were found to not have toxic characteristics near the expected concentration of discharge, their contribution to risk could be more related to the candidates' bioaccumulation and biodegradation potential. The determination of the bioaccumulation potential values was made earlier in this thesis (section 2.3.2.2) as part of the literature review on experiments conducted when shortlisting the candidates (3–5). As seen in the third column of Table 22, the PITT candidate with the lowest bioaccumulation potential was 2,3-Dimethylpyrazine followed by 2,6-Dimethylpyrazine. The pyrazine candidates generally ranked above the other candidates throughout the assessments. However, the biodegradation potential of 2,3-Dimethylpyrazine, seen in column two, was found to be the lowest of all the shortlisted PITT candidates, meaning that it is the most persistent compound of the seven candidates (34). 2,6-Dimethylpyrazine was more biodegradable but was ranked lower than 4-Methoxybenzyl alcohol and Pyridine. These two, 4-Methoxybenzyl alcohol and Pyridine, were the only candidates that could be regarded as readily biodegradable as more than 60% had been

biodegraded (9). Simultaneously, these candidates are among the three most bioaccumulating candidates. However, none of the candidates were found to potentially bioaccumulate to a large extent in organisms (9). Therefore, the general area of concern regarding risk during PITT applications is more related to biodegradability potential rather than their toxicity or bioaccumulation.

Chapter 5: Conclusion

The aim of this ERA was to characterise and then compare the risk of the seven shortlisted PITT candidates in PITT applications. This was achieved by first determining the bioaccumulation potential, the biodegradation potential, and the toxicity characteristics (EC_{50}), of all candidates before running a 30-day simulation on DREAM of the potential discharge scenario with a real produced water profile. This was done to find the candidates' contribution to EIF which was then related to their risk.

None of the candidates were found to have a significant bioaccumulation potential based on calculations of results acquired from experiments conducted when shortlisting the candidates. The biodegradability of the candidates was found to be the biggest contributor to potential risk. Pyridine and 4-Methoxybenzyl alcohol were the only readily biodegradable candidates during this ERA. The remaining candidates were found to be below the threshold of readily biodegradable but above the threshold of persistent compounds, with 2,3-Dimethylpyrazine being the least biodegradable candidate. All candidates were found to be non-toxic for PITT applications because their EC_{50} concentrations were significantly higher than the expected concentration of the candidates in a produced water profile. The most toxic candidates were 4-Chlorobenzyl alcohol and 2,6-Dichlorobenzyl alcohol and the least toxic were 2,3-Dimethylpyrazine, 2,6-Dimethylpyrazine, and 3,4-Dimethoxybenzyl alcohol.

The 30-day DREAM simulation was run using the highest expected concentration for each PITT candidate in the produced water discharge, 0.003 ppm. Based on this assumption, the risk was characterised as "acceptable" for all seven PITT candidates in PITT applications as they had no contribution to EIF. The risk of each PITT candidate was compared by increasing the concentrations and observing their contribution to EIF. The PITT candidate with the least contribution to EIF, and risk, at higher concentrations was 2,3-Dimethylpyrazine thereafter followed by 2,6-Dimethylpyrazine, 3,4-Dimethoxybenzyl alcohol, 4-Methoxybenzyl alcohol, Pyridine, 2,6-Dichlorobenzyl alcohol, and 4-Chlorobenzyl alcohol.

The risk of all PITT candidates was therefore successfully characterised and compared during this ERA. A selection of a candidate for PITT applications can be made by observing their overall performances in this ERA as well as their tracer properties. However, based on the environmental perspective highlighted above, Pyridine and 4-Methoxybenzyl alcohol are the overall best choices as they obtained acceptable outcomes in all categories assessed. In the

cases where other candidates exhibit better tracer properties, the results of this ERA indicate that these candidates can also be deemed to have obtained acceptable results depending on the emphasis put on each of the tested aspects. In such cases these findings will allow for risk-informed decisions to be made over the shortlisted candidates to be taken further for PITT applications.

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Appendix

This appendix provides supplemental documents containing the raw data measured and calculated for the following: biodegradation potential (Appendix A), toxicity tests on algae (Appendix B), and toxicity tests on fish cells (Appendix C).

Appendix A: Biodegradability Data

Table A-1, A-2, and A-3 show the dissolved oxygen measured periodically over 28 days for all PITT candidates. Each table has the PITT candidates with their corresponding blank measurements, these were used to calculate the BOD.

Table A-1: Raw BOD data for Pyridine, 4-Chlorobenzyl and 2,6-Dichlorobenzyl alcohol

	Dissolved Oxygen Measured (mg/l)							
	Day 0		Day 5		Day 15		Day 28	
Compound	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2
Pyridine	6.2	6.2	6.2	6.1	4.6	4.8	1.1	0.9
4-Chlorobenzyl alcohol	6.2	6.2	6	5.5	5.3	5.2	2.8	2.5
2,6-Dichlorobenzyl alcohol	6.2	6.2	6.1	5.9	5.6	5.5	2.6	2.3
Blank	6.2	6.2	6.1	5.7	4.9	4.5	3.7	3.1

Table A-2: Raw BOD data for 2,3-Dimethylpyrazine, 4-Methoxybenzyl and 3,4-Dimethoxybenzyl alcohol

	Dissolved Oxygen Measured (mg/l)							
	Day 0		Day 5		Day 15		Day 28	
Compound	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2
4-Methoxybenzyl alcohol	6.0	6.1	5.4	5.2	2.5	1.9	1.6	1.8
3,4-Dimethoxybenzyl alcohol	6.0	6.0	5.8	5.9	4.0	4.4	3.2	3.4
2,3-Dimethylpyrazine	6.0	6.0	5.5	5.6	4.3	5.0	4.0	4.0
Blank	6.0	6.1	5.6	5.8	5.0	5.1	4.7	4.7

Table A-3: Raw BOD data for 2,6-Dimethylpyrazine

	Dissolved Oxygen Measured (mg/l)							
	Day 0		Day 5		Day 15		Day 28	
Compound	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2
2,6-Dimethylpyrazine	6.2	6.4	5.7	5.6	4.6	4.7	4.2	3.9
Blank	6.6	6.5	6.0	6.2	6.1	6.2	6.1	6.1

Appendix B: Toxicity on Algae Data

Tables B-1 to B-7 show the fluorescence units (FSU) measured using a fluorimeter on all candidates at varying concentrations during the toxicity tests on the algae *Skeletonema costatum*. The FSU was used to find the specific growth rates and the consequent growth inhibition. The growth inhibition data was put into the HEPB program. The output from the program was the regression data as well as the EC_{50} values expressed in Table 19. The regression data from HEPB was used on the y-axis of the dose-response curves (Figure 23 and 24) as the growth inhibition percentage.

Table B-1: Algae toxicity data for: Pyridine.

Pyridine	FSU hour 0		FSU hour 48		Specific growth rate (d^{-1})		Growth inhibition (%)		Regression data from
(mg/L)	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	HEPB (%)
0	19000	23000	180000	190000	1.12	1.05	0	0	4.97E-06
100	19000	19000	160000	200000	1.07	1.18	5.24	< 0	7.122588
167	20000	20000	110000	120000	0.85	0.90	24.2	15.1	18.09174
278.89	20000	23000	82000	77000	0.71	0.60	37.2	42.8	38.88191
465.74	25000	21000	47000	47000	0.32	0.40	71.9	61.8	64.6927
777.79	19000	20000	30000	35000	0.23	0.28	80.0	73.5	84.06986
1298.91	21000	17000	20000	18000	-0.02	0.03	> 100	97.3	93.8272
2169.19	18000	16000	5200	5800	-0.62	-0.51	> 100	> 100	97.76686

Table B-2: Algae toxicity data for: 2,3-Dimethylpyrazine.

2,3-Dimethyl pyrazine (mg/L)	FSU hour 0		FSU hour 48		Specific growth rate (d^{-1})		Growth inhibition (%)		Regression data from HEPB (%)
	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	
0	15000	20000	350000	440000	1.57	1.55	0	0	7.57E-17
300	17000	21000	360000	370000	1.53	1.43	3.08	7.18	0.288012
429	18000	22000	290000	300000	1.39	1.31	11.8	15.5	1.414339
613.46	16000	18000	230000	260000	1.33	1.34	15.4	13.6	6.651109
877.26	19000	17000	200000	190000	1.18	1.21	25.3	22.0	26.13967
1254.00	17000	16000	61000	59000	0.64	0.65	59.4	57.8	63.69922
1793.91	18000	12000	7000	8200	-1.06	-0.73	> 100	> 100	89.72313
2565.29	20000	16000	6600	5800	-1.33	-1.59	> 100	> 100	97.74587

Table B-3: Algae toxicity data for: 2,6-Dimethylpyrazine.

2,6-Dimethyl pyrazine (mg/L)	FSU hour 0		FSU hour 48		Specific growth rate (d^{-1})		Growth inhibition (%)		Regression data from HEPB (%)
	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	
0	15000	16000	370000	420000	1.60	1.63	0	0	6.58E-05
75	16000	16000	400000	350000	1.61	1.54	< 0	5.58	2.3939
123.75	17000	20000	410000	410000	1.59	1.51	0.70	7.57	5.1625
204.19	14000	16000	280000	310000	1.50	1.48	6.54	9.39	10.7930
336.9	17000	14000	160000	170000	1.12	1.25	30.1	23.6	21.2416
555.9	17000	16000	110000	110000	0.93	0.96	41.7	41.0	37.7114
917.23	15000	12000	65000	61000	0.73	0.81	54.3	50.2	57.9910
1513.43	13000	15000	38000	40000	0.54	0.49	66.5	70.0	76.5626
2497.17	17000	20000	15000	21000	-0.06	0.02	> 100	98.5	89.4895

Table B-4: Algae toxicity data for: 4-Methoxybenzyl alcohol.

4-Methoxy benzyl alcohol (mg/L)	FSU hour 0		FSU hour 48		Specific growth rate (d^{-1})		Growth inhibition (%)		Regression data from HEPB (%)
	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	
0	15000	13000	140000	180000	1.12	1.31	0	0	3.64E-13
20	20000	18000	170000	170000	1.13	1.20	< 0	< 0	0.00113
40	15000	13000	190000	160000	1.27	1.26	< 0	4.48	0.01966
80	16000	14000	160000	150000	1.15	1.19	< 0	9.76	0.34171
160	13000	18000	120000	150000	1.11	1.96	0.49	19.3	5.6428
320	18000	12000	57000	45000	0.58	0.66	48.4	49.7	51.0528
640	13000	15000	10000	14000	-0.13	-0.03	> 100	> 100	94.7893
1280	14000	9300	1400	1300	-1.14	-1.00	> 100	> 100	99.6858

Table B-5: Algae toxicity data for: 3,4-Dimethoxybenzyl alcohol.

3,4-Dimethoxy benzyl alcohol (mg/L)	FSU hour 0		FSU hour 48		Specific growth rate (d^{-1})		Growth inhibition (%)		Regression data from HEPB (%)
	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	
0	14000	14000	260000	230000	1.46	1.40	0	0	3.09E-10
100	14000	13000	260000	240000	1.46	1.41	< 0	< 0	0.54942
200	16000	17000	190000	240000	1.23	1.32	15.3	5.42	4.47496
300	16000	18000	180000	230000	1.21	1.27	17.2	8.98	14.0585
450	15000	12000	74000	69000	0.80	0.87	45.4	37.5	36.3551
675	13000	14000	48000	53000	0.65	0.67	55.3	52.4	66.6072
1012.5	16000	13000	8400	6500	-0.32	-0.35	> 100	> 100	87.4454
1518.75	12000	13000	1500	1000	-1.05	-1.30	> 100	> 100	96.0509

Table B-6: Algae toxicity data for: 4-Chlorobenzyl alcohol dissolved in DMSO

4-Chloro benzyl alcohol (mg/L)	FSU hour 0		FSU hour 48		Specific growth rate (d^{-1})		Growth inhibition (%)		Regression data from HEPB (%)
	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	
0	15000	13000	270000	260000	1.53	1.50	0	0	1.42E-06
2.1	14000	14000	300000	300000	1.53	1.53	< 0	< 0	0.00617
3.88	13000	14000	310000	380000	1.59	1.65	< 0	<0	0.03341
7.14	16000	15000	250000	200000	1.37	1.30	4.90	13.5	0.17871
13.13	15000	13000	240000	230000	1.39	1.44	4.07	4.09	0.94820
24.17	17000	12000	240000	210000	1.32	1.43	8.41	4.46	4.88243
44.84	16000	13000	140000	110000	1.08	1.07	25.0	28.7	21.9480
81.84	14000	11000	58000	47000	0.71	0.73	50.8	51.5	59.5594
150.59	15000	14000	16000	10000	0.03	-0.17	97.8	> 100	88.7490
277.09	12000	12000	2300	2400	-0.82	-0.80	> 100	> 100	97.6877

Table B-7: Algae toxicity data for: 2,6-Dichlorobenzyl alcohol dissolved in DMSO

2,6-Dichloro benzyl alcohol (mg/L)	FSU hour 0		FSU hour 48		Specific growth rate (d^{-1})		Growth inhibition (%)		Regression data from HEPB (%)
	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	Flask 1	Flask 2	
0	15000	13000	270000	260000	1.53	1.51	0	0	-
15.28	15000	16000	220000	190000	1.34	1.24	7.09	17.4	-0.27972
24.91	15000	17000	200000	240000	1.30	1.32	10.4	11.6	8.2306
40.61	17000	14000	130000	150000	1.02	1.19	29.6	20.8	23.7307
66.19	12000	13000	93000	86000	1.02	0.94	29.2	36.9	45.6839
107.9	12000	14000	47000	32000	0.68	0.41	52.8	72.4	67.8741
175.88	9300	15000	8700	5200	-0.03	-0.53	> 100	> 100	83.7348
286.68	11000	12000	1600	1100	-0.94	-1.18	> 100	> 100	92.5485

Appendix C: Toxicity on Fish Cells Data

Tables C-1 to C-7 show the fluorescence units (FSU) measured using a fluorimeter on all PITT candidates after exposing the RTgill-W1 cells to varying concentrations. The FSU was used to find the percentage cell viability after exposure by comparing the exposed and non-exposed cells. The cell viability percentage was then added to the HEPB program together with the respective concentrations to produce the regression data and EC_{50} values expressed in Table 20. The regression data from HEPB was used on the y-axis of the dose-response curves (Figure 25 and 26) as the percentage cell viability. Some readings were nullified and were not considered, these are expressed as "-".

Table C-1: Fish cell toxicity data for: Pyridine.

	Measured FSU			Cell viability (%)			Regression data from
Pyridine (mg/L)	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	HEPB (%)
No cells	15834991	16860366	16808604	0	0	0	-
0	31587698	33857588	33059088	100	100	100	99.99816
100	32517146	33435498	33053784	>100	97.5	99.9	96.20076
145	33069406	33622088	31888572	>100	98.6	>100	94.37086
210.25	29514506	32171066	30638878	86.8	90.1	85.1	91.73703
304.86	30787448	32413502	31465870	95.0	91.5	90.2	88.03091
442.05	28491946	-	29541276	80.3	-	78.4	82.97817
640.97	26842800	-	28433664	69.9	-	71.5	76.37939
929.41	24567978	28854374	-	55.4	70.6	-	68.22801
1347.64	27200690	-	25818464	-	60.8	55.4	58.82448
1954.08	24860388	23915014	-	57.3	51.3	-	48.79481
2833.42	-	25070072	24733464	-	50.8	48.8	38.95418
4108.46	23325343	24215324	-	32.5	31.0	-	30.05889
5957.28	-	20288254	-	-	20.2	-	22.59444
8638.05	16175720	-	19560910	2.2	-	16.9	16.7119
12525.1	17083114	17296444	17169640	7.9	2.6	2.2	12.30126

Table C-2: Fish cell toxicity data for: 2,3-Dimethylpyrazine.

2,3-Dimethylpyrazine (mg/L)	Measured FSU			Cell viability (%)			Regression data from HEPB (%)
	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	
No cells	16380629	15494219	15433171	0	0	0	-
0	33317240	31609774	31381676	100	100	100	99.99998
100	31652102	29867918	31514162	90.2	89.2	89.4	98.94403
500	27592722	26302678	28167302	66.2	67.1	79.8	87.90541
1000	28476878	28761940	-	71.4	73.1	-	70.73273
1230	-	26563568	25182254	-	68.7	61.1	63.49696
1512.9	26636954	25934374	-	60.6	64.8	-	55.59533
1860.86	-	23547908	24200496	-	50.0	55.0	47.40031
2288.86	-	23301492	20379010	-	48.4	31.0	39.34281
2815.3	23950232	21307650	-	44.7	36.1	-	31.82625
3462.82	22874120	20602440	20205044	38.3	31.7	29.9	25.15033
4259.27	20366324	16348057	16082473	23.5	5.3	4.1	19.47473
5238.9	18840554	16385957	16158402	14.5	5.5	4.5	14.82624
6443.85	16625072	16570431	16537164	1.4	6.7	6.9	11.13389
7925.94	16730346	16645870	15829665	2.1	7.1	2.5	8.271781
9748.91	15731573	15602894	15930245	-3.8	0.67	3.1	6.094955

Table C-3: Fish cell toxicity data for: 2,6-Dimethylpyrazine.

2,6-Dimethylpyrazine (mg/L)	Measured FSU			Cell viability (%)			Regression data from HEPB (%)
	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	
No cells	15494219	16380629	15433171	0	0	0	-
0	31609774	31381676	33317240	100	100	100	100
210.25	31521512	31029708	-	99.5	97.7	-	93.68668
304.86	32793376	30619886	-	> 100	94.9	-	87.14085
442.05	27962070	25819558	-	77.4	62.9	-	75.57675
640.97	24762226	24513688	-	57.5	54.2	-	58.55936
929.41	23280598	20613974	-	48.3	28.2	-	39.22007
1347.64	20059366	18259488	-	28.3	12.5	-	22.76011
1954.08	18796372	18012052	-	20.5	10.9	-	11.86004
2833.42	18106706	17252634	-	16.2	5.8	-	5.788897
4108.46	17263400	16822262	-	11.0	2.9	-	2.729336
5957.28	17002874	15936379	-	9.4	-2.96146	-	1.265102
8638.05	16135208	16000828	-	4.0	-2.53183	-	0.581706
12525.1	15701172	15653524	-	1.3	-4.84703	-	0.26648

Table C-4: Fish cell toxicity data for: 4-Methoxybenzyl alcohol.

4-Methoxybenzyl alcohol(mg/L)	Measured FSU			Cell viability (%)			Regression data from HEPB (%)
	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	
No cells	13486637	14076207	14662223	0	0	0	-
0	30661986	28225476	28201024	100	100	100	99.99996
26.56	31391790	28255290	29842764	> 100	> 100	> 100	99.60509
44.81	29083650	28191114	29652300	90.8	99.8	> 100	99.06102
75.61	28173780	29717660	28715276	85.5	> 100	>100	97.78355
127.55	28490530	28187928	28182854	87.4	99.7	99. 9	94.86027
215.18	26600266	25981646	27181000	76.4	84.1	92.5	88.53279
363.01	21907300	25895880	25182316	49.0	83.5	77.7	76.35742
612.4	21584636	23248752	23871138	47.1	64.8	68.0	57.46554
1033.13	21464642	21775048	21559916	46.5	54.4	50.9	36.10877
1742.89	14627996	15956664	15845484	6.6	13.3	8.7	19.12133
2940.26	12698633	12929848	12882112	-4.6	-8.1	-13.1	8.9999
4960.23	12855609	12824123	12860712	-3.7	-8.8	-13.3	3.972839

Table C-5: Fish cell toxicity data for: 3,4-Dimethoxybenzyl alcohol.

3,4-Dimethoxybenzyl alcohol(mg/L)	Measured FSU			Cell viability (%)			Regression data from HEPB (%)
	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	
No cells	15834991	16860366	16808604	0	0	0	-
0	33053784	33435498	32517146	100	100	100	100
100	33059088	33059088	33693204	> 100	97.7	> 100	99.98901
500	28450614	-	28773718	73.3	-	76.2	98.47451
1230	28215640	29077168	-	71.9	73.7	-	80.21464
1512.9	29033952	26648398	-	76.7	59.1	-	68.20653
1860.86	26194352	25991480	-	60.2	55.1	-	53.1649
2288.86	24248540	25248540	-	48.9	50.6	-	37.5254
2815.3	20798838	19798792	-	28.8	17.7	-	24.11748
3462.82	15493568	17330866	-	-2.0	2.8	-	14.39632
4259.27	15112580	15502994	-	-4.2	-8.2	-	8.171567

Table C-6: Fish cell toxicity data for: 4-Chlorobenzyl alcohol dissolved in DMSO.

4-Chlorobenzyl alcohol(mg/L)	Measured FSU			Cell viability (%)			Regression data from HEPB (%)
	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	
No cells + DMSO	15960271	15053184	16482229	0	0	0	-
0	29258940	25305858	28960566	100	100	100	100
8.41	27418844	27325204	-	86.2	100	-	99.94015
15.98	25773450	24932662	27850344	73.8	96.4	91.1	98.92222
30.36	26730870	24380928	26958364	81.0	91.0	84.0	83.46525
57.68	17088092	18422876	-	8.5	32.9	-	21.72974
109.6	15369330	15392439	15587483	-4.4	3.3	-7.2	1.502972
208.25	15289823	14997504	15194968	-5.0	-0.5	-10.3	0.083808

Table C-7: Fish cell toxicity data for: 2,6-Dichlorobenzyl alcohol dissolved in DMSO.

2,6-Dichlorobenzyl alcohol(mg/L)	Measured FSU			Cell viability (%)			Regression data from HEPB (%)
	Well 1	Well 2	Well 3	Well 1	Well 2	Well 3	
No cells + DMSO	15874094	15662556	15477167	0	0	0	-
0	27081082	26615410	26258412	100	100	100	99.9999
4.43	30183226	24849590	24707656	> 100	83.9	85.6	99.5449
8.41	26229274	24976486	-	92.4	85.0	-	98.13208
15.98	25984428	25301168	25196394	90.2	88.0	90.1	92.65694
30.36	-	23095930	22989408	-	67.9	69.7	75.19604
57.68	22270418	21563148	20851636	57.1	53.9	49.9	42.14192
109.6	16472230	16179795	15072212	5.3	4.7	-3.8	14.88928
208.25	14734712	14648831	14508177	-10.2	-9.3	-9.0	4.032521
395.68	14304932	13909956	13770464	-14.0	-16.0	-15.8	0.973397