NTNU – Trondheim
Norwegian University of
Science and Technology

Effects of Produced Water on the Marine Phytoplankton *Phaeodactylum tricornutum*

A study of the effects Produced Water, from the Petroleum Industry, can have on Marine Phytoplankton, and the possibilities of a simple yet highly modifiable experimental design

Hans Henriksen Marki

Environmental Toxicology and Chemistry
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Supervisor: Bjørn Munro Jenssen, IBI
Co-supervisor: Murat Van Ardelan, IKJ

Norwegian University of Science and Technology
Department of Biology

Abstract

Produced Water (PW) is a complex mixture of many chemical compounds, ranging from heavy metals and dispersed oil to Polycyclic Aromatic Hydrocarbons (PAHs), Alkylphenols (APs), and even production chemicals. In the following study the effects of PW discharged from the Norwegian Petroleum Industry was studied on the marine phytoplankton *Phaeodactylum tricornutum*, a species of marine diatoms. The experimental design of this study was focused on simplicity, and the ability to modify the methods for future studies was discussed. The results from this study shows effects of PW as a mixture of all the compounds mentioned above. The toxic effects, seen in the results as growth inhibition or effects on different parameters of algal growth, was compared to effects seen in early life-stages of fish, zooplankton and other microalgae species. The many different parameters measured in this experiment could all be an indication of growth, but as they all indicated growth by different reactions or factors in algal growth, the results were hard to discuss and any direct conclusions or correlations was difficult to justify. The most interesting results was found in a delay in pH change, visible in the pH results from the exposure group with the highest PW concentration, 10% PW. When this lack of pH increase, which was unexpected with algal growth, occurred, while other tests showed growth in all cultures, a possibility of growth and even photosynthesis without the use of CO2 was suggested. This result was in part correlated with a study on volatile hydrocarbons and their effect on Lipid : Chlorophyll a ratio in algal cells, although any definite conclusions was not justifiable based on this study alone. The differences between the results from all tests show that the ability to test and consider many factors and parameters are important when studying microalgae. Many earlier studies assume that algal growth rates can be directly extracted from one parameter measuring growth , but this study suggest heavy considerations of the actual chemical reactions behind results from a growth experiment are required to properly understand what any result actually show.

Keywords: Produced Water, *Phaeodactylum tricornutum*, BTEX, PAHs, Alkylphenols, Chlorophyll-a, Fluorescence, North Sea Petroleum Industry.

Sammendrag – Norwegian/Norsk

Produsert Vann (PW) er en kompleks blanding av mange kjemiske stoffer, blant annet tungmetaller, løste oljepartikler, Polysykliske Aromatiske Hydrokarboner (PAHs), Alkylfenoler (APs) i tillegg til kjemikalier brukt under produksjon. I dette studiet ble effektene av PW fra den Norske Petroleumsindustrien studert på den marine fytoplanktonet *Phaeodactylum tricornutum* (*P. tricornutum*), en marin diatomeer. Dette studiets eksperimentelle design var fokusert på enkelhet og evnen til å modifisere metodene for fremtidige studier ble diskutert. Resultatene fra dette studiet viser effektene av PW som en blanding av alle stoffene nevnt over. De toksiske effektene, som vist i resultatene som veksthemning eller effekter på forskjellige parametere av algevekst, ble sammenlignet med effekter vist på tidlige livsstadier hos fisk, zooplankton, og andre arter microalger. De mange forskjellige parameterne målt i dette forsøket kunne alle indikere vekst, men siden alle indikerer vekst basert på forskjellige reaksjoner eller faktorer i algevekst, var resultatene vanskelige å diskutere og direkte konklusjoner eller korrelasjoner var vanskelige å rettferdiggjøre. De mest interessante resultatene ble funnet i form av en forsinket pH forandring, som kunne sees i pH resultatene fra kulturene eksponert for den høyeste konsentrasjonen PW, 10% PW. Når den manglende pH økningen, som normalt er forventet ved algevekst, viste seg, mens andre tester viste vekst i alle kulturene, kunne en mulighet for vekst og kanskje også fotosyntese uten bruk av CO_2 forslås. En delvis korrelasjon mellom dette resultatet og en studie på flyktige hydrokarboner, og deres effekt på Lipid : Klorofyll a forhold i algeceller ble diskutert, men noen definitiv konklusjon var ikke rettferdiggjort av resultatene fra dette studiet alene. Forskjellene mellom alle testene i dette studiet viser at evnen til å teste mange faktorer og parametere er viktig når man studerer mikroalger. Mange tidligere studier antar at vekstraten til alger kan direkte måles fra en parameter som måler vekst, men dette studiet forslår at en må ha god forståelse rundt kjemien og betrakte alle de kjemiske reaksjonene som står bak resultatene fra et vekstforsøk.

Abbreviations

AP	Alkyl phenol
AH	Aromatic Hydrocarbon
BTEX	Benzene, Toulene, Ethylbenzene, and Xylenes
^{0}C	Celsius
CO_2	Carbon Dioxide
Chl-a	Chlorophyll-a
Chl. Fluor.	Chlorophyll Fluorescence
cm	Centimeter
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DEG	Diethylene Glycols
dH_2O	Distilled Water
FRI	Fluorescence Response Index
Ft	Fluorescence Yield (Actual Fluorescence)
F-QY	Fluorescence Quantum Yield
Fv/Fm	Potential Photosystem II efficiency
H_2O	Water
HCl	Hydrogen Cloride
HD-PE	High-Density Polyethylene
LD-PE	Low-Density Polyethyelene
MEG	Mono ethylene glycols
NaOH	Sodium Hydroxide
nm	Nanometer
OLF	The Norwegian Oil Industry Association
OGP	International Association of Oil and Gas Producers
PAH	Polycyclic Aromatic Hydrocarbon
PS Cap.	Photosynthetic Capacity
PW	Produced Water
mL	Milliliter
mm	Millimeter
TOC	Total Organic Carbon
µg	Microgram
µm	Micrometer

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Chapter 1. Introduction

1.1 Produced Water

Produced Water (PW) is a term used for any water used in production of a product. It is used in the petroleum industry where it is a term for the water created within an oil well and the water that follows the oil from drilling to the refinery (Neff, 2002). Legislation surrounding PW is mainly focused on oil-content, but it is known that PW is a complex mixture of oil droplets, other hydrocarbons, alkylphenols (APs), organic acids, and heavy metals. In addition to all these chemical components, chemicals used during production, transport, and treatment of the oil or the PW itself can be found in PW at varying levels (Neff, 2002). Using data from the public report from Statoil's Åsgard oilfield it is shown that between 2002 and 2012 a 900 000 000 m^3 of Produced Water was released from the combined production at the Åsgard oilfield (Sekkesæter and Myrhaug, 2013). Noting that this report consist of data from 10 years of production, other studies measure a total amount of PW discharged into the North Sea to be more than 500 000 000 m^3 per year (OLF 2011, OGP 2011). Although these numbers sound high, it is discussed in both reports (OLF 2011 and OGP 2011) that even though the amount of discharged PW increases, more and more PW is re-injected before shutdown of oil wells, and the total oil content in PW is reduced in the later years. It is also worth noting that the levels and content of PW discharged to the North Sea is not the worst compared to for example North America, Africa or the Middle East (*See OGP 2011 Figure 4.1c*). Produced Water from the North Sea Petroleum Industry is interesting when studying the total environmental impact from the petroleum industry, because the PW is dispersed and spread to fisheries in Norway, the UK, and Skagerrak. The effects of PW on fish have been studied on fish from larvae stages to adult fish and effects have been seen in larvae at even low concentrations, while adult fish experience a toxic effect in the highest concentrations closest to the offshore facilities (Meier et al., 2010). In many reports it is also stated that the major studies performed on toxic effects from PW discharge show lesser to no effects on fish on a population level, and that the rapid dispersion caused by waves and water-flow around the offshore facilities lead to non-toxic concentrations even at just >100m away from the source of discharge. It is therefore also found in most studies that effects on community levels are low or non-existent for fish and zooplankton throughout the North Sea. However, even though the effects of PW have been studied in fish, only a few studies have tried looking for toxic effects in the lowest stage of the food chain, the phytoplankton.

As mentioned above, PW is a complex mixture with many chemical components and production chemicals, and many of these single components have shown both toxic and helpful effects on the growth of algae. It is commonly known that release of wastewater, from urban localities or other industries, can lead to algal blooms and have harmful effects on both freshwater and marine environments.

1.2 Toxicity testing: Growth Experiment

The toxicity tests performed on Produced Water samples in earlier studies have been performed by micro assays, captured or placed individuals of fish, and most often by preparing test solutions based on predicted or calculated levels of single components from Produced Water. This study uses a basic growth experiment with different concentrations of PW to measure different parameters of growth and assess what effects can be seen in a marine phytoplankton. The goal of the experimental setup was to create a basis for an experiment, which could be modified heavily, and be performed easy and cheap. The effects of PW, visualized as growth inhibition could be studied, and the multiple parameters tested all measured growth. The key however, was that since each parameter studied was based on different reactions within the chemistry of microalgae, many different findings could be suggestive of many different conclusions. The experimental design has its problems, and lacks the precision of a well-funded multidisciplinary experiment, but its simplicity leads to an ability to modify all parameters and test for the almost unlimited amount of effects that one could expect a complex mixture like PW to have on a relatively sensitive algal culture. The growth experiment itself can therefore be seen as a pilot study on growth of microalgae in natural concentrations of PW. The concentrations used in this experiment was adopted from a study on early life stages of fish, where the highest concentration was 10% and resembled a radius very close to the offshore facility, while the lowest concentration of 0,01% resembled a more general ecosystem-wide chronic effect of PW (Meier et al., 2010). The focus on relevance to the natural environment was hindered a bit by the lack of funding and time, but the improvements and further possible studies are discussed fully in Section 4.5.

1.3 Aims

The aims of the study was focused around simplicity and based on the theory of a pilot project. The first goal was to examine if the natural concentrations of PW, mentioned in section 1.2, could have any effect on microalgae. Earlier studies have shown effects on fish in multiple stages, but the official reports from OLF 2011 and OGP 2011 suggest low to non-existent toxic effects, especially in the lowest parts of the food chain. This means that the first goal of the study was to disprove this assumption that Produced Water does not affect the lowest parts of the food chain. The second goal of the study was to review literature and earlier studies on Produced Water to examine if any correlations between the results found in the growth experiment on *P. tricornutum* and effects on larvae-stadium fish, zooplankton, or other microalgae, could be found. Finally, it was important for me to look at the ability to create a cheap project, which was highly modifiable, and which could be used to study further effects of PW on microalgae, and possibly study how the findings of such studies could be used in methods surrounding PW treatment or Bio-fuel production.

Chapter 2. Materials & Methods

2.1 Experimental design

The tanks was set up according to Table 1. Each exposure group consisted for 3 LD-PE tanks, which was treated the exact same way throughout the experiment. The growth experiment lasted for 7 days after inoculation. Each day from Day 1-7 pH, Turbidity, and all the fluorescence tests were performed on a sample from all 15 tanks. The tests were not performed randomly or blindly, but the test-order was chosen following the simple rule of starting with the presumed lowest concentration. This is a technique used to reduce the chance of higher concentration samples affecting lower concentration samples, if the washing steps between each tests are not performed correctly. For the Chlorophyll a analyses, 1 tank was chosen from each exposure group, and a sample from these tanks were used for filtration throughout the experiment.

Table 1: Experimental Setup for growth of P. tricornutum in Produced Water

Tank number:		1-2-3	4-5-6	7-8-9	10-11-12	13-14-15
Produced Water (%):		10	1	0.1	0.01	0
Produced Water (ml):		222	20,2	2,002	0,2	0
F/2 Medium (ml):		2000	2000	2000	2000	2000
Volume PW+Medium:		2222	2020,2	2002,002	2000,2	2000
P. tricornutum culture (ml):		5	5	5	5	5
Volume Total:		2227	2025,2	2007,002	2005,2	2005
Light intensity (nm):		710	710	710	710	710
Temperature (ºC)		5	5	5	5	5

Optimization of growth was assured by the use of a good medium and high light intensity. The experimental setup was designed to ensure that the major limiting factors on the growth of *P. tricornutum* would be the result of possible contaminants from the Produced Water. The only limiting factor on growth-rate is self-shading with increased cell-counts, and together with turbidity this is taken into account in the *Discussion*. Other than self-shading, the medium and high light intensity is assumed to provide enough nutrients and energy for maximum growth throughout the growth period.

Overall, the goals of this experimental design was to be able to establish a fast growth of *P. tricornutum*, and examine the effects of Produced Water on this growth early. It was also a goal for this experiment to be easy and cheap, so one person could perform it over a short time, and on a small budget.

2.2 Sampling and preparation

The Produced Water sample was collected at the oil refinery at Mongstad, Norway. On-site technicians, who were following a sampling-procedure provided by Me, performed the sampling. The sample was collected at an “entry-point” where the sample would most closely resemble the produced water released on an offshore facility. This means that the water was not stored for days/weeks/months, or treated before sampling. The sampling procedure was as follows:

A 5L High-density Polyethylene (HD-PE) tank was filled almost up to the rim of the tank, to reduce oxygen-sample interaction. It was then shipped overnight in a sealed container with cooling elements. This ensured that neither light nor heat would affect the sample during the overnight shipping. This procedure was designed after consulting papers surrounding multiple similar experiments (Thomas et al., 2003, Brendehaug et al., 1992, Dórea et al., 2007).

In the laboratory, the sample was kept at 5 ºC before utilization. The sample was carefully mixed before use, by turning the tank upside down multiple times, but without excessive use of force.

The medium was prepared using a standard medium recipe for the laboratory at the Trondhjem Biological Station, which was derived from a standard F/2 medium recipe (*See Appendix 1)*. Seawater was collected from the Trondheim Fjord by underwater cable. The seawater was filtered, and sterilized by autoclaving overnight. It was cooled down to room temperature before the addition of the media stocks. The finished medium was stored at 5 ºC before utilization, and kept sterile by covering the top of the tank (*See Appendix 1 for full recipe*).

Inoculation took place the same day as the Produced Water sample arrived at the laboratory. The medium was distributed into the 15 LD-PE tanks, and the correct levels of Produced Water was added to create a medium with the correct PW concentration (*See Table 1*).

After addition of PW, 5 ml of concentrated *P. tricornutum* culture was added to each tank. The tanks were set up so that equal light intensity hit each tank. This was made possible by the use of sheets of paper to cover the lamps, and distribute the light more even throughout the room where the growth experiment was performed (*See Appendix 3*).

The choice of HD-PE over Glass-, Amber-, or Teflon-tanks for transport and LF-PE for the growth-experiment, was made after consulting literature, surrounding sampling of oil-waste (Thomas et al., 2003, Brendehaug et.al. 1992). In relation to Diatom growth, Glass and Amber-glass was deemed not fit due to its ability to react with the sample, and releasing metals and silicates into the medium during the experiment. The release of silicates into the sample could positively interact with the diatom growth rates. Teflon, although preferred by some scientists and laboratories, was deemed not fit for this experiment due mainly to its price, and to the fact that LD-PE and HD-PE has been proven not to have a huge impact, especially regarding overnight transport, cooled sample during transport, and the short growth time of the experiment (*See Appendix 2*).

Day 0 results were collected by measuring pH, Turbidity, and In Vivo fluorescence in small samples from each exposure group before addition of *P. tricornutum*.

2.3 Produced Water chemical composition

Statoil ASA perform bi-weekly tests on the chemical composition of the Produced Water where the PW sample was taken from. This data was supplied to me, and protected under a non-disclosure agreement. However, the average of some components from 1 year of testing was allowed to be published with this study, and is presented in *Figure 1.*

There is however, no information about how the chemical composition was determined, so no further procedures or details regarding these tests are presented or discussed. However, the results are taken into account when discussing possible toxicity in the Discussion. It is assumed that the results from the chemical composition tests are reliable, and that the described content of the Produced Water sample is sound.

2.4 Chlorophyll a-analysis

The Chlorophyll a-analysis was performed using a standardized method (Mackinney, 1941). Each day, 1 tank from each exposure group (chosen at day 1, based on proximity to average within the exposure group) was tested for Chlorophyll a-levels. At day 1, 100 mL from each tank was filtered using water pressure, and the filters were dry-frozen until the analyses could be performed. After day 1, as the concentration of cells increased, less water could be filtered before the filter filled up. The reduced amount of filtered water was taken into account in the calculations.

Chlorophyll a was extracted from the filters by adding 5 mL 85% Acetone, leaving them over night, before re-filtrating the extract through a 0.45 µm syringe-filter. The filtrated extract was then measured for absorbance in a spectrophotometer. The spectrophotometer was used instead of a fluorimeter (which is normally used), as the samples had levels beyond the fluorimeters range of detection. (Mackinney, 1941).

The amount of Chlorophyll a in each extracted sample was calculated using the following formula:

Formula 1 - $$\mu g\ chl\ a / L = ((Abs665 - Abs750)\ E*1000)/\ (74.5*L*F)$$

Where Abs665 and 750 is absorbance measured at 665 nm and 750nm in a spectrophotometer. E equals the extraction volume in milliliter, while F was the filtered amount in Liter. L is the length-way of light in the cuvette, normally 1 cm. The data was finally calibrated against a 0-test, and presented in *Figure 2.*

2.6 pH-analysis

The pH-analysis was performed using a new, but standardized pH-meter. The pH-meter was calibrated each day before testing, using a built-in multi-point calibration procedure with 3 buffer solutions (pH 4, 7, 10). Each day, pH-levels were measured for all tanks in all exposure groups, and the results are presented in *Figure 4.*

2.5 Turbidity

Turbidity (Absorbance) was tested using a standard spectrophotometer at 750 nm light intensity. The absorbance measured at 750 nm was calibrated against a 0-test consisting of distilled H2O. Turbidity was tested for all tanks in all exposure groups every day. It was measured using a standard 50 mm quartz cuvette, which was washed well with water and distilled water between each use. The results from the turbidity tests are presented in *Figure 3.*

2.7 Fluorescence analyses

The fluorescence tests was performed using the AquaPen-C designed by Photon System Instruments. It is a handheld fluorimeter, where you can perform many tests using a 10 mm cuvette. It is equipped with a Blue LED emitter, which is optimal for algal cultures (Photon Systems Instruments, 2015). All the tests performed using the AquaPen was done by adding 5ml of the sample to a quartz cuvette after dark adaptation, in a dimly lit room. Some of the tests are light sensitive, to a less or more extent, so depending on the tests and sample, dark adaptation times varied.

Instantaneous Chl. Fluorescence was measured by adding 5ml of sample (before dark adaptation) to a cuvette and running the Ft program on the AquaPen. This program runs for a few seconds, before the number stabilizes and the result is presented as fluorescence yield (Ft) or minimum (actual) fluorescence (Šlapakauskas and Ruzgas, 2005). The results from these tests are presented in *Figure 5.*

Fluorescence Quantum Yield (F-QY) was measured by adding 5ml of sample (after dark adaptation) to a cuvette and running the QY program on the AquaPen. The program runs for a few seconds, before the number stabilizes and the result is presented as Fv/Fm, which is an estimation of potential Photosystem II efficiency (Kitajima and Butler, 1975). The results from these tests are presented in *Figure 6.*.

Photosynthetic Capacity is a measure derived from a study suggesting a relationship between low Fluorescence Response Index (FRI) and diminished Photosynthetic Capacity (Cullen and Renger, 1979). It is of special interest to this study because other studies have suggested a good correlation between DCMU-induced fluorescence and photosynthesis levels (Samuelsson and Öquist, 1977). These relationships are more fully discussed regarding the pH results in *Chapter 3.2: Algal growth*, and in the Discussion).

The Photosynthetic Capacity is derived from the FRI, which is calculated as a ratio between minimum fluorescence and a DCMU-induced increase in fluorescence. It is performed using the same method as for Instant. Chl. Fluorescence above, but with the addition of another similar test where you add a few drops of DCMU (*3-(3,4-dichlorophenyl)-1,1-dimethylurea*) to the sample before testing. DCMU is a known photosynthesis inhibitor, and interrupts the photosynthetic electron transport chain. The addition of DCMU ultimately provides a visible increase in fluorescence, which is a picture of the absorbed light energy normally used for photosynthesis.

The ratio between Minimum or Actual fluorescence, which is presented above, and the DCMU-induced fluorescence can be presented as a Fluorescence Response Index, which can be related to Photosynthetic Capacity, and hence also Photosynthesis. The FRI is presented in *Figure 7*, and represents the DCMU-induction ratio.

2.8 Presentation of results

The results from all tests are presented as graphs, where the trend lines represent the average between the triplicates of each exposure group. The graphs from all tests except Chlorophyll a also include error bars, which are determined from the standard deviation between the triplicates. All the results have been corrected against Day 0 results and versus 0-controls like dH_2O.

Chapter 3. Results

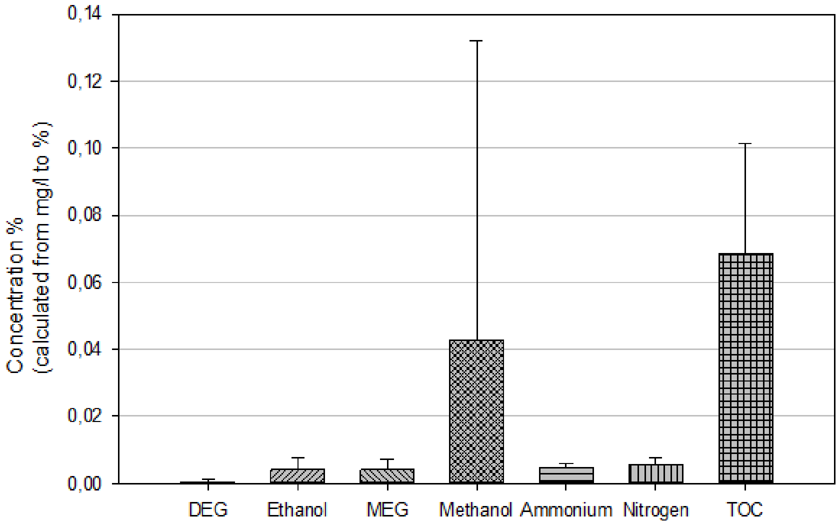
3.1 Chemical composition of Produced Water sample

The Produced Water sample used for this project is tested for chemical composition 2 or 3 times a month by an independent laboratory on order from Statoil ASA. The data for 1 year of these tests were supplied, and have been presented in *Figure 1*. I did not perform the tests, and the details of the results or analyses cannot be disclosed in this report (*See Materials & Methods*). However, the averages from 1 year of test results show two predominant chemical components, which are of interest to this project, Methanol (CH_3OH) and Total Organic Carbon (TOC).

Methanol content is important for growth of diatoms, due to its inhibitory and stimulatory effects on biomass production. Dewes et al. 2003 suggests that the effects of methanol on growth is dependent on concentration and exposure time (Dewez et al., 2003). Methanol can act as a very effect solvent for organic molecules including Chl-a, which means that a high methanol concentration can affect buildup of Chl-a and other vital organic molecules in the cells (Dewez et al., 2003). From the results of the chemical analyses, we note that the average concentration of Methanol in this Produced Water source is 0.42%, with a 0.89% possible deviation. This means that this source of PW can have a maximum concentration of ca 1.3%.

Total Organic Carbon (TOC) content is also important for growth of diatoms, as it can affect uptake of CO2 and Dissolved Inorganic Carbon, and directly affect Photosynthesis, and hence also growth (Goldman et al., 1971). The TOC concentration in the Produced Water source is on average 0.68%, with a standard deviation of 0.32% giving a possible max of 1%.

Figure 1: Chemical Composition of Produced Water Sample from Mongstad, Norway.

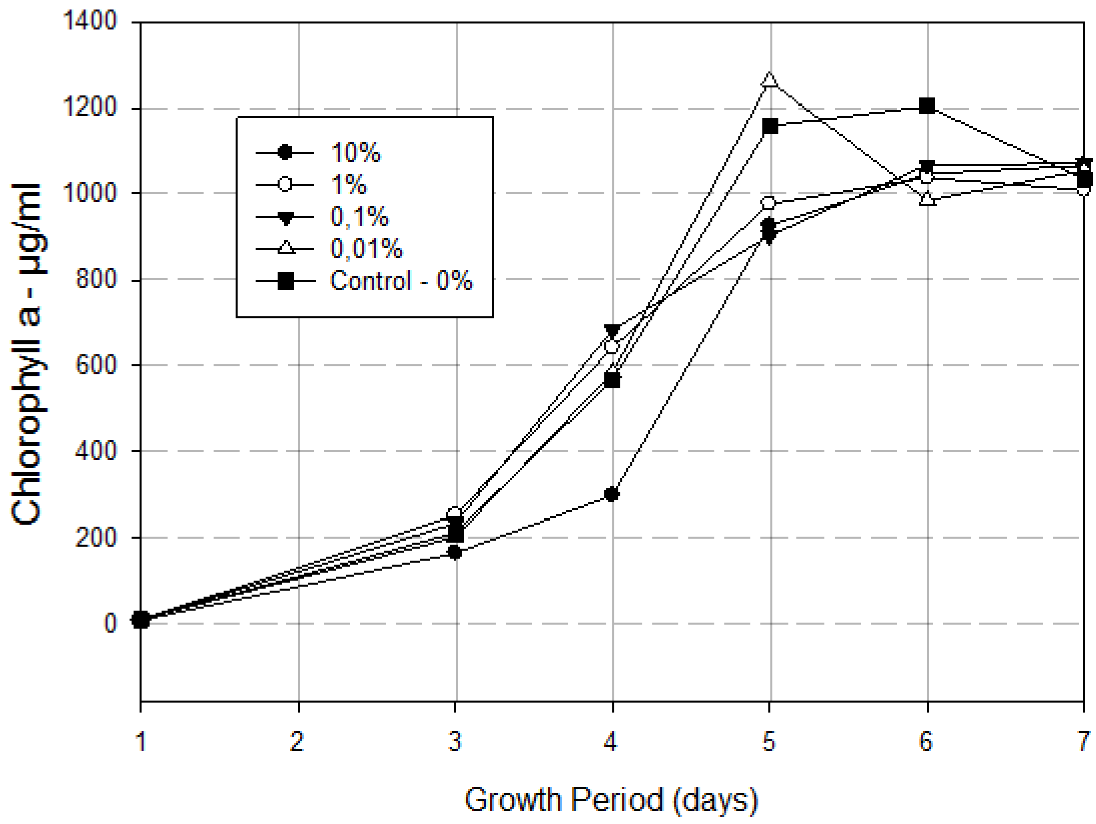


3.2 Algal Growth (Chlorophyll a, Turbidity and pH)

Many of the tests performed in this project can indicate Algal Growth. I have chosen the results from the Chlorophyll a (Chl-a), Turbidity and pH tests to describe the bigger picture of the results which is further discussed in the Discussion.

Chlorophyll a (Chl-a) content in µg/ml from each exposure group is presented in *Figure 2*. Chl-a can be used as an indication of algal growth, and is often used to determine the "health" of a body of water, and predict or monitor deadly algal blooms. However, studies also suggest that Chl-a is not a good indicator for biomass (Ramaraj et al., 2013). I therefore use the Chl-a concentrations presented in *Figure 2*, to assess if the growth presented in *Figure 3* and *Figure 4*, is an actual representation of *P. tricornutum* growth, and not contamination of organic or inorganic matter, or an indication of other chemical reactions than photosynthesis.

Figure 2: Chlorophyll-a results from growth experiment on *P. tricornutum* in Produced Water

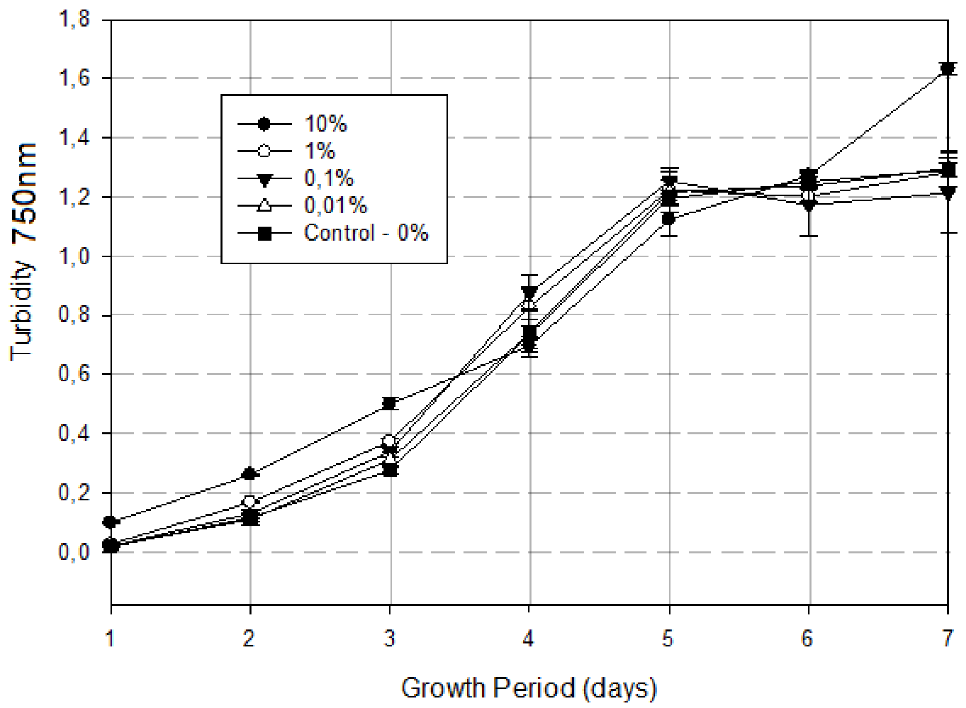


In Figure 2, we see that the Chl-a content follows a growth-trend, which indicates a slow exponential growth with a carrying capacity indicated around Day 5 at concentrations between 900-1200 µg/ml.

The highest Chl-a concentrations are present at Day 5, in exposure groups 0.01%-PW and Control. This maximum is similar to a traditional "overshoot" of carrying capacity (Whittaker and Likens, 1973). It is worth noting that all exposure groups hits the same carrying capacity at around 1050-1100 µg/ml at the end of the experiment, and that the level at days 3 and 4 indicates a slightly lower Chl-a content in exposure group 10%-PW. Chl-a content of each exposure group is also an indication of the algal health, which is presented in *Section 3.3*, and more deeply discussed in the *Discussion*.

Turbidity in each exposure group is presented in *Figure 3*, as Turbidity (absorption) at a wavelength of 750 nm. Turbidity indicates algal growth by giving a representation of light-absorbing matter, or particle concentration in a sample. The results in Figure 3 was presented after correcting each result against a standard (distilled water), and Day 0 levels of turbidity (0.05 in 10%-PW, and 0.002 in 0.01%PW) to account for the turbidity in the added Produced Water.

Figure 3: Turbidity results from growth experiment on *P. tricornutum* in Produced Water



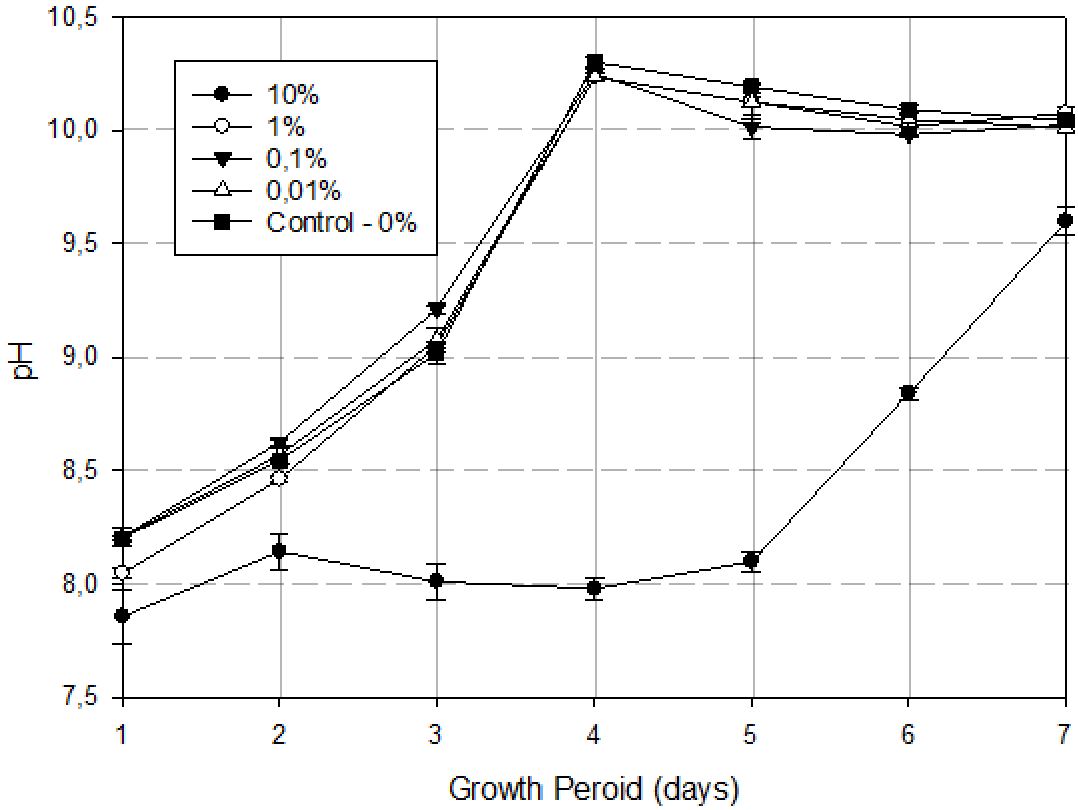
The results from the turbidity tests show a similar trend to that of Chl-a, and the visible correlation between these results suggest that growth of *P. tricornutum* in each exposure group can be indicated by either of the results.

However, the 10%-PW exposure group (indicated in figure 3 by black-dots/solid-line), continues to increase after the final day of growth. The other 4 exposure groups show a carrying capacity, and stabilization of turbidity/growth at day 5 around a Turbidity of 1.2. This can be an indication that particles, which are not representing cells with Chl-a, is present and growing in the 10%-PW after Day 6.

pH-levels in each exposure group throughout the experiment is presented in *Figure 4*. The results were corrected with pH-levels from 10%-PW/media and 0%-PW/Media, to account for potential pH-increases by higher PW concentrations. The pH-levels presented in figure 4 is among the most exciting results from this experiment. The pH results together with Photosystem II (PSII) efficiency suggest that there are multiple underlying reactions within the culture, both performed by the algae and the media+PW combination, which are affecting each other.

pH is widely used as an indicator for Algal growth in Aquatic and Marine environments because, pH in a culture changes with phytoplankton uptake of carbon for photosynthesis (Axelsson 1988, Hofslagare et al. 1985). This means that the pH results in this experiment can be discussed in relation to both diatom growth presented in this section, and photosynthetic activity/health presented in *Section 3.3*.

Figure 4: pH results from growth experiment on *P. tricornutum* in Produced Water



From *Figure 4*, we can see that Exposure groups 1%,0.1%,0.01%, and 0%-PW show a similar trend of exponential growth, and stabilizes at Day 4 around pH 10.2-10.3. This is not in correlation with the trends presented in figure 2 and 3, which indicated a stabilization (carrying capacity) around Day 5. Even more interesting is the fact that exposure group 10%-PW shows a slow almost stabilizing trend until Day 5, before growing linearly upwards until the end of the experiment, where it ends around pH 9.5-9.6. at Day 7. An explanation of what is shown in *Figure 4* is the addition of H-ions from the PW. Together with the removal of H-ions caused by the uptake of CO_2 reach steady state around Day 2, and this steady state is probably changed around Day 5 as the amount of photosynthesis remove more H-ions than what was added with the PW at Day 0.

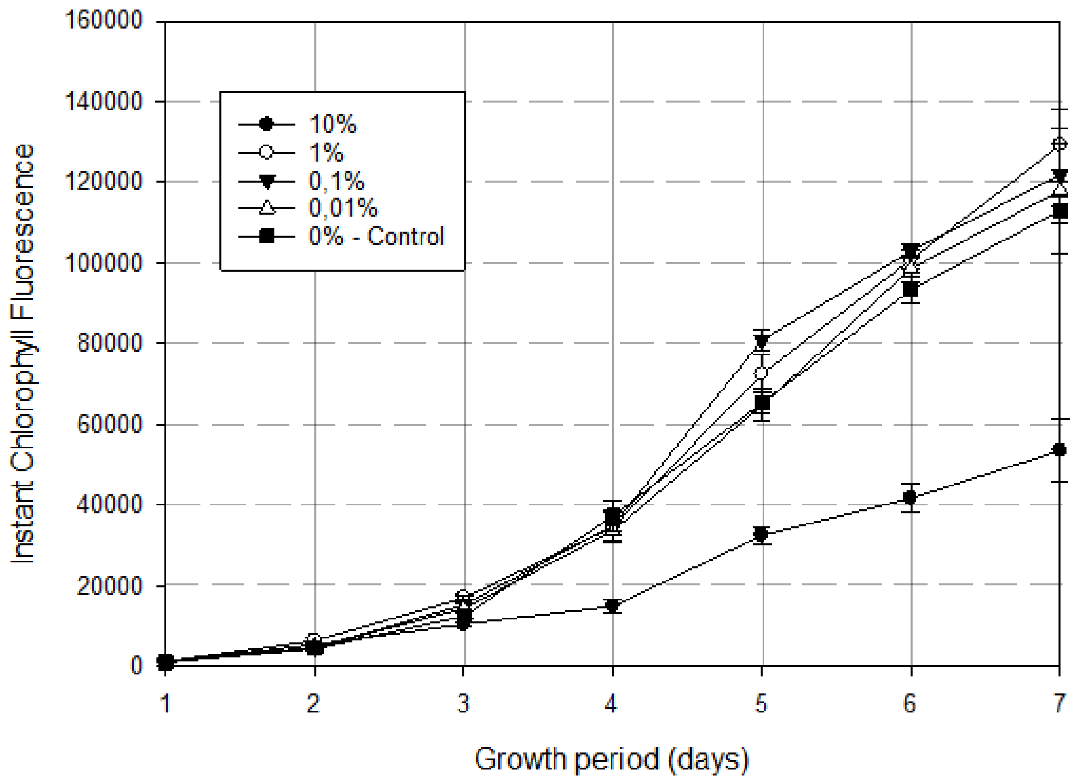
A short summary of this section is that the results from the Chl-a and Turbidity tests indicate a slow semi-exponential growth from Day 1-5 with a stabilization at Day 5. The pH results indicate that 4 of the exposure groups (from 0-1%-PW) growth exponentially to Day 4 before stabilizing (if pH changes are assumed to be an indicator of growth), while the highest exposure group (10%-PW) does not show any high increase in pH before Day 5 where it linearly grows until the end of the experiment. This growth could be indicated in the turbidity tests where the 10%-PW exposure group continues to increase after day 6. Finally, it is worth noting that the 0.01%-PW exposure group along with the control-group seem to “overshoot” the carrying capacity based on the Chl-a test at Day 5 but ultimately stabilizes along with the rest.

3.3 Fluorescence (Instant. Chl-Fluorescence, Fluorescence-Quantum yield, Photosynthetic Capacity)

Since the pH results show a different trend compared to the Chl-a and Turbidity tests, the fluorescence tests presented in this section are meant to give further indication of what happens, in relation to photochemistry, in the exposure groups. Based on Photosynthetic activity, during the growth period. The term “Photosynthetic Activity” is based, mostly on assumptions and iteration from references, more than actual calculations and definitions. This concept coincides with my choice to focus more on the big picture in the results, and less on calculations and statistics.

Instantaneous Chlorophyll Fluorescence was measured using the PSI-AquaPen-C (See *Materials and Methods*), and indicates steady-state yield of fluorescence in light (Maxwell and Johnson, 2000). The results are presented in *Figure 5*, and was corrected against a standard (Distilled Water). *Figure 5* shows a slow trend from near-zero to 110000-130000 at the end of the experiment, for the four lowest exposure groups (1%-0%-PW). *Figure 5* also shows a slower increase from near-zero to ca. 55000 for the 10%-PW exposure group, which may indicate a higher rate of photochemistry, or even a lower cell count. The Instant. Chl. Fluorescence was used to for calculations in the Photosynthetic Capacity. The Instant Chl. Fluorescence also shows that there is photosynthetic activity in the 10%-PW exposure group, which means that the pH results showing a H-ion steady state must be the reason for the lack of pH increase, and not growth of non-photosynthetic cells. This finding supports the theory that there are growing cells which are producing Chlorophyll a, and performing photosynthesis, but that the H-ion steady state hinders the pH increase normally seen with photosynthetic activity.

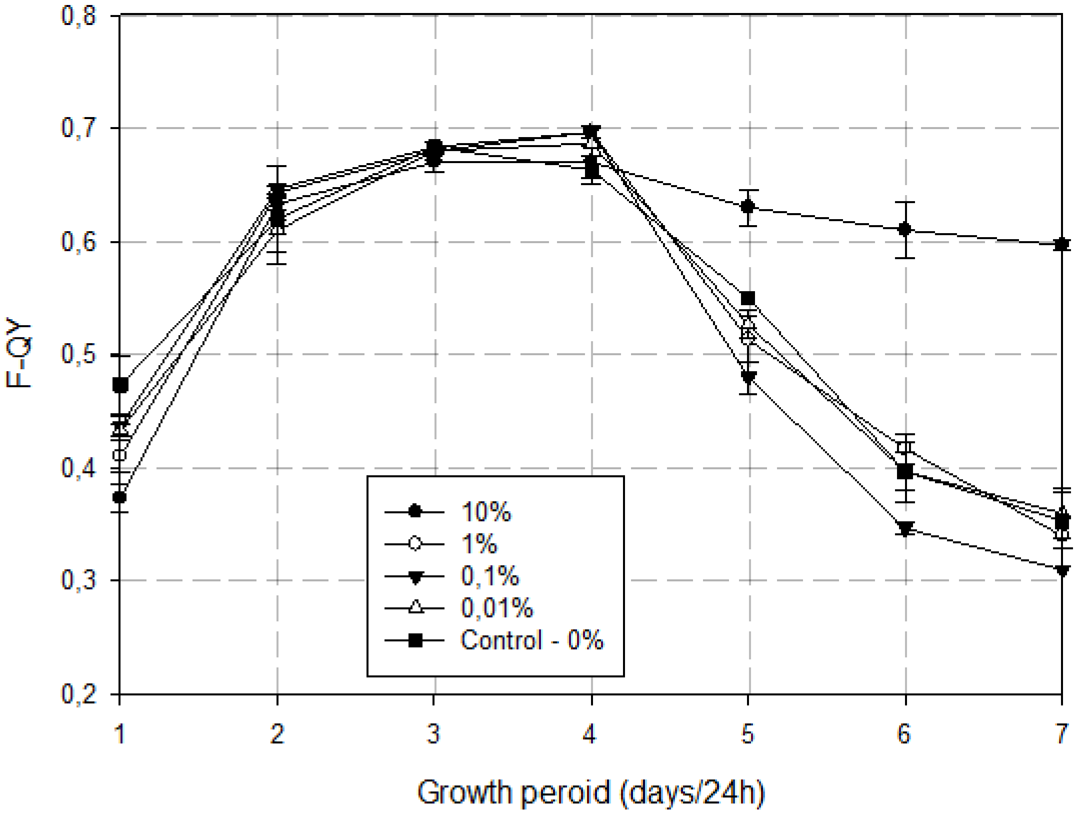
Figure 5: Instantaneous Chlorophyll Fluorescence (measured as Intensity) from growth experiment on *P. tricornutum* in Produced Water



Fluorescence-Quantum Yield (F-QY) was also measured using the QY-program of the PSI-AquaPen-C. F-QY is an indication of Photosystem II (PSII) efficiency, and can indicate; levels of damage on PSII or levels of Quenching by light-damage or chemical inhibition (Maxwell and Johnson, 2000). The results are presented in *Figure 6*, and was corrected against a standard (Distilled Water). *Figure 6* shows that F-QY increases strongly from day 1 to day 2, and then increase slower towards day 3 and 4, with a maximum of around 6.5-7. At day 5, F-QY has decreased to around 0.47-0.55 for the lowest exposure groups (1%-0%-PW), and only decreased to about 6.2 for the 10%-PW exposure group. All groups decrease further until the end of the experiment, but while the four lowest exposure groups decrease all the way to 3.1-3.7, the 10%-PW exposure group only decrease to 0.6. All exposure groups seem to be stabilizing at these final levels, although further testing would have given a clearer picture of this. The four lowest exposure groups decline from day 4 to day 7, to a level lower than Day 1. This may indicate that stress (possibly related to high cell-counts/competition), or damage to PSII can have affected these exposure groups. However, F-QY can also, during laboratory experiments, give a measure of linear electron transport, and indicate overall photosynthesis (Maxwell and Johnson, 2000).

F-QY can be related to carbon fixation during photosynthesis in PSII. From this we can suggest that the slower decline in F-QY seen in the 10%-PW exposure group can be correlated with the pH-increase happening from Day 5 (*See Section 3.2*), although this is an interesting suggestion, it is only a speculation which will be discussed fully in the Discussion

Figure 6: Photosystem II Efficiency presented as Fluorescence Quantum Yield (F-QY) from growth experiment on *P. tricornutum* in Produced Water



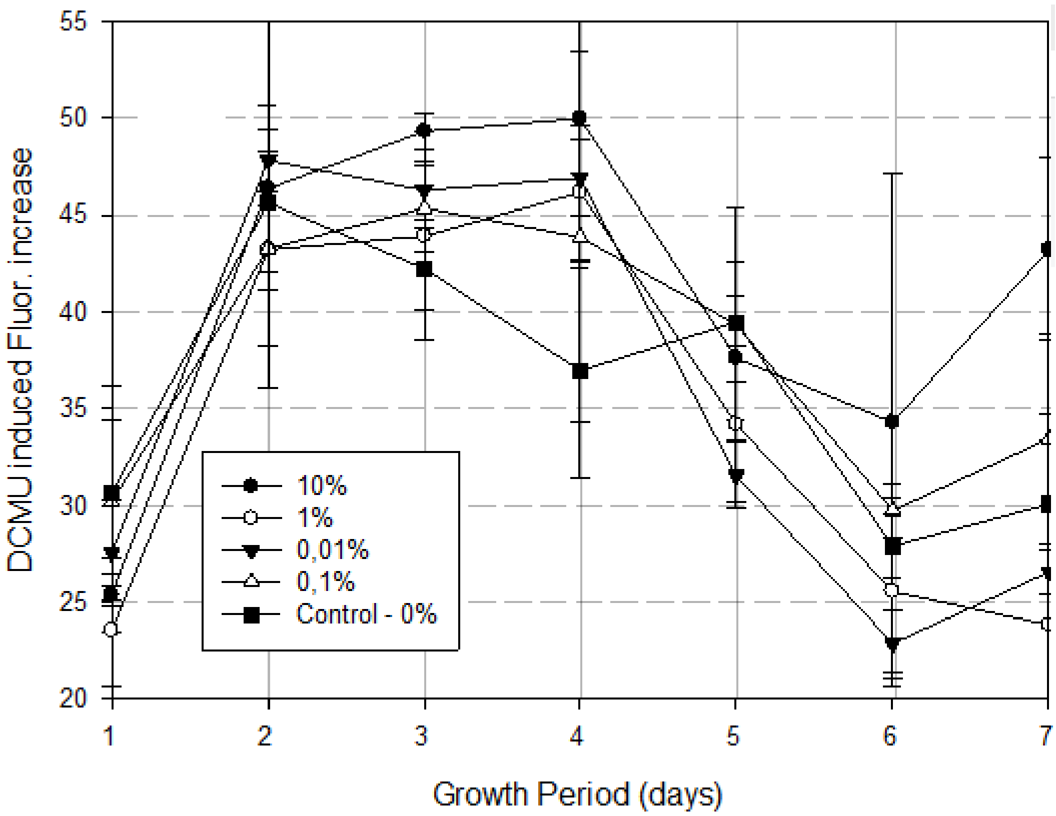
Photosynthetic Capacity is another parameter, which can indicate the health of a phytoplankton cell. In this experiment, Photosynthetic Capacity is iterated from a Fluorescence Response Index, which is based on DCMU-Induced increase in fluorescence. The results were based on the Instant. Chl. Fluorescence, before and after addition of DCMU to the sample. The results from these analyses are presented in *Figure 7*.

DCMU-Induced increase in fluorescence can indicate the levels of cells, which are currently actively performing photosynthesis (Cullen and Renger, 1979). Cullen and Renger suggests that a DCMU-induced increase in fluorescence can be linked to the health of a phytoplankton culture, and indicate the level of non-photosynthetic material or “dead cells” in a culture.

From *Figure 7,* we found that all exposure groups follow almost the same trend. They increase rapidly from day 1 to 2 up to a level of 43-48, and then stabilizes (more or less in the same fashion) until day 4/5, before the all decrease until day 6. The results show many scattering results, high deviations and errors, and no real dose-dependency.

However, Maxwell and Johnson, as well as Cullen and Renger mention that these kind of tests have many variables, and require a near perfect experiment-setup and performance (*See Chapter 2) (*Maxwell and Johnson 2000, Cullen and Renger 1979). This is a reasonable explanation for the high standard deviations. However, it is still possible to see a good trend where all exposure groups follow a similar trend, except the 10%-PW which plateau at a higher level around day 3 and 4, and ends the experiment at a significant higher level than the rest of the exposure group. This result, together with the F-QY, suggest that the Photosynthetic activity in the 10%-PW exposure group become better towards the end of the experiment, compared to the rest of exposure groups, which seem to fall off in both capacity and quantum yield.

Figure 7: Photosynthetic Capacity presented as DCMU Induced Fluorescence increases from growth experiment on *P. tricornutum* in Produced Water



3.4 Visual and Physical observations

I want to point out, for further reference, multiple visible and sensible changes throughout the experiment. The main visible change was a change of color in all 15 tanks. The change of color was distinct from clear to brownish water (*See Appendix 3*). This change was parallel to an increased amount of downfall, presumably from organic matter. In addition to the visible changes, there was a distinct “gasoline”-smell in the 10%-PW tanks.

This smell, although project to bias, seemed to reduce in power through the growth period. Although this observation is strictly based on my previous visual experiences, it is assumed that the change of color throughout the experiment could be a good indication of growth in all 15 tanks. The slight lighter color visible in tanks 1-2-3 (Appendix 3) could also be an indication of reduced growth in these tanks, although this is also speculation.

The reduction of “gasoline”-smell present in the highest 10%-PW exposure group could be an indication of volatile BTEX components escaping through the seal, as the plastic seal on a tank is not airtight. The change in smell could also be an effect of *P. tricornutum* absorbing, chemically altering, or removing BTEX compounds and possibly oil-droplets, from the media, hence reducing the amount of gases given off during sampling. It is again worth noting that these results are based on observations and not quantifiable. However, they are discussed further in the Discussion.

Chapter 4. Discussion

4.1 Toxicity of Produced Water

Toxicity of Produced Water was assessed, by comparing the dose-response in the results, with the chemical composition of the Produced Water sample. From the results, presented in *Chapter 3. Results*, no visible dose-response between all 5 concentrations was found in any of the tests. However, the 10%-PW exposure group deviates from the rest of the exposure groups in all tests (to some degree).

From earlier studies (presented in the Introduction) it is suggested that many of the chemicals used in the petroleum industry, and other components found in PW, can have toxic effects in high concentrations on higher marine organisms, especially mussels and fish (Meier et al., 2010, Brooks et al., 2011). However, both Meier et al. 2011 and Brooks et al. 2011 suggest that the highest effect of PW discharge is detected in organisms located close to the point of discharge, which in relation to this study represent the 10%-PW exposure group. Previous studies focus mainly on effects of Alkylphenols (APs), Heavy Metals, and Aromatic Hydrocarbons (AHs) including BTEX compounds (Benzene, Toluene, Ethylbenzene and Xylenes). Levels of Heavy Metals, BTEX cmpounds, and APs can be extracted from Statoil Reports including composition of Produced Water effluents from North Sea petroleum activities (*See Introduction*).

Alkylphenols have shown effects on the endocrine and reproductive systems in fish and other vertebrates (Meier et al., 2010, Brooks et al., 2011). These effects are based on the chemistry of APs, and for example their ability to mimic effects of sex hormones and inhibit or induce endocrine- or reproductive processes. In phytoplankton a study on 4-Nonylphenol, a compound in the AP family, showed different effects on phytoplankton cultures, based mainly on species and concentration (Hense et al., 2003). A similar but longer study on the ecotoxicology of 4-nonylphenol mixtures included a diatom (*Melosira Sp.*). However, in the study, a species of fathead minnows was also studied, and the only publications from this study are based on the effects on the fish. The effects on the diatom remains unpublished, but taken into account in this study. This support the hypotheses that organisms in higher trophic levels remain the focus of studies and publications.

The study does mention one result from the diatom part of the experiment. It states that the results suggest that 4-nonylphenol alters the Lipid : Chlorophyll a ratio in the phytoplankton cells (Schoenfuss et al., 2004-2006).
The Lipid : Chlorophyll a ratio is an indicator of stress, where both the production of Chlorophyll a and storage/depletion of lipids can indicate different types of stress-coping mechanisms. In relation to the current study, the Lipid : Chlorophyll a ratio is interesting to discuss, because the pH results (*Figure 4*) indicate low carbon uptake (which can be an indication of lipid storage, growth rate etc.), while the chlorophyll a (*Figure 2*) and Instant Chl. Fluor. (*Figure 5*) results indicate a lower growth rate in the 10%-PW exposure group. It is also interesting to relate the knowledge of APs effects on Lipid : Chlorophyll a ratios to the turbidity results (*Figure 3*), because turbidity can be an indication of increased particulate lipids, increased cell size (increased lipid-storage), or light permeability of cell membranes, all of which can be related to the lipid : chlorophyll a ratio. Sadly, the publications from the mentioned study only focus on the results from the freshwater fish species. The correlations mentioned in this section regarding the diatom part of the study, was derived from speculation and should therefore be considered as assumptions, more than conclusions or suggestions.

Heavy Metals have shown effects on growth, duration of log phase, and motility in phytoplankton. A study focusing on a mixture of heavy metals, also found results suggesting how different concentration of multiple heavy metals in mixtures can lead to both synergistic and antagonistic growth inhibitory effects on phytoplankton, including *P. tricornutum* (Thomas et al., 1980). The effects of heavy metals on the more delicate processes inside of phytoplankton cells are unknown or poorly studied, which makes relating heavy metal content to results from the specialized Fluorescence tests (F-QY and PS Capacity) difficult to justify. However, as heavy metals, both singular and in mixtures, have shown toxic effects on phytoplankton it is interesting to relate the study from Thomas et al. 1980 to the lower growth rates shown in the Chlorophyll a and Instant. Chl. Fluor. tests. Another study focusing on precipitation of heavy metals, suggest that heavy metal concentrations alone does not correlate with toxicity (growth inhibition), but this study does mention heavy metals aggregate to larger particles (Azetsu-Scott et al., 2006). Although this study by Azetsu Scott et al. 2006 suggest that heavy metals alone exempt no toxicity towards phytoplankton, and hence works against the hypotheses suggested by Thomas et al. 1980 it does state a need for more precise measurements.

The results from Azetsu-Scott et al. 2006 do suggest another hypotheses; that the turbidity results, which suggest a similar growth rate in all exposure groups, may be affected by increased particulate matter. Since the 10%-PW exposure group could have more particulate matter in the form of oil droplets or other agglomerates, the results suggest that the heavy metal content in the 10%-PW exposure group could increase turbidity by heavy metals aggregating to these larger particles, and affecting the turbidity results.

BTEX compounds found in effluents related to the petroleum industry have shown both inhibitory and inducing effects on phytoplankton growth. A study focusing on the effects of BTEX mixtures, and especially mixtures containing volatile hydrocarbons, showed the different effects on 4 species of microalgae (Dunstan et al., 1975). The study performed by Dunstan et al. 1975 show that a diatom (*Skeletonema costatum)* showed no growth enhancement in any mixture or concentration, but showed an inhibitory effect in low concentrations of the mixture containing volatiles. As the current study was performed using a screw-capped plastic bottle, and not plastic-capped glass bottle as the Dunstan et al. 1975 study, a suggestion might be made towards the pH results, showing a delayed growth in the 10%-PW exposure group, being a result of volatile hydrocarbons leaving the culture through gaps in the plastic screw-cap cover. As the volatile hydrocarbons leave the culture, the inhibitory effect of low-concentrations shown by Dunstan et al. 1975 might be reduced. However, the BTEX compound, which showed the biggest influence on the diatom from Dunstan et al. 1975, was Xylene, which we from the Åsgard 2012 Statoil report know to be one of the lesser BTEX compounds released. The results from this study on the BTEX compounds effects on diatoms support the hypotheses that growth is inhibited in 10%-PW, but to a lesser extent that APs and Heavy Metals. The BTEX levels in produced water can also be related to particle matter, Total Organic Carbon from the Chemical Composition tests, and turbidity, but any direct toxicity from BTEX is not assumed present in this study. As mentioned before, the results part of this study show that the pH results deviate the most from the other tests. The 10%-PW exposure group show a delayed change until day 5 (*Figure 4*), which should suggest a delayed growth. The Instant Chlorophyll Fluorescence results suggest a slower growth rate (*Figure 5*), and the Chlorophyll a results show a similar, but less distinct, effect around day 4 (*Figure 2*). This comparison support the theory that *P. tricornutum* growth is slower in the 10%-PW exposure group, which would be an indication of the toxicity of produced water suggested by the compounds above.

The results which indicate similar growth rate, and in some instances higher growth rate, in the 10%-PW exposure group consist of; F-QY (*Figure 6*), Photosynthetic Capacity (*Figure 7*), and Turbidity (*Figure 3*). However, the F-QY and Photosynthetic Capacity results are based on ratios between two data points, and not direct quantifiable numbers. This means that these results only give an indirect indication of actual growth, and instead indicate photosynthesis efficiency (or algal health). The turbidity results, although commonly used to assess algal growth, can also be highly influenced by cell-structure, cell-size, contaminants (for example oil-droplets dispersed in water), and dead organic matter, a theory that is supported by the toxicity assessments above. This means that although the turbidity results support a similar growth rate in all exposure groups, it is reasonable to assume that the results are affected by many confounding factors.

In summary, the results, although dependent on the assumptions made above, suggest a slower growth rate in the highest concentration 10%, but as the 1%-PW exposure group show no visible effects no dose response between 0% to 1% was found. It is therefore assumed that the produced water tested in this experiment have toxic effects on the growth of *P. tricornutum* in the highest environmentally relevant concentration (10%-PW). This suggestion is mainly based on the Instantaneous Chlorophyll Fluorescence, and pH results. It is however interesting to note that although the pH of the 10%-PW exposure group is negative and almost stagnant between day 2 and 4, the rest of the results suggest slow but visible growth. This suggestion that growth occur in all exposure groups, while pH stays stagnant in the 10%-PW exposure group, is supported by physical/visual observations made during the experiment (*See Section 3.4).* This suggest that either; Growth occur in the 10%-PW exposure group without the use of CO2 between day 2 and 4, or that compounds from the Produced Water act as a pH buffer negating the expected pH changes normally observed with algal growth at a concentration level of 10%.

4.2 Produced Water Impacts on Marine Phytoplankton

Produced Water impacts on Marine Phytoplankton was brought up in the early hypotheses of this study. The question was if and how, the results from this experiment, and from earlier and future studies, could be related to the actual impacts produced water have on marine phytoplankton in the natural environment. In *Section 4.1*, the toxicity of produced water suggested by the results from this experiment is discussed. In this section however, the discussion centers on how produced water is released, and react, in the environment.

Finally, the discussion focus on determining how the marine phytoplankton *P. tricornutum* can be related to communities of phytoplankton in the actual environment.

Release of Produced Water occur, as discussed in the introduction before, during, and after the production part of an industry. The Produced Water, which this study focus on, is from the Petroleum Industry in the North Sea. For the results in this study to be relatable to the natural environment, it is necessary to discuss how the exact sample of PW used in this experiment relate to the PW actually released into the ocean. The PW, which was sampled at Mongstad, was collected at a point of entry, where (according to sources in Statoil ASA) the produced water would be the best representation of the water discharged on an offshore facility. Knowing this, it is still a few important points to consider when relating the results to the actual environment. First, is the time the produced water sample used in the oil-pipelines, where it endured different temperatures and interaction with metals and sedimentation from the pipes themselves. The time spent in the pipeline is unknown, but it is assumed that both interaction with oil, oxygen, and perhaps seawater, is natural in those conditions. The PW that is released into the ocean from and offshore facility will, in most cases, undergo a treatment process before discharge. This process focus on removing the biggest oil-particles, as this is where the legislation is focused (Durell et al., 2006). The PW used in this experiment could therefore be assumed to contain more oil-droplets, which has diffused into the water from interaction with the oil in the pipelines, and has not been removed through a treatment facility. The PW used in this experiment will also have had a longer interaction with oxygen (if present in pipes), and some reactions could have changed the composition and chemistry of the Produced Water. However, it is still assumed (based on the statements from Statoil employees), that the produced water used in this experiment is relatable to the water discharged offshore, as long as the differences mentioned above is taken into consideration.

Phaeodactylum tricornutum was chosen for this experiment, because of its reliability, its ability to growth fast and in many different media/mixtures. The fact that *P. tricornutum* also have been studied thoroughly was important for this study. As mentioned in *Section 4.1*, there was a study by Dunstan et al. 1975, which studied effects of BTEX components of petroleum origin on 4 different microalgae. The results from this experiment shows that there is a species difference in toxicity of PW (Dunstan et al., 1975). This species difference was suggested based on toxicity of Alkylphenols (Hense et al., 2003). It is therefore important to examine how *P. tricornutum* relate to a community of phytoplankton, and discuss how the results from this experiment can be an indication of possible effects PW has on the marine environment. This study suggest that PW as a mixture has a small effect on algal growth, if the growth is assessed by Chlorophyll a levels and Instant Chlorophyll Fluorescence. This study also suggest that CO2 levels are not changing in the highest concentration exposure group until later in the experiment based on the pH results. As both Instant Chlorophyll Fluorescence and pH are results derived from chemical reactions related to photosynthesis within the cells of the phytoplankton it is possible to suggest that PW discharged from an offshore oil-facility will have an effect on the *P. tricornutum* community located close to the point of discharge. However, since the results from this experiment is based on PW as a total mixture, while earlier studies focus on different components of PW it is hard to relate the results seen in this experiment to the results of the other studies. It is also hard to determine how other species would react to the PW used in this study. The question related to Produced Water impacts on a natural phytoplankton community is therefore based on the theory behind a mixture of chemicals and pollutants, and the shown variance in composition and chemistry of Produced Water. The suggestion from this study as an experiment, and a literature review, is therefore that Produced Water as a mixture of many components can provide both important nutrients and be a source of toxic chemicals to marine phytoplankton. The results from many of the tests in this study can therefore be of importance when discussing the legislation on Produced Water effluents, not only from the petroleum industry, but when discussing any discharge of water used in production.

4.3 Presentation and Statistics

Earlier in the study, it has been mentioned that the lack of statistics and the simplicity of the results was important to me. The results part of the study starts by showing the chemical composition of Produced Water throughout a year of testing. The discussion sections above also focus heavily on the lacks of this study, and how it is hard to relate results from this study and previous study to the natural environment, and actual effects of PW on phytoplankton communities. The reason statistics are used in scientific experiments is to be able to visualize data, and to be able to find hidden correlations between different results and different tests. In this study, each single test is its own result. Each graph presented in Chapter 3 can be a picture of growth, and in many studies only one of these tests are used to determine growth. It is therefore not important to this study that each graph and each data point be statistically analyzed against each other. It is more important that the big pictures each graph show is discussed in related to what each test actually mean. As an example from this study, it is not the growth rates between days 1 and 2 that is important. It is not how the pH on day 4 relate to the Chlorophyll a levels on day 6. The study is not looking for correlations between each test, but is trying to show a simple graph, and then relating what this graph actually show to results from previous studies.

Another important part of statistics in scientific studies is the ability to reproduce the results, and for other scientists to be able to relate their data to the results from this experiment. However, as mentioned before the Produced Water used in this experiment was the PW arriving at Mongstad at the day of sampling. If the sampling was delayed 2 weeks, it is possible that the results from some of the tests in this experiment would be different. Questions that may rise when discussing the variability of Produced Water discharge can be; how any experiment using produced water can be relatable to the actual natural environment? Alternatively, how can anyone working with produced water or marine phytoplankton can use the findings of this study in their discussion? The simple answer is that I would not recommend any use of any data points or results from this study directly in discussion of other studies. However, if one use other sources from similar studies, and have a wide knowledge of the differences between those studies, the results from this study, especially regarding photosynthetic activity and pH, can be of great use to future studies surrounding PW or any industrial effluent, in regards to algae.

The results from this study also show the importance of focusing on the chemical reactions leading up to a result in a test, and not only looking at the data point from the test itself and especially not after the data points have been subjected to many different statistical programs.

4.4 Further Studies

Many improvements can be made to a study like this. With more funding and more time, it would be possible to look at many parameters of phytoplankton growth, like CO2 uptake, cell count, cell size, and perhaps the uptake of oil- or heavy metal-particles in the phytoplankton cell. This study can in sort, be seen as a pilot study on phytoplankton and produced water. The results of this study give an indication of possible effects, but many factors must be taken into account, and more parameters could be studied. The results also suggest the possibility of Produced Water influencing reactions within the cells of the diatoms, especially related to photosynthesis. Possible future studies based on the results of this experiment could be:

Checking for bacterial growth within the culture. Even though actions were taken to try to keep the experiment from being contaminated from bacteria, it is possible that there would be bacterial growth throughout the experiment, which could affect the results.

Test of PW effects on a phytoplankton community. As mentioned earlier in the discussion, the environmental relevancy of this experiment is lacking due to the choice of only 1 phytoplankton species. If one were to examine the effects on a community scale, it could be possible to understand more deeply how PW affect the natural environment of marine phytoplankton.

Perform a similar experiment in a larger scale to examine the results, which suggest that the 10%-PW exposure group seem to be doing better than the others towards the end of the experiment. With the addition of PW, a lot of possible nutrients and toxicants were added, and as shown by the experiment in this study, a longer study on a bigger scale would give more results that are more detailed and possibly show more trends that are interesting after Day 7.

The discussions brought up in this study can also be important for further discussions surrounding the increasing use of statistics, and the importance of discussing all factors within a study as a whole, from planning phase to conclusions.

Chapter 5. Conclusion

The findings of this study on Produced Water effects on marine phytoplankton was presented in *Chapter 3*, and discussed in *Chapter 4*. The goals of the study, have been examined throughout both the experiment and the literature review. The natural concentrations adopted from Meier et al. 2011 did not show any dose-response, other than a few tests giving very interesting results for the highest concentration 10%-PW. The goal was to examine if these natural concentrations would have any effect on *P. tricornutum*. It is possible to say, based on the literature review and comparison to the results from this study, that in high concentrations of PW the natural community of marine phytoplankton could be affected. The highest concentrations do however represent a low environmentally relevant radius around an offshore facility. The pH results differ greatly from the rest of the tests, and together with the Photosystem II Efficiency (PSII) and Chl-a data, a suggestion can be made that *P. tricornutum* is growing from Day 1, but the addition of 10%-PW creates a H-ion steady state which is causing the pH to stay low, while growth continues. At Day 4, the pH rises, and the PSII efficiency in the 10%-PW exposure group does not fall with the other groups to a level lower than Day 1. This could support the theory that the H-ion steady state is holding off the expected pH increase. The results of the Instant Chl. Fluorescence and F-QY together with this pH trend, could suggest that the 10%-PW exposure group is actually doing better than the other groups, towards the end of the experiment. This is a theory, which should be studied further, and could have importance related to PW treatment and Bio-Fuel production. The final goal is intertwined in the methodology of the study and the final discussions surrounding presentation and statistics. The small conclusion presented in *Section 4.4* shows that a simple experiment with a cheap and highly modifiable methodology can have many possibilities for future studies.

Overall, the study takes the form of a pilot study, and the results are interesting and provides multiple points to think about and discuss. This study shows interesting results regarding pH changes in relation to industrial effluents, and the photosynthetic rate and efficiency of marine phytoplankton in polluted waters. This shows the importance of looking more deeply at the lowest parts of the food chain.

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Appendix 1 – F/2 Medium Recipe

f/2 Medium

Stocks per liter

(1) $NaNO_3$ 75g
(2) $NaH_2PO_4.2H_2O$ 5.65g
(3) Trace elements (chelated)
NA_2 EDTA 4.16 g
$FeCl_3.6H_2O$ 3.15 g
$CuSO_4.5H_2O$ 0.01 g
$ZnSO_4.7H_2O$ 0.022 g
$CoCl_2.6H_2O$ 0.01 g
$MnCl_2.4H_2O$ 0.18 g
$Na_2MoO_4.2H_2O$ 0.006 g
(4) Vitamin mix
Cyanocobalamin (Vitamin B_{12}) 0.0005 g
Thiamine HCl (Vitamin B_1) 0.1 g
Biotin 0.0005 g

Medium per liter
$NaNO_3$ 1.0 ml
$NaH_2PO_4.2H_2O$ 1.0 ml
Trace elements stock solution (1) 1.0 ml
Vitamin mix stock solution (2) 1.0 ml

Make up to 1 liter with filtered natural seawater. Adjust pH to 8.0 with 1M NaOH or HCl. For agar add 15g per liter Bacteriological Agar. Sterilize by autoclaving for 15 minutes at 15 psi and use when cooled to room temperature.

Appendix 2 – HD-PE Resistance Charts

HDPE Chemical Resistance Chart

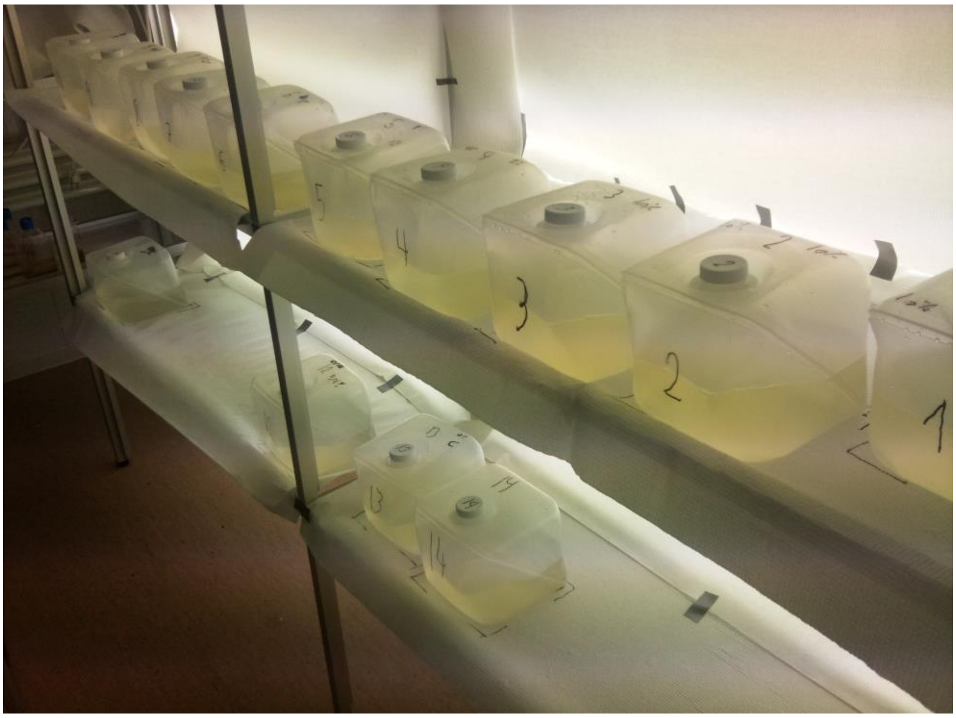
Chemical Name	Resistance Level 20oC(68F)	60oC(140F)	Chemical Name	Resistance Leve 20oC(68F)	60oC(140F)
Acetaldehyde	R	R	Carbon disulphide	R	NR
Acetic acid (10%)	R	R	Carbonic acid	R	R
Acetic acid (glac./anh.)	R	R	Carbon tetrachloride	NR	NR
Acetic anhydride	R	R	Caustic soda & potash	R	R
Acetone	R	R	Cellulose paint	R	R
Other ketones	R	R	Chlorates of Na, K, Ba	R	R
Acetonitrile	R	R	Chlorine, dry	LR	NR
Acetylene	R	R	Chlorine, wet	LR	NR
Acid fumes	R	R	Chlorides of Na, K, Ba	R	R
Alcohols	R	R	Chloroacetic acid	R	R
Aliphatic esters	R	R	Chlorobenzene	NR	NR
Alkyl chlorides	R	R	Chloroform	NR	NR
Alum	R	R	Chlorosulphonic acid	NR	NR
Aluminium chloride	R	R	Chromic acid (80%)	R	NR
Aluminium sulphate	R	R	Citric acid	R	R
Ammonia, anhydrous	R	R	Copper salts (most)	R	R
Ammonia, aqueous	R	R	Cresylic acids (50%)	R	R
Ammonium chloride	R	R	Cyclohexane	NR	NR
Amyl acetate	R	R	Detergents, synthetic	R	R
Aniline	R	R	Emulsifiers, concentrated	R	R
Aromatic solvents	R	NR	Esters	ND	ND
Ascorbic acid	R	R	Ether	R	R
Beer	R	R	Fatty acids (>C6)	R	R
Benzaldehyde	R	ND	Ferric chloride	R	R
Benzoic acid	R	R	Ferrous sulphate	R	R
Boric acid	R	R	Fluorinated refrigerants	R	NR
Brines, saturated	R	R	Fluorine, dry	NR	NR
Bromide (K) solution	R	R	Flourine, wet	NR	NR
Butyl acetate	R	LR	Fluorosilic acid	R	R
Calcium chloride	R	R	Formaldehyde (40%)	R	R

Chemical Name	Resistance Level 20oC(68F)	60oC(140F)	Chemical Name	Resistance Level 20oC(68F)	60oC(140F)
Formic acid	R	R	Mercuric chloride	R	R
Fruit juices	R	R	Mercury	R	R
Gelatine	R	R	Methanol	R	NR
Glycerine	R	R	Methylene chloride	LR	NR
Glycols	R	R	Milk products	R	R
Glycol, ethylene	R	R	Moist air	R	R
Glycolic acid	R	R	Molasses	R	R
Hexamethylene diamine	R	R	Monoethanolamine	ND	ND
Hexamine	R	R	Naptha	NR	NR
Hydrazine	R	R	Napthalene	R	ND
Hydrobromic acid (50%)	R	R	Nickel salts	R	R
Hydrochloric acid (10%)	R	R	Nitrates of Na, K and NH3	R	R
Hydrochloric acid (conc.)	R	R	Nitric acid (<25%)	R	R
Hydrocyanic acid	R	R	Nitric acid (50%)	R	NR
Hydrofluoric acid (40%)	R	R	Nitric acid (90%)	NR	NR
Hydrofluoric acid (75%)	R	R	Nitric acid (fuming)	NR	NR
Hydrogen peroxide (30%)	R	R	Nitrite (Na)	R	R
Hydrogen peroxide (30 - 90%)	R	NR	Nitrobenzene	NR	NR
Hydrogen sulphide	R	R	Oils, diesel	R	NR
Hypochlorites	R	R	Oils, essential	R	NR
Hypochlorites (Na 12-14%)	R	R			
			Oils, lubricating + aromatic additives	R	R
Iso-butyl-acetate	ND	ND	Oils, mineral	R	R
Lactic acid (90%)	R	R	Oils, vegetable and animal	R	NR
Lead acetate	R	R	Oxalic acid	R	R
Lead perchlorate	ND	ND	Ozone	R	LR
Lime (CaO)	R	R	Paraffin wax	R	R
Maleic acidR			Perchloric acid	R	R
Manganate, potassium (K)	R	R	Petroleum spirits	R	R
Meat juices	R	R	Phenol	R	R
Phosphoric acid (50%)	R	R	Phosphoric acid (20%)	R	R

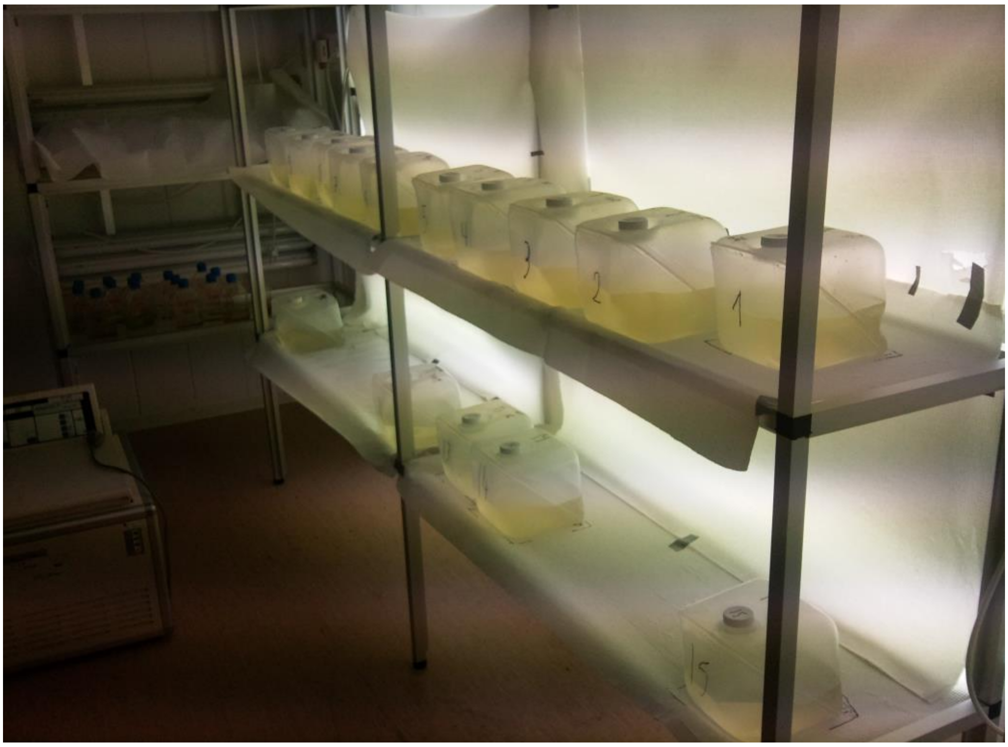
Appendix 3- Growth Experiment Pictures

Appendix 3.1 – Day 0: all 15 tanks with light setup and slight coloration in tanks 1-3.

Appendix 3.2 - Day 2: all 15 tanks with coloration in all tanks, but slightly less in tanks 9-15



Appendix 3.3 - Day 4: All 15 tanks visible growth based on coloration, almost similar coloration in all 15 tanks.



Appendix 3.4 – Day 5: All 15 tanks with great coloration in tanks 4-15, while tanks 1-3 have more bottom waste and less coloration.

Appendix 3.5 – Day 7 (End of experiment): All 15 tanks with coloration and bottom waste, but with reduced coloration in tanks 1-3.

Pictures Taken by Hans Henriksen Marki before sampling throughout the experiment.