Temperature dependence of naphthalene biodegradation in seawater (0.5-15ºC)

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Abstract

The polar areas are very vulnerable to exposure to contaminants. An oil spill in polar areas could be a disaster to large areas. The recovery from contamination takes longer time in arctic areas than in more temperate areas due to the low temperatures.

In this research a relationship between growth factor (k_1) and temperature for bacterial degradation of naphthalene in seawater is being found. Different analyses was utilized to monitor degradation: Chemical analysis for substrate concentration, automated OxiTop® method for BOD monitoring, and DAPI bacterial cell counting.

The consistency between the results from the different analysis methods was mostly good, even for the biological analysis. But the bacterial cells were very different in size, which made it difficult to make good estimations of biomass concentration in the system. The k_1 value was found to be $0.021d^{-1}$ at 0.5ºC, $0.035d^{-1}$ at 4ºC, $0.056d^{-1}$ at 8ºC and $0.112d^{-1}$ at 15ºC. A relation between k_1 and temperature (t) can then be written like this: $k_1 = 0.0002t^2 + 0.0029t + 0.0196$. These results gives some information regarding how much faster the naphthalene biodegradation process goes at high than at low temperature within the temperature area of the research.

Acknowledgements

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Contents

1. Introduction

Petroleum is a great energy source, which has benefited billions of people, especially during the last hundred years. Petroleum are making life easier in areas like transportation and heating. But the use of fossil fuels creates a lot of trouble. Most of the problems are problems that we who burn fossil fuels pile up for future generations. One such problem is related to energy. It is the problem that the modern society utilizes fossil fuels in a tempo that by far exceeds the rates by which such fossil fuels are being created. And it is simple mathematics that it is impossible that such over exploitation of natural resources can continue.

Another concern related to the great use of petroleum in the society of today, is the fear that extensive release of carbon dioxide to the atmosphere could change the climate and cause a lot of trouble.

However, the main focus of this research is on the challenges that petroleum components may cause when they are released to the marine environment. While there are several sources to release of oil to sea, 70% of all oil released from the petroleum industry in Norway originates from produced water [24]. There are more environmental hazardous chemicals that have been released to the sea in other ways, but the largest quantities of oil released from the production units of the Norwegian petroleum industry, are being released with the produced water.

The petroleum industry are searching for petroleum in new areas. Many of the old wells are soon depleted. The world`s population does also have a growing demand for energy. Total population is rapidly growing, and huge nations are experiencing a financial boom.

The need for energy and the desire for money push the petroleum industry into more harsh climatic condition. Even arctic areas are experiencing growing activity from the petroleum industry. Arctic ecosystems are very vulnerable to chemical contamination. Low temperatures causes partition coefficients from atmosphere to rise dramaticly, resulting in additional transportation of organic contaminant to arctic ecosystems. [25]

Biological processes progress slower at low temperature, including the biodegradation of contaminants.

This research is about temperature dependence of naphthalene biodegradation in seawater. Naphthalene is a quantitatively important fraction of produced water. It is a toxic chemical, but not among the most toxic compounds found in petroleum. The main objective is to find a relationship between the growth rate and the temperature for bacteria growing on naphthalene as its sole carbon source in seawater.

2. Background and theory

2.1 Petroleum well compositions

Crude oil is believed to originate from dead organisms that were trapped in cavities millions of years ago. The age estimates vary between reservoirs and regions.. In addition to the age, many other properties also vary between different reservoirs and reservoir-zones. For example pressure, temperature, pH, and the native chemical composition of the organic matter that was trapped. Because of this, the crude oil that is being produced from each well has its distinct composition, and there can be significant differences. Also each crude oil is a complex mixture of hydrocarbons and other organic compounds, including organometallic constituents (primarily vanadium and nickel complexes). [17] Some wells produce primarily heavy, asphalt-like, oil, while other wells produce mainly gas condensate. Some wells produce a large portion of aromatic organic molecules, while others produce mainly straight chain alkanes. According to Marshall and Rodgers, more than 20000 distinct elemental compositions of organic molecules had been found in different crude oils in 2004 [5]. And the number of distinct, documented organic species is still growing. The different well streams do also have different contents of various inorganic substances.

Although the diversity is great between wells, there are some trends in chemical composition of crude oil based on the geographical location. For example North sea crude oils are generally lighter than Middle-East crude oils. But neighbouring wells may also have very different chemical composition.

2.2 Polycyclic aromatic hydrocarbons (PAHs)

2.2.1 PAHs in general

Hydrocarbons are molecules that consist of hydrogen and carbon. Aromatic hydrocarbons are hydrocarbons that are based on aromatic rings with delocalized double bonds. Delocalized double bonds mean that electrons are moving from place to place in a molecule, forming temporary double bonds. This is also called a

resonance structure. Unsaturated organic molecules are normally quite reactive, but an aromatic structure changes the reactivity of molecules, making them unusually stable. [2]

Most aromatic molecules are composed of one or more six-membered benzene ring. The simplest six carbon ring aromatic hydrocarbon is called benzene. The six carbon atoms in the benzene molecule have three single bonds and two double bonds between them. But the double bonds are delocalized, which means that the electrons making the π-bonds continuously moves to other positions. This gives benzene two "stable" resonance structures, as shown in figure 1:

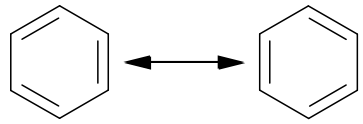


Figure 1: *Benzene resonance structures.*

Aromatic hydrocarbons that consist of more than one aromatic ring are called polycyclic aromatic hydrocarbons. They are built up of aromatic rings that are fused together.

Different PAHs are known to be carcinogenic, mutagenic and teratogenic, in addition to their acute toxicity. But the toxicity varies very much, even among PAHs that are very similar in chemical structure and molecular size. There are some characteristics related to the chemical structure of the most hazardous PAHs, but toxicity tests for relevant species are needed to conclude over the ecotoxological threat that a specific PAH may pose, if released in the nature.

Benzo [a] pyrene is one of the most potent carcinogens among the PAHs. And it was among the first chemical carcinogens to be discovered.[2, 4] When PAHs are biodegraded, the first step are normally to make them more water soluble by oxygenation, catalysed by oxygenase enzymes. Oxygenation makes sense, as it makes the molecules more available for further biotransformation. Because its easier for microorganisms to get in an attack that can break up ring structures and carbon

chains when the molecules are oxygenated. But in higher organisms, higher water solubility is also a property of the molecules that could make excretion happen more easily. Because a more water soluble molecule will not be as much attracted by an organisms tissue as a less water soluble one.

But in the case of benzo [a] pyrene, the oxygenising can lead to bioactivation of the molecule, and produce a DNA-adduct, that will bond to DNA and disturb normal replication. In this way cancer can develop. Figure 1 shows the transformation of benzo [a] pyrene via diol epoxide to DNA-adduct. The “bay-structure” on the upper left corner of the benzo [a] pyrene molecule contributes in making the molecule a more potent carcinogen than for example pyrene. [4,7]

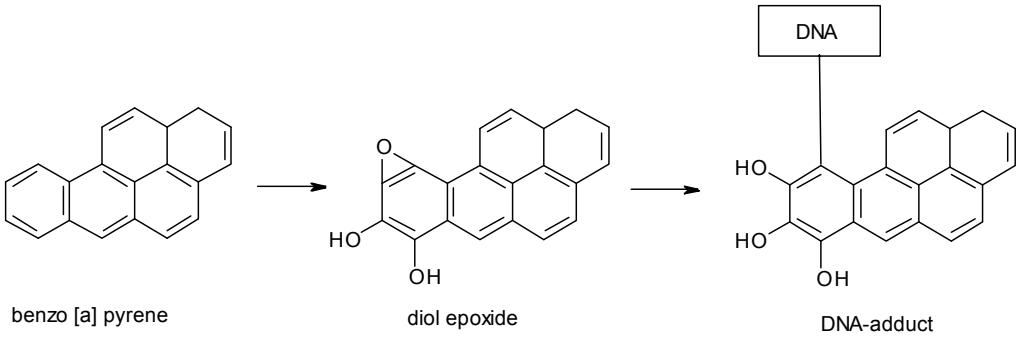


Figure 2: *Simplified scetch of the transformation from benzo [a] pyrene to DNA-adduct.* [2]

Benzo [a] pyrene and similar sized PAHs are often generated as a result of uncomplete combustion of organics. The PAH-fraction of crude oil does normally primarily consist of lighter PAHs (although heavier PAHs are found at lower concentrations). The lightest PAHs are generally not as carcinogenic as the more heavy PAHs. Therefore PAH induced carcinogenic effects in the nature are normally more related to industry sites with heavy combustion of fossile fuels than to direct releases of petroleum, e.g. from petroleum production or transportation units. [7]

Produced water from offshore petroleum production units is a major source of the pollution from petroleum release. For production at Norwegian oilfields there have been put strong limitations on the amount of dispersed oil that is allowed in the produced water, with a maximum limit of 30 ppm. [16] But there are also some

remainders of petroleum that are truly dissolved in the water phase, not included in the dispersed oil limitation. The dissolved petroleum in produced water is primarily the crude oil components that are most soluble in water. And the single component concentration decreases with decreasing water solubility, in addition to other factors like the concentration of each component in the relevant crude oil. Like other organic molecules that do not have polar groups, PAHs have very low solubility in water. Big PAHs are even less water soluble than the small PAHs. Therefore the PAH-fraction of produced water has lower mean molecular weight than the PAH-fraction of crude oil. Naphthalene is one of the smallest PAHs, and it is therefore one of the quantitatively important petroleum constituents in produced water.

2.2.2 Naphthalene

Naphthalene consists of two benzene rings *ortho*-fused together [3], and is therefore a very small molecule compared to the other PAHs. (*Ortho*-fusion means that two aromatic rings have two carbons and one bond in common.) The molecular formula of naphthalene is $C_{10}H_8$. Figure 2 shows the chemical structure of the naphthalene molecule.

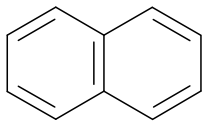


Figure 3: *Naphthalene molecular structure.*

Naphthalene has a molecular weight of 128.19g/mole. It appears as a white, crystaline solid at standard conditions. It has a boiling point of 218ºC, and a melting point of 80.2ºC. Naphthalene is quite volatile, and when it sublimes it leaves a strong aromatic smell. The odor threshold is about 0.038ppm. Since naphthalene is both volatile and quite toxic, it should be kept under a lid, or a vent as much as possible, to minimize inhalation. It is also important to keep any container of naphthalene closed to minimize loss off the chemical under lab research.

Figure 4 shows the NFPA 704 color code for napthalene. NFPA 704 is a color code for identification of hazardous materials. The numbers range from 0 to 4, where 4 is the most extreme. The blue square symbolizes toxicity, the red flammability, and the yellow instability/reactivity. The white square is for a special notice, but it is not used in the case of naphthalene. [8]

Figure 4: *NFPA 704 color code for napthalene.* [8]

As the digit 2 in the blue square of figure 2 indicates, naphthalene is a quite toxic compound. In fact all chemicals are toxic when in sufficient concentrations or amounts, but the chemicals that are normally regarded as “toxic”, are chemicals that are able to cause harm even at low level of exposure. The digit 2 indicates that naphthalene is not among the most toxic chemicals, but it is toxic. Naphthalene MSDS shows that oral consumption of naphthalene gives a 50% lethal dose (LD50) at 490mg naphthalene/kg body mass for rats, 533mg/kg for mouse and 1200 mg/kg for guinea pig. Exposure to vapourised Naphthalene in atmosphere has given an acute LC50 of 170 ppm for 4 hours for rat. [8]

A research on the marine copepod *Paracartia grani* from Barcelona, Spain, by Calbet et. al. (2006) found that that naphthalene concentration needs to be high (2.5mg/l) to reach LD50 on acute toxicity tests.

The sciencelab.com MSDS for naphthalene states this about potential acute health effects: “Very hazardous in case of ingestion. Hazardous in case of eye contact (irritant), of inhalation. Slightly hazardous in case of skin contact (irritant, permeator). Severe over-exposure can result in death.”[8]

When it comes to chronic health effect caused by naphthalene, there is no available evidence of neither carcinogenic, nor mutagenic, nor tetratogenic effects of

naphthalene. But both blood, kidneys, the nervous system, the reproductive system, liver, mucous membranes, gastrointestinal tract, upper respiratory tract and the central nervous system can become damaged by repeated or enduring exposure to naphthalene [8]

Naphthalene is among the important components of the water soluble fractions of crude oil and of fuel oils. [10] Leakage of fuel to water and soil is a problem of ecotoxicological concern. [13]

Napthalene is not a hydrophilic molecule. But because of its small size, and its aromatic structure, it is much more soluble than many of the other petroleum components. Therefore naphthalene is relevant when it comes to environmental considerations related to produced water from petroleum production.

2.3 Biodegradation

2.3.1 Petroleum exposure and the environment

Natural environments have been introduced to petroleum exposure in times long before man started its first utilization of petroleum products. Transformation of biological matter, driven by pressure, heat and time, has created “petroleum molecules” in rock and soil. And petroleum that is not trapped in completely sealed cavities has continuously been seeping out into different natural environments. Sealed bodies of petroleum has also naturally been released as a result of earthquakes and more slow going movement of tectonic plates or more local movement or cracking of rock and soil. Since water is heavier than petroleum, water may also float under and around natural traps that keeps petroleum enclosed. Two phase diffusion between oil phase and water phase can be a way for petroleum molecules to be released, for example to sea, if the water phase is less isolated than the oil phase.

Bacteria that are able to degrade petroleum components are broadly distributed in nature. Genes that catalyze degradation of petroleum compounds is believed to have evolved in nature adaptively as a response to natural seepage of petroleum.

Microbial degradation is the main mechanism in ecological recovery of contamination from PAHs [11] and other petroleum components. But the degradability is very different among the different oil components. The most easily degradable components available are degraded first. When the concentration of the most easily degradable components gets so low that such compounds are not available in sufficient amounts, the bacteria starts degrading compounds that are less degradable. The most easily degradable components of crude oil are the short chain n-alkanes, then follows isoalkanes and higher molecular weight n-alkanes, olefins, and then aromatic compounds, with the aromatic compouds with lowest molecular weight as the most easy degradable. The petroleum components that are most difficult for microorganisms to break down are asphaltenes, resins and highly condensed cycloalkanes. [15]

Some bacteria can utilize low molecular weight PAHs as their only carbon source. Naphthalene is only a diaromatic hydrocarbon, and is therefore readily degraded compared to the other PAHs.

Drill cuttings that are contaminated by different drilling fluids are regarded as the main source of contamination of the marine environment from the Norwegian petroleum industry. Especially cutting piles from the early days of Norwegian offshore petroleum production have high concentrations of environmentally harmful chemicals. The Norwegian pollution control authority (SFT), have made a system with color codes to classify chemicals used in the petroleum industry with regard to environment pollution potential. The system has got four colors: black, red, yellow and green, where the most hazardous chemicals are labeled black, the second most hazardous, red, the chemicals that are only slightly toxic, yellow, and the chemicals regarded as non-hazardous, green. The petroleum industry has agreed to stop or minimize the use of black and red chemicals as a result of the target of zero harmful discharge of pollutants. The discharge of chemical hazard additives has been reduced from 4161 tonnes to 24 tonnes per year between 1997 and 2007. Therefore cutting piles from drilling of newer wells are far less contaminated than the older ones. It should also be mentioned that the petroleum industry now contributes less than 3% of the total discharge to sea of the chemicals on the SFT priority list [16].

The discharge of petroleum components with produced water is now the main focus of Norwegian authorities when it comes to environmental concerns related to discharge from the offshore petroleum industry. There has been a lot of research and discussion on environmental issues related to produced water in Norway. But the final conclusions has yet to be made about to what extent the release of produced water that is released from offshore petroleum production units in Norwegian waters is harmful to the environment. The Institute for Marine Research (Havforskningsinstituttet) in Bergen has found that produced water has both lethal and sublethal effects on cod larvas and eggs at unrealisticly high concentrations (1% and 4% produced water in seawater). But the research showed no effect at more realistic concentrations of produced water in a production area (0.1% and 0.01%). [14]

2.3.2 Different approaches to biodegradation

Biodegradation is removal or transformation of a chemical compound that involves living organisms. But there are different ways to approach biodegradation. One could define the biodegradation as plainly the removal of a compound involving microorganisms. But such a point of view would exclude the focus on further transformation of the compound. It would only focus on the first step of biological uptake of the compound. This is the focus of the analyst when he uses a chemical analysis that measures the concentration or mass of a substrate in a biodegradation research.

Another approach to biodegradation is to regard all the steps between the undegraded compound and full mineralization as biodegradation. Mineralization is the end-station of biodegradation, where organic molecules have been turned into inorganic carbon (mainly CO_2). The sum of all the steps involved in the biodegradation, is called a biodegradation pathway. [23]

2.3.3 Naphthalene biodegradation pathway

The stability of the aromatic ring structure of the PAHs means that microorganisms have to utilize metabolic strategies that can overcome the high activation energy that is needed to split up a ring. The biological degradation system that is normally used

in degradation of naphthalene and other low molecular weight PAHs is called the napthalene dioxygenase system. Naphthalene dioxygenase is a multicomponent enzyme that generally includes an NADH oxioreductase, a ferredoxin, and an oxygenase component that contains the active site. [11]

Figure 5 shows the naphthalene biodegradation pathway from naphthalene to catechol and gentistate. Catechol is well known as a “crossroad metabolite”, which reflects that it is a well-known intermediate that appears in many different metabolic pathways. Gentistate is a central intermediate in tyrosine metabolism. The further degradation of gentistate splits in two directions, of which one goes through fumarat to the citric acid cycle, and the other goes through pyruvate to the pyruvate metabolism. [6]

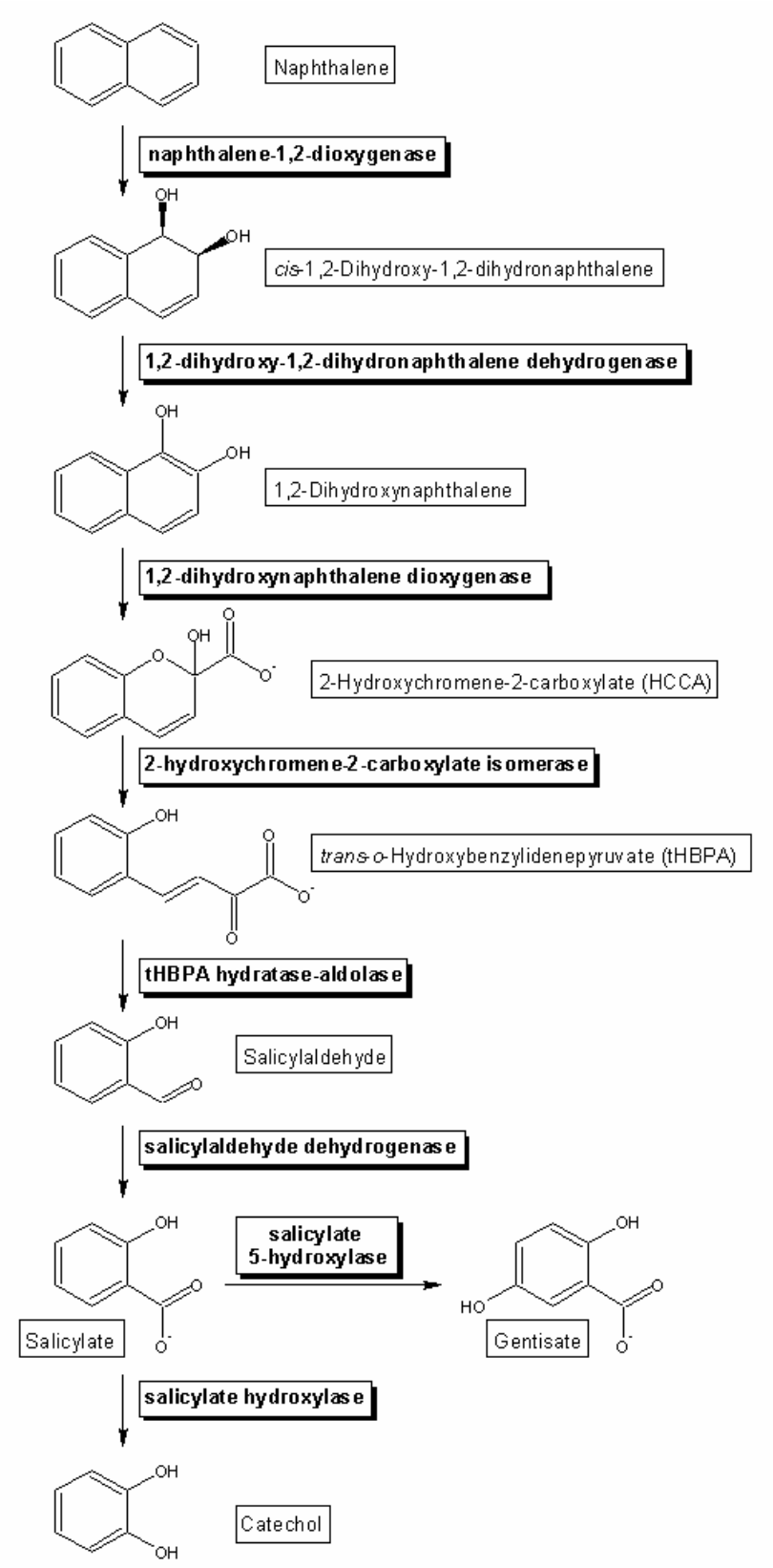


Figure 5: *Degradation pathway for naphthalene. Extracted from University of Minnesota, Biocatalysis/Biodegradation Database [6]*

2.3.4 Kinetics

The kinetics of microbial growth is controlling the utilization of substrate, which leads to the production of biomass. [19] The kinetics of microbial reactions has been mathematically modelled in many different ways. Models are simulation formulas that are simple and incomplete in complexity, comparing to the actual processes they describes. The different models have different approaches, and different degree of complexity. The challenge when dealing with a problem is to find a model that is simple enough to handle within the limits of present economic situation, time, knowledge and experience available among the people working on a problem, and the computing power of available computers. But the model should not be so simple that it fails to describe the situation of interest with the relevant precision either.

For biological systems simple first order and zero order kinetics have been used, but also more complex kinetic models, like the Monod model have been utilized. The first order and the zero order kinetic models are non-autocatalytic, which means that they do not recognize the influence of biomass on degradation rate. [9]

Monod kinetics is based on Monods equation: $\mu = \mu_{\max} \frac{C_S}{K_S + C_S}$, where:

μ = Specific growth rate
μmax = Maximum specific growth rate (1/d).
CS = Concentration of substrate

The petroleum industry in the North Sea is using first order kinetics for microbial growth, because that is what is implemented in the MEMW (Marine Environmental Modelling Workbench) environmental risk assessment model. This model is previously known as DREAM, and is now incorporating the DREAM model. [9]

The original plan for this Master's thesis was to develop a model in the Aquasim 2.0 modeling software based on Monod kinetics. But since the research proved itself to

be even more time consuming than expected, the plans had to be reduced to a simple non-autocatalytic analysis, based on half reaction times.

The first order kinetics was chosen: $C = C_0 \cdot e^{-k_1 \cdot t}$, where

t = time

C = COD potential of organic substrate at time, t

C_0 = COD potential of organic substrate at t=0

k_1 = growth coefficient

At $t_{1/2}$, the time when half the COD potential of the organic substrate has been utilized, the formula can be rewritten like this:

$k_1 = \frac{\ln(2)}{t_{1/2}}$, which means that k_1 can be calculated based on the half reaction time.

2.3.5 Stochiometry

An autocatalytic and a non-autocatalytic stochiometric description of biodegradation that is suitable for this experiment is shown below (energy is left out). Stochiometric coefficients are not specified, because a yield factor ($Y_{x/s}$) for naphthalene biodegradation was not found in literature. The yield factor would have be equal to the value of d (recalculated into molar units), which is the amount of biomass produced per mol of naphthalene consumed. [9]

Autocatalytic:

$C_{10}H_8 + aO_2 + bNO_3^- + cHPO_4^{2-} \rightarrow dBiomass + eCO_2 + fH_2O + gH^+$

Non-autocatalytic:

$C_{10}H_8 + aO_2 \rightarrow bCO_2 + cH_2O$

$Organic\ matter + aO_2 + bNO_3^- + cHPO_4^{2-} \rightarrow dC_5H_7NO_2P_{0.1} + eCO_2 + fH_2O + gH^+$

2.3.6 Abiotic factors influencing marine biodegradation

There are many factors that are influencing microbial degradation of organic contaminants in marine environment. Examples are temperature, oxygen availability, nutrient availability, hydrostatic pressure, pH-value, degradability of contaminant, availability of competing carbon sources, bioavailability of contaminant, contaminant concentration and the history of the contaminated site. [7, 11, 15]

The history of the contaminated site is important because it is crucial when it comes to the composition of bacteria found on site. Not all bacteria are degrading petroleum contaminants, and when for example an oil spill hits an area, it will take some time before the number of degrading bacteria have become so large that extensive degradation takes place. [15]

2.3.6.1 Limiting factor for growth

Different bacteria have different windows of tolerance towards the different abiotic factors. Too much or too little can stop or inhibit growth. Liebig`s law of the minimum describes how everything is dependent on the limiting factor. The limiting factor is the growth factor that is least fulfilled, and therefore is limiting faster growth. This can be explained by an example from car production: If you own a factory that is able to produce 200 engines per year, and 600 wheels per year, and you have abundant amounts of all the other parts, your factory will only be able to produce 150 cars per year, even though you produce 200 engines, and only need one engine per car. That is because you need 4 wheels per car, and 600 wheels is therefore not sufficient for more than 150 cars. In the same way different microorganisms have many different demands for growth, and all of them needs to be met if growth are to take place. And if more growth is needed in a system, the best way to increase growth will be to change the limiting factor so that it is closer to the optimum value for the microorganisms of interest. If we turn back to the car factory, the recipe to increase production would not be to increase the number of engines produced to 300, but to increase the number of wheels to 800. If more than 800 wheels are produced, engines would become limiting factor.

The knowledge of limiting factors can be used in nature to keep environments healthy, or to heal contaminated sites. For example oxygen depletion in lakes can be prevented by reducing supply of organic matter, nitrogen or phosphorous. Reduction of the limiting factor will reduce growth, and thereby reduce demand for oxygen. If a lot of nutrients are stored in a compartment of a lake, it will take more time for the lake to heal than if the microorganisms are depending on external supply of nutrients.

One example of where this knowledge has been used to heal a contaminated site, is the recovery after the large l oil spill by the tanker *Exxon Valdez* in Alaska in March 1989. Nutrients were then added to contaminated areas to prevent the low concentration of nutrients in sea to be limiting degradation of oil. [20]

2.3.6.2 Effects of temperature

Temperature is the abiotic factor that has the main focus of this thesis. Microbial degradation is well known to be a temperature-dependent process. [12] But although it is interesting to see how much influence a change in temperature in fact has on the specific system being studied. Especially since the experimental setup is designed to have relevance to marine degradation of petroleum components.

When temperature is reduced, physical and chemical parameters change, and the changes are unfavorable to microorganism growth. At low temperature viscosity increases, volatility increases, and water solubility of hydrocarbons decreases. [21]

Bacteria have different tolerance to temperature. Some species of bacteria have their growth optimum at just above 0ºC, while others have their growth optimum at well over 100ºC. But the literature agrees on that psychrophilic bacteria have a lower growth rate than most other bacteria, and that biodegradation goes slower at low temperatures than at moderate temperatures. [12, 20, 22]

2.4 Objectives

The main objective of this research was to find the temperature effect on the growth rate of the microorganisms degrading naphthalene in a specific temperate seawater inoculum. The objective is achieved using a closed bottle BOD test in glass bottles. In

addition to the results about the direct oxygen consumption in the system, substrate concentration is being measured using gas chromatography, and TOC measurement of control samples. Furthermore cell concentration is being monitored by taking microscope pictures of DAPI stained cells, and quantifying the cells using a a MatLab program for cell counting (Austvoll, I and Kommedal, R. (2007) pers. com.). 50% degradation time based on BOD results are used to find growth rates for each temperature, assuming first order kinetics.

The idea of the thesis is to contribute a little piece to the puzzle that will give better understanding of the challenges related to temperature effects on the degradability of different components of crude oil in seawater. This could have relevance to environmental aspects concerning the fact that the petroleum industry is putting drilling and production into areas with increasingly harsh conditions concerning wind, depth and temperature.

3. Materials and Methods

3.1 Experimental setup

The experiment was carried out in 1000 ml Scott Duran glass bottles with OxiTop® pressure measuring heads, and adapters to fit the bottles to the head. There was also put a magnet in each bottle. And two pellets of NaOH was put into a rubber container inside the OxiTop® head adapters, and a little water was added to make a saturated NaOH sulution. This is illustrated in Figure 6. The bottles were placed on stirring plates in refrigreators, after the sample solution was added. In each refrigerator 10 bottles were placed. Figure 6 gives a glance inside one of the four refrigerators used for the experiment. The refrigerators was set to 0.5, 4 , 8, and 15ºC. There was not enough adapters for the OxiTop® heads, and therefore another type of Scott Duran bottles had to be used for the samples at 0.5ºC. This other type of bottles had another two smaller openings slanting to each side of the main opening. On these smaller openings the OxiTop® heads fitted directly. The two remaining openings was capped with screw caps. The volume of the bottles with 3 openings was 1170ml, while the volume of the bottles with one opening was 1111ml.

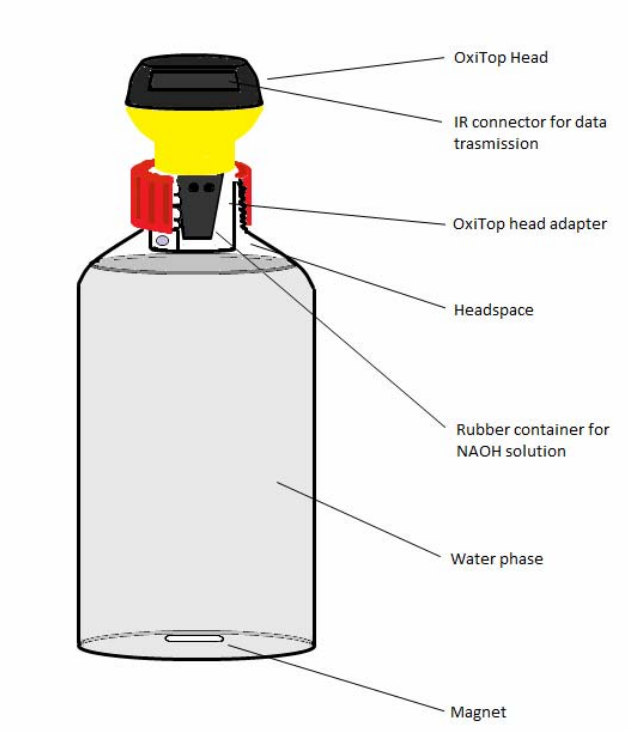


Figure 6: *Sample bottle with OxiTop® head.*

Figure 7*: Experimental setup. Sample bottles in refrigerator.*

The 10 bottles that was prepared for each temperature, were 6 naphthalene sample bottles, 2 positive controls with sodiumbenzoate, 1 blank sample without any additional carbon. And 1 bottle containing a negative control with the same contents as the positive controls, but the negative control samples was autoclaved, and later added 2.5 g/l of sodium azide for biological inhibition.

A 100mg/l sodium benzoate stock solution was made for the control samples, and a 50 mg/l naphthalene stock solution was made for the naphthalene samples. It is not possible to dillute that much naphthalene in water at room temperature, so the stock solution was heated to about 70ºC, and kept at that temperature and stirred until the solids were dissolved. The stock solutions was made in artificial seawater.

200 ml of stock solution was then added in 850 ml of natural test seawater. For the blank samples, 200 ml of artificial seawater was added instead of stock solution, to leave the blank sample with the same concentration of inoculum seawater (and thereby bacteria concentration) as the naphthalene and positive control samples. This means that the concentration of naphthalene in the naphthalene sample bottles

was supposed to be 9.5 mg/l, and benzoate concentration in control sample bottles was supposed to be 19 mg/l (which corresponds to 11.5mg/l TOC).

To each of the sample bottles there was added different mineral solutions. The first salt solutions that was added was based on the Bushnell-Haas Marine salts contributed these salts to the sample bottles: K_2HPO_4, NaH_2PO_2, $FeCl_3$, $NaNO_3$, $MgSO_4$ and $CaCl_2$. In addition was a solution based on Balch trace mineral solution added. This solution contained the following salts: EDTA, $MgSO_4$, $MnSO_4$, NaCl, $FeSO_4$, $Co(NO_3)_2$, $CaCl_2$, $ZnSO_4$, $CuSO_4$, $AlK(SO_4)_2$, H_3BO_3, Na_2 MoO_4, Na_2SeO_3 and $NiCl_2$. Totally 4 ml of mineral solutions was added to each bottle. And also 100µL of vitamin solution (RPMI 1640 Amino Acids Solution, Sigma). Minerals and vitamins were added to make the substrate the limiting factor for bacterial growth.

3.2 Inoculum

The inoculum seawater was gathered through an inlet 80 meters below sea surface at IRIS Akvamiljø at Mekjarvik just north of Stavanger, Norway. The seawater was prefiltered through a 10 µm polypropylene filter (Opticap, XL, Millipore^{TM}, MA, USA), to remove algae and zooplankton, which could influence prokaryotic biodegradation.

3.2 Startup

Following final preparation of all sample bottles they were placed in their respective refrigerator, and left for 24 hours for temperature equilibration. Following equilibration flasks was vented, and reclosed. After venting BOD logging was started.

Experiment startup time was February 17. between 17.00 and 21.45. The lowest temperatures were prepared first.

BOD logging started February 18. from 12.15 onwards, also here starting with the lowest temperature.

3.3 Sampling and analysis

3.3.1 Sampling procedure

There were four different analytic methods for which samples were taken in this experiment. That were the GC naphthalene concentration analysis, the TOC analysis that was used for determination of benzoate concentration in the control samples, the DAPI staining and fluorescence microscoping for cell counting, and finally the DGGE samples that were sent to Trondheim for further analysis by a PhD student. The results from the DGGE analysis will not be presented in this thesis. All the sampling for this research was done with glass syringes with metal pins to reduce the chance for organic substrate to stick to a plastic pipette tip, and for such a tip to release organic matter and contaminate sample bottles.

The practical sampling for each kind of analysis was done as follows:
GC naphthalene analysis:
1 ml of the sample solution was transferred to a GC Headspace vial, and conserved with 100µl 2M HCl

TOC benzoate analysis:
4 ml of sample solution was transferred to a clean and autoclaved TOC vial. 250µl 2M HCl was added for conservation

DAPI cell counting:
2 ml sample solution was transferred to a teflon bottle with screw-cap. 100 µl borate buffered formalin was added for conservation.

All sampling was done at similar temperatures as the refrigerator used for each sample bottle. And the volumes that were removed from the sample bottles were replaced with native seawater to avoid disturbance of the calculated oxygen consumption based on headspace pressure. (Input about liquid and headspace volume would have to be changed if the calculations should remain correct after actual volumes had been changed) The exception from this were the volumes removed for DGGE analysis. The DGGE analysis demanded so large sampling volumes that replacement of the volumes with native seawater would have disturbed

the composition of the samples quite a bit. The sample bottles where DGGE sampling was done, were instead regarded non reliable when it came to results obtained after DGGE sampling.

Figure 8 shows a sketch of an ideal timing plan for sampling. The figure shows how we would want the sampling to be done, and not how it actually went with the practical research.

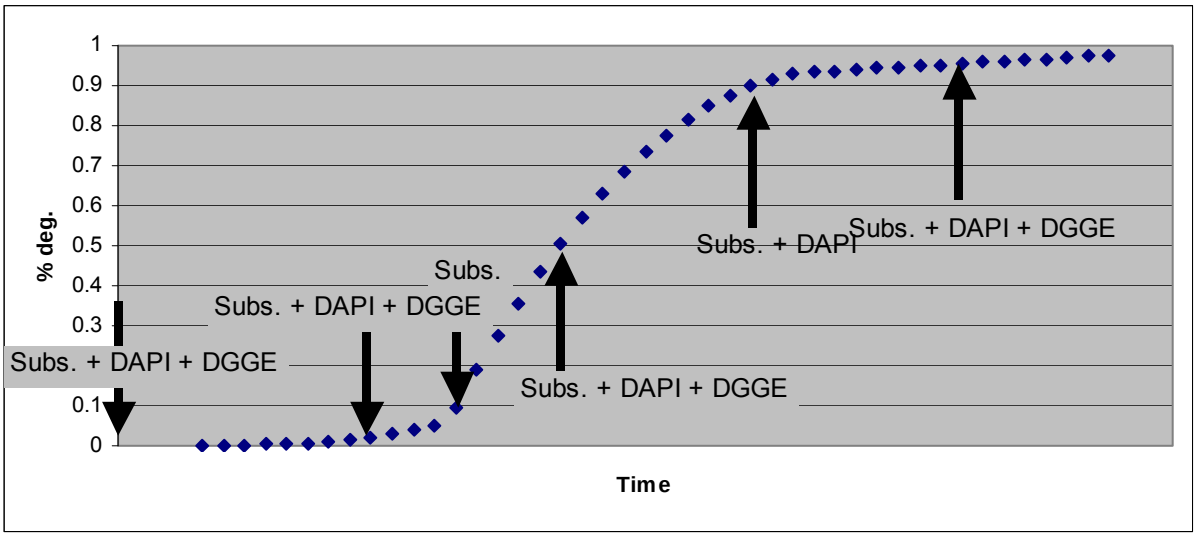


Figure 8: *Sample timing plan.*

3.3.2 Substrate analysis

3.3.2.1 Naphthalene analysis

Napthalene concentration in sample bottles was monitored through repeated sampling and subsequent gas chromatography (GC). The GC instrument was an Agilent 6890N, utilizing a flame ionization detector (FID). The column was a Supelco Equity 1, fused silica capillary column, dimensions was 10m*200µm, with a 1.2 µm film (Sigma-Aldrich, Oslo). Instrument control parameters was optimized for good and fast separation of naphthalene. The GC analysis was run under the following conditions:

- Carrier gas: Nitrogen (N_2)
- Flow rate: 0.7 ml/min
- Injection mode: Splitless
- Inlet temperature: 260 □C

- Oven temperature program:
    - Initial temperature: 60 □C, kept for 0.2 min
    - Ramp: 70 □C per minute
    - Final temperature: 240 □C
- Run time: 4.0 min

Injection was done automaticly by a Gerstel MultiPurpose Sampler. Method used was headspace injection. Sample volume was 500 µl. The sample vials were incubated for 5 minutes at 65 □C before sample volumes were transferred to GC inlet.

The response registered at the FID, produced a response curve like the one viewed in Figure 9. The response was automaticly integrated and given a value of naphthalene concentration based on a calibration curve. The calibration curve was made as a part of the preparation for the research, with known concentrations of naphthalene.

The time on the curve in Figure 9 is elution time, which is the time each compound uses to pass the coloumn. Different compounds are separated based on their elution time.

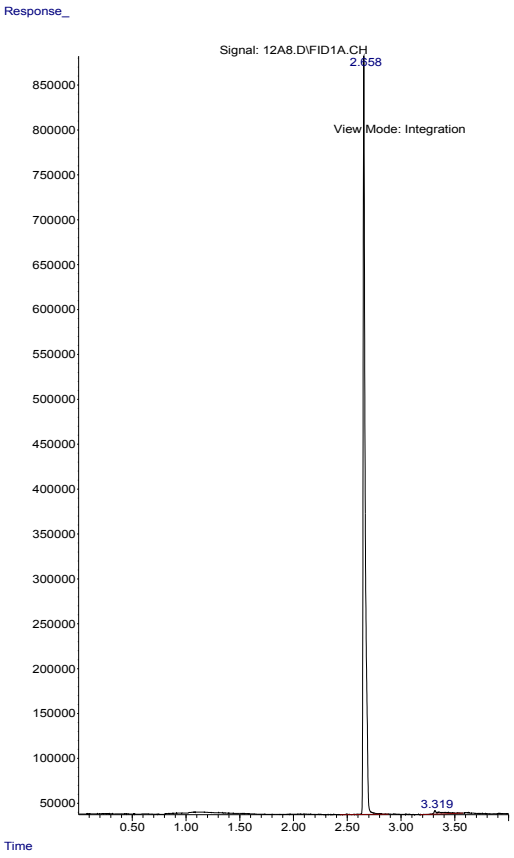


Figure 9: *Example of a GC response curve.*

3.3.2.2 Benzoate analysis

Benzoate concentration in control sample bottles were determined by TOC analysis. The instrument was an Analytik Jena multi N/C 3100. The samples was acidiced as described in chapter 3.3.1, and purged with nitrogen (N_2) for removal of inorganic carbon before TOC analysis took place. The acid turns inorganic carbon like carbonate and bicarbonate into carbon dioxide, and the purge gas strips away both the CO_2 and the purgeable (volatile) organic carbon. Organic carbon that is left in the solution after purging is burnt, and the CO_2 produced by this burning are sent to the detector. This is commonly referred to as total organic carbon. This analysis method is not usable for quantification of volatile organic compounds. [23]

3.3.3 DAPI cellcounting

Biomass in the system was monitored by filtering, DAPI staining and microscoping of bacteria. The analysis method was adapted Sherr, B et al. in the book of Paul, J. H., *Methods in Microbiology – Marine microbiology* [18].

Polycarbonate, 0.22 micron, black filters, 25 mm diameter from GE Water & Process Technologies was used for the filtration, on a Sartorius filter apparatus. Bacterial DNA staining (4,6-diamino-2-phenylindole, DAPI) was used as staining.

A Leica DMLS microscope, equipped with a Leica DC100 camera, and a fluorescent lamp was used to capture images of randomly picked areas on the filter surface. 15 images was taken for each sample. Bacterial cells was quantified using a MatLab program for cell counting (Austvoll, I and Kommedal, R. (2007) pers. com.). Figure 10 shows an example of how the progam counts the bacteria on an image on the upper two images. All the red crosses on the picture are objects that have not been counted as cells, while the green circles are marking objects recognized as cells. The red area with a green square just above the center of the image have been counted as a group of cells, and the nuber of cells are written in the green square. It is obvious that lots of cells in this picture did not become recognised by the program. That is probably because of the blue light from the background. By experience with the microscope, by changing software settings, or by changing amount of immersion oil, or kind of oil, it is easier to obtain pictures that are easily readable for the program, like the lower one in Figure 10.

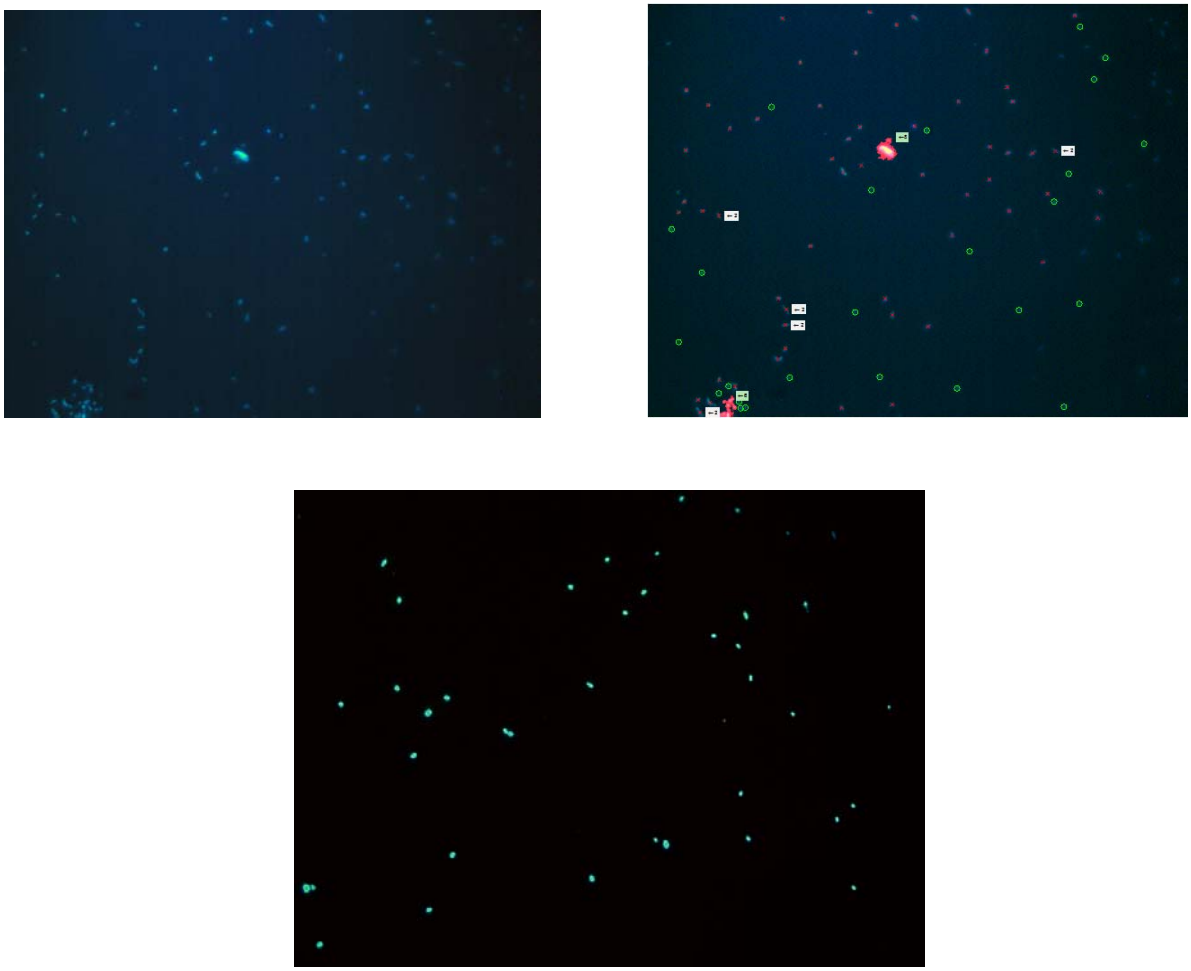


Figure 10: *The two upper pictures shows an example of a picture of DAPI stained bacteria to the left, and the same picture counted by the MatLab program used in this research to the right. The lower picture is a quite sharp and clear microscope picture of DAPI stained marine bacterial cells.*

3.3.4 BOD OxiTop® method

Oxygen demand in the system was monitored by measurement based on headspace pressure in the sample bottles, monitored by the OxiTop® heads. The oxygen demand was calculated based on the following formula:

$$BOD = \frac{M(O_2)}{R \cdot T_M} \cdot \left(\frac{V_t \cdot V_l}{V_l} + \alpha \cdot \frac{T_M}{T_0} \right) \cdot \Delta p(O_2)$$

, where:

BOD = Biological Oxygen Demand

$M(O_2)$ = Molecular weight (32000mg/mol)

R = Gas constant (83.144 l·mbar/mol·K)

T_0 = Reference temperature (273.15 K)

T_M = Measuring temperature (273.65, 277.15, 281.15 and 288.15 K for this experiment)

V_t = Bottle volume (1111 ml and 1170ml for different bottles in this experiment)

V_l = Sample volume (1054.1 ml for this experiment)

α = Bunsen absorption coefficient (0.03103)

$\Delta p(O_2)$ = Difference in oxygen partial pressure (in millibar)

Data could be collected for examination from the OxiTop® heads at any time during the experiment, with a OxiTop® handheld control unit, by infrared signal transmission. It was important to keep an eye on the development of the OxiTop® BOD measurement, because the BOD curve also told when the correct time was for sampling for the other analysis methods.

4. Results

4.1 Results 0.5ºC

The refrigerator that was set to 0.5ºC, did not keep that temperature stable. The low temperature made it do de-icing by rising the temperature for a while. There were approximately 24 hours between each de-icing period.

4.1.1 Samples 0.5ºC

Figure 11 and 12 shows the BOD curves for naphthalene samples at 0.5ºC. Figure 11 is presented without blank ajustment. The graphs in Figure 12 is adjusted by subtracting the blank value from the sample values.

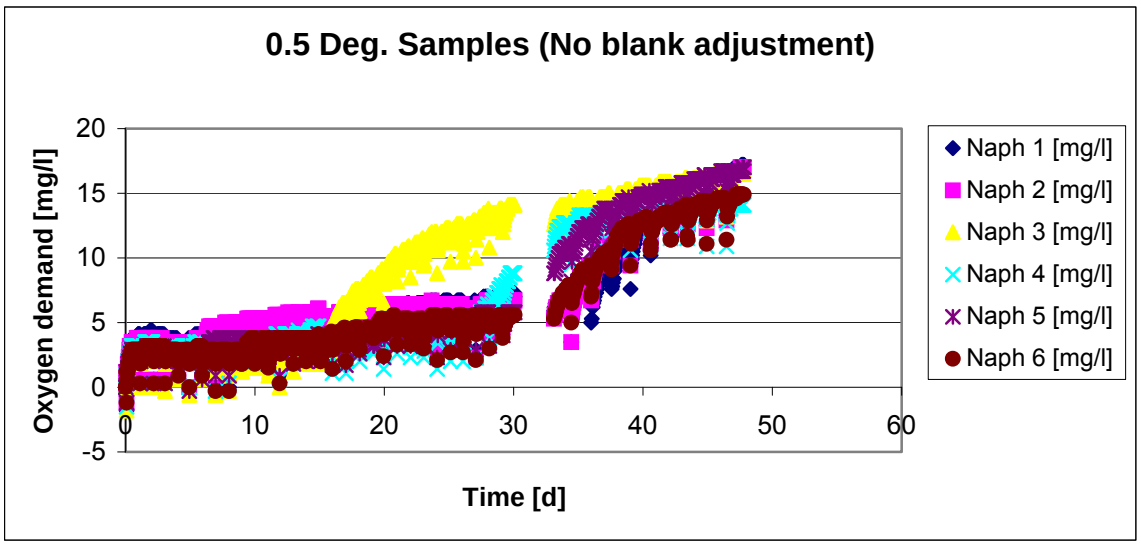


Figure 11: *Results for naphthalene samples, OxiTop®, 0.5ºC, no blank ajustment.*

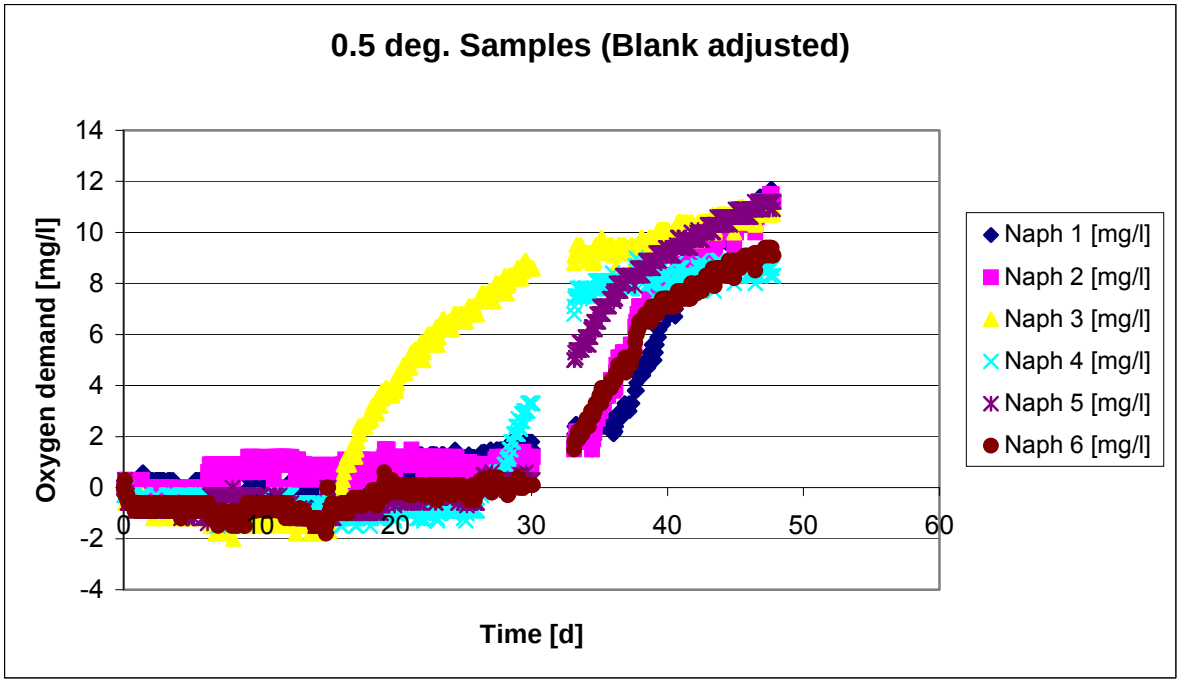


Figure 12: *Results for naphthalene samples, OxiTop®, 0.5ºC, blank adjusted.*

Figure 13 presents the data from GC analysis for naphthalene samples at 0.5ºC.

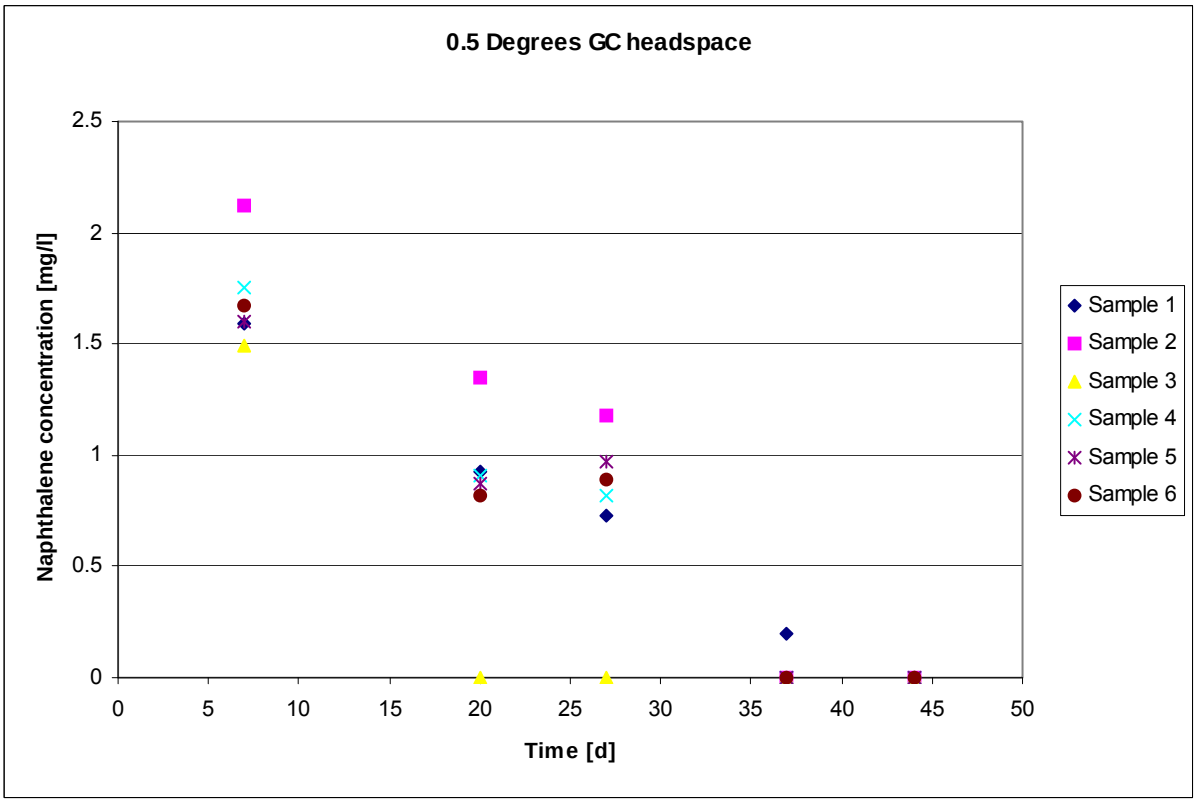


Figure 13: *Results for naphthalene samples, GC headspace, 0.5ºC.*

The results from DAPI cell counting for naphthalene samples at 0.5ºC is presented in Figure 14. Figure 15 is based on the same data as Figure 14. It is a estimation of COD concentration based on the assumption that the cells in all samples had a mean mass of $2 \cdot 10^{-10}$g/cell. Images showed that the size of cells was anything but uniform. Some cells was many times larger than other cells, and some samples contained primarily large cells, and others primarily small cells. Some had similar amounts of small and big cells. Therefore COD estimations based on cell number and a standardized cell mass was not found to give a very good estimation of real biomass concentration, and similar COD curves will not be presented for other samples.

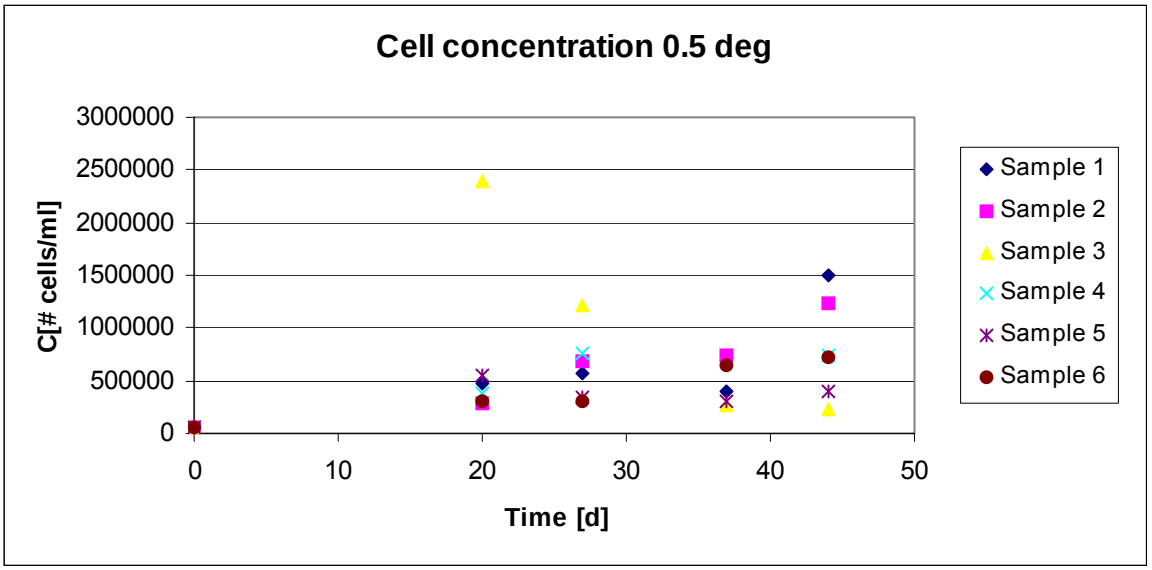


Figure 14: *Results for naphthalene samples, DAPI cellconting, 0.5ºC.*

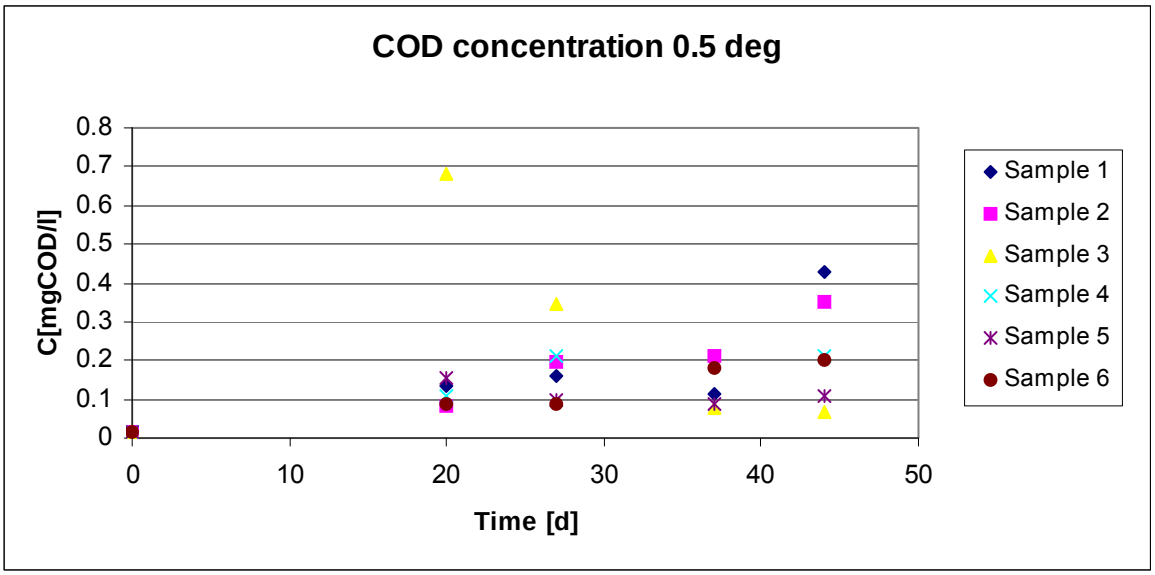


Figure 15: *COD concentration in naphthalene samples estimated based on cell concentration, 0.5ºC.*

Table 1 presents the 50% degradation time for naphthalene samples at 0.5ºC.

Table 1: 50% degradation time and degradation constant, 0.5ºC

Sample no	**$t_{1/2}$ [d]**	
1	39.25	
2	37.29	
3	21.87	
4	31.50	
5	33.54	
6	36.60	
Mean 0.5 deg:	33.34	
St. dev.:	6.26	
St. dev. Mean	2.6	
k_1:	0.021	d^{-1}
st. dev. K1	0.002	

4.1.2 Controls 0.5ºC

Figure 16 and 17 shows the BOD curves for control samples at 0.5ºC. Fig. 17 is blank adjusted. The negative control curve in Fig. 17 shows a odd behavior. It could maybe be a chemical related to sodium azide that caused the headspace pressure to vary. Similar effect is found for the negative control at 8ºC, while the curve is stable around zero at 4 and 15ºC.

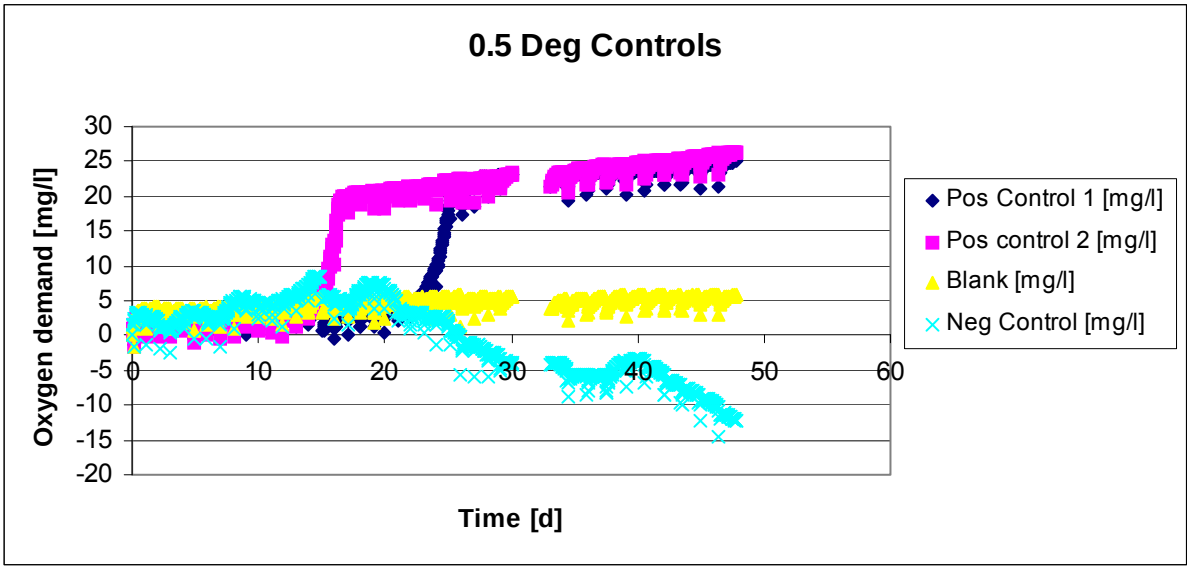


Figure 16: *Results for controls, OxiTop®, 0.5ºC.*

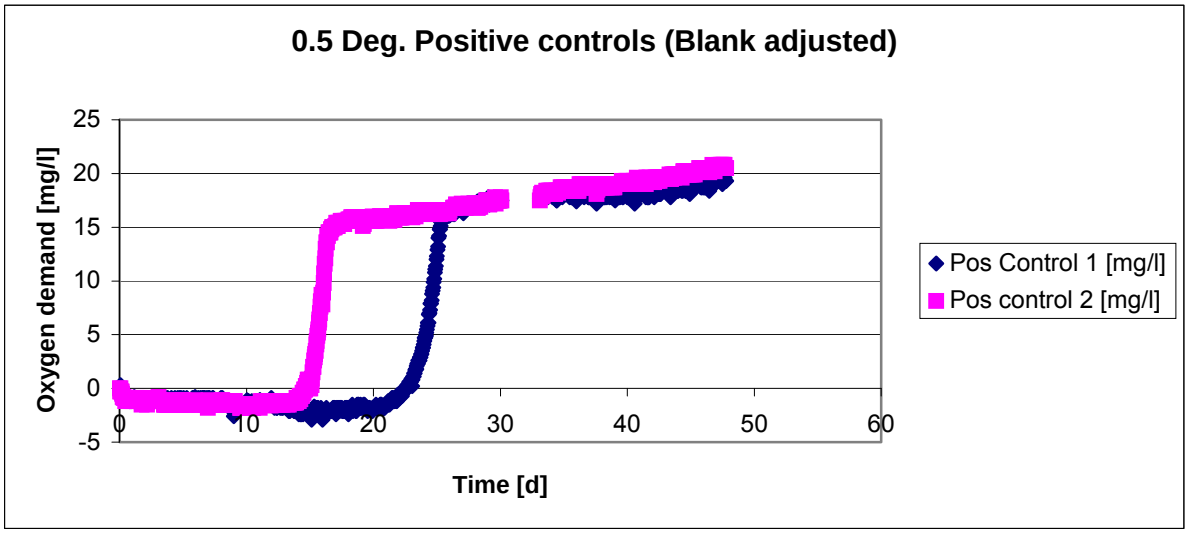


Figure 17: *Results for controls, OxiTop®, 0.5ºC, blank adjusted.*

Figure 18 presents TOC concentration in control samples at 0.5ºC. The data are blank adjusted, which is the reason why some concentrations is registered below zero.

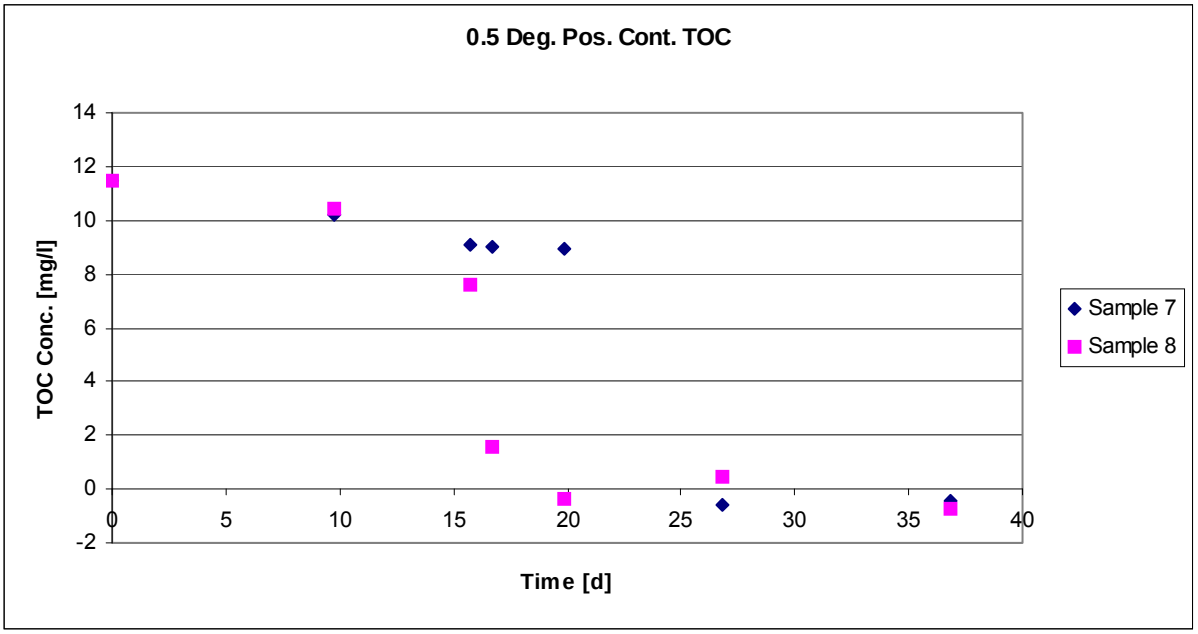


Figure 18: *Results for controls, TOC analysis, 0.5ºC, blank adjusted.*

Figure 19 presents data from DAPI cell count for control samples at 0.5ºC.

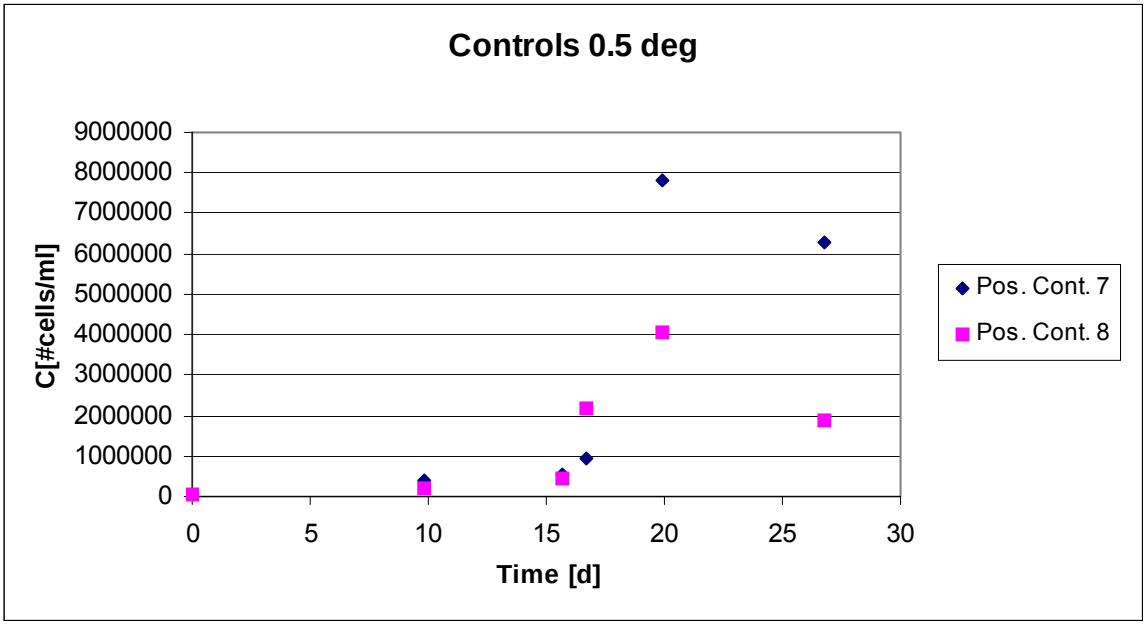


Figure 19: *Results for control samples, DAPI cellconting, 0.5ºC.*

4.2 Results 4ºC

4.2.1 Samples 4ºC

Figure 20 presents the blank adjusted BOD curves for naphthalene samples at 4 ºC.

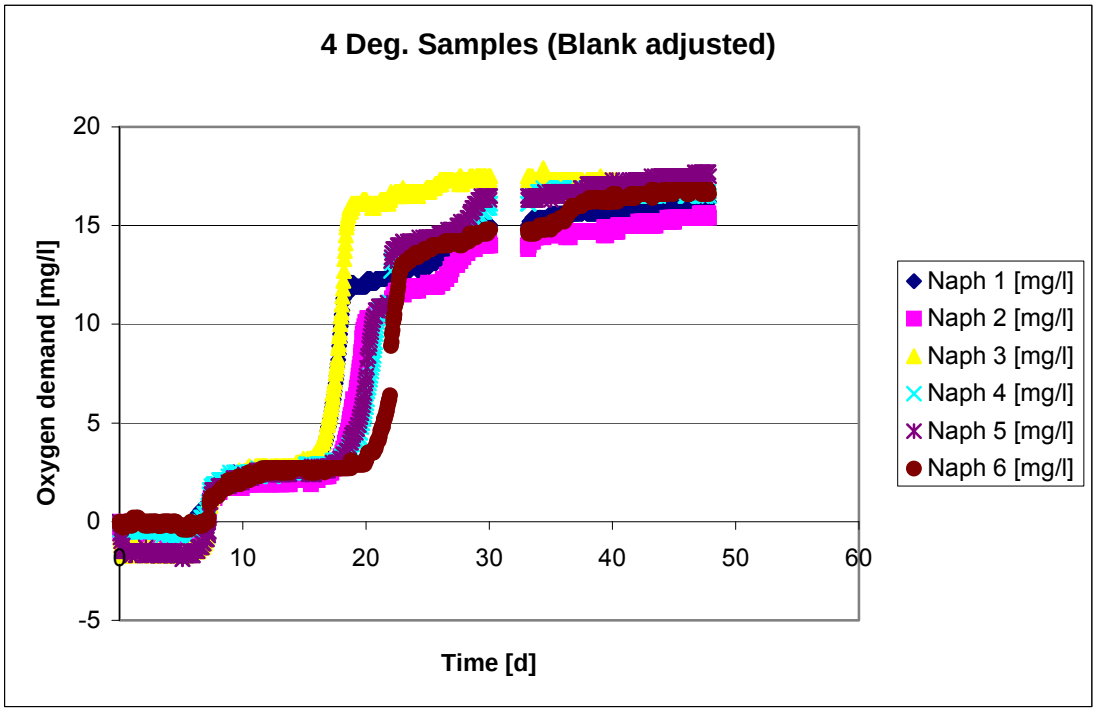


Figure 20: *Results for naphthalene samples, OxiTop®, 4ºC, blank adjusted.*

Figure 21 presents the data from GC analysis for naphthalene samples at 4ºC.

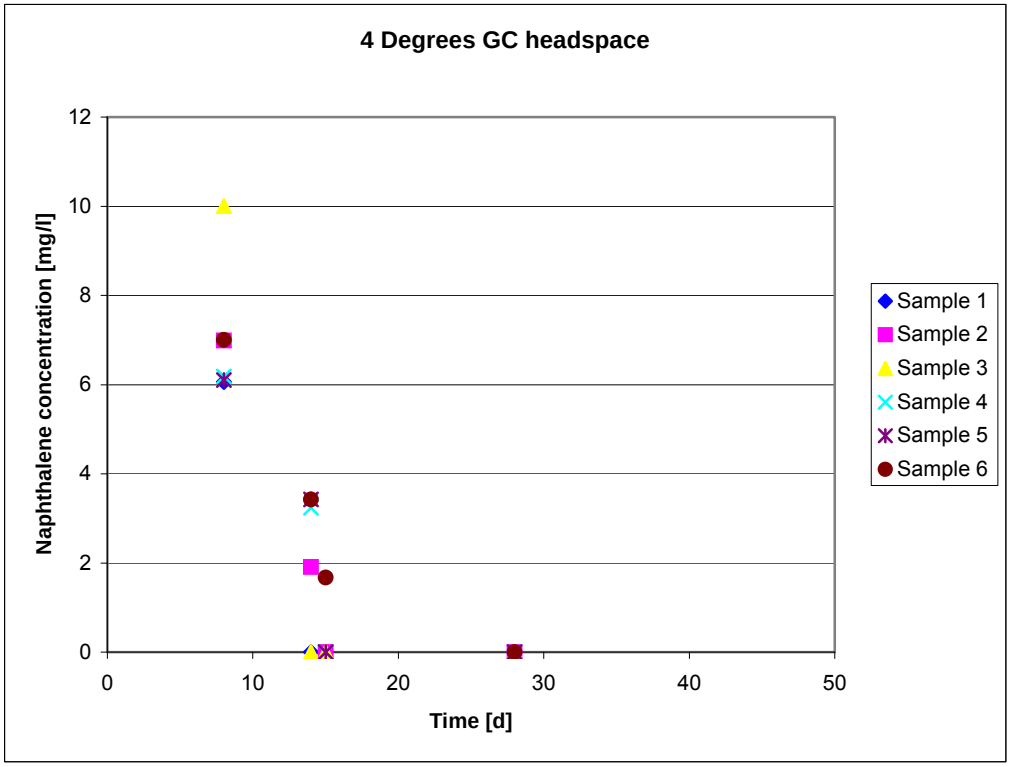


Figure 21: *Results for naphthalene samples, GC headspace, 4ºC.*

The results from DAPI cell counting for naphthalene samples at 4ºC is presented in Figure 22.

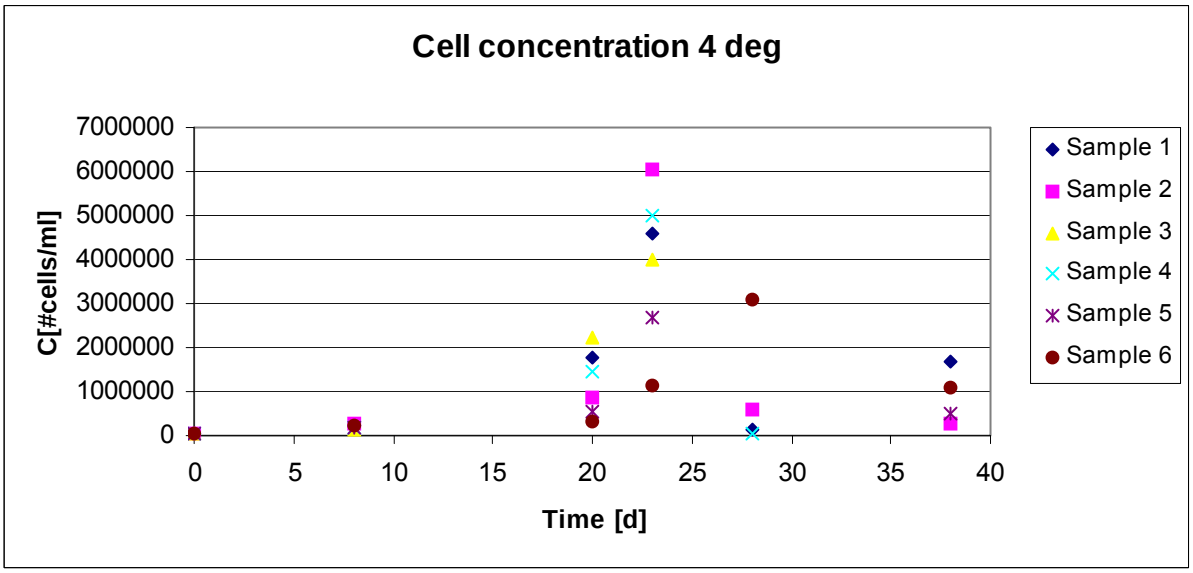


Figure 22: *Results for naphthalene samples, DAPI cellconting, 4ºC.*

Table 2 presents 50% degradation times and degradation constant, k_1, at 4ºC.

Table 2: 50% degradation time and degradation constant, 4ºC

Sample no	$t_{1/2}$ [d]	
11	17.75	
12	19.50	
13	17.75	
14	20.62	
15	20.25	
16	22.04	
Mean 4 deg:	19.65	
St. dev.:	1.69	
St. dev. Mean	0.7	
k_1:	0.035	d^{-1}
st. dev. K1	0.001	

4.2.1 Controls 4ºC

Figure 23 and 24 shows the BOD curves for control samples at 4ºC. Fig. 24 is blank adjusted.

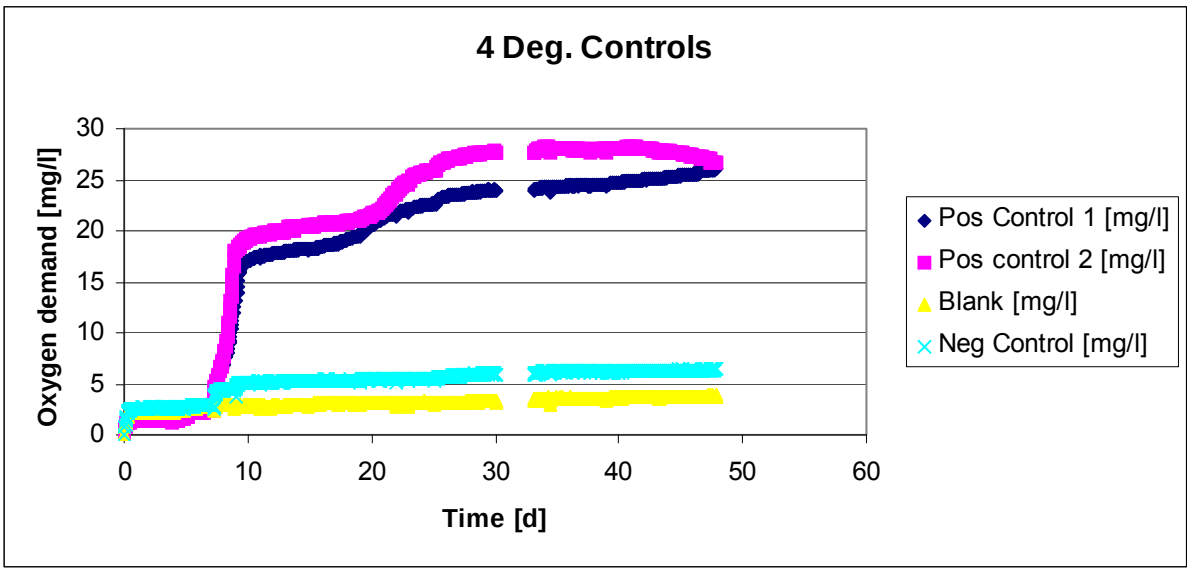


Figure 23: *Results for controls, OxiTop®, 4ºC.*

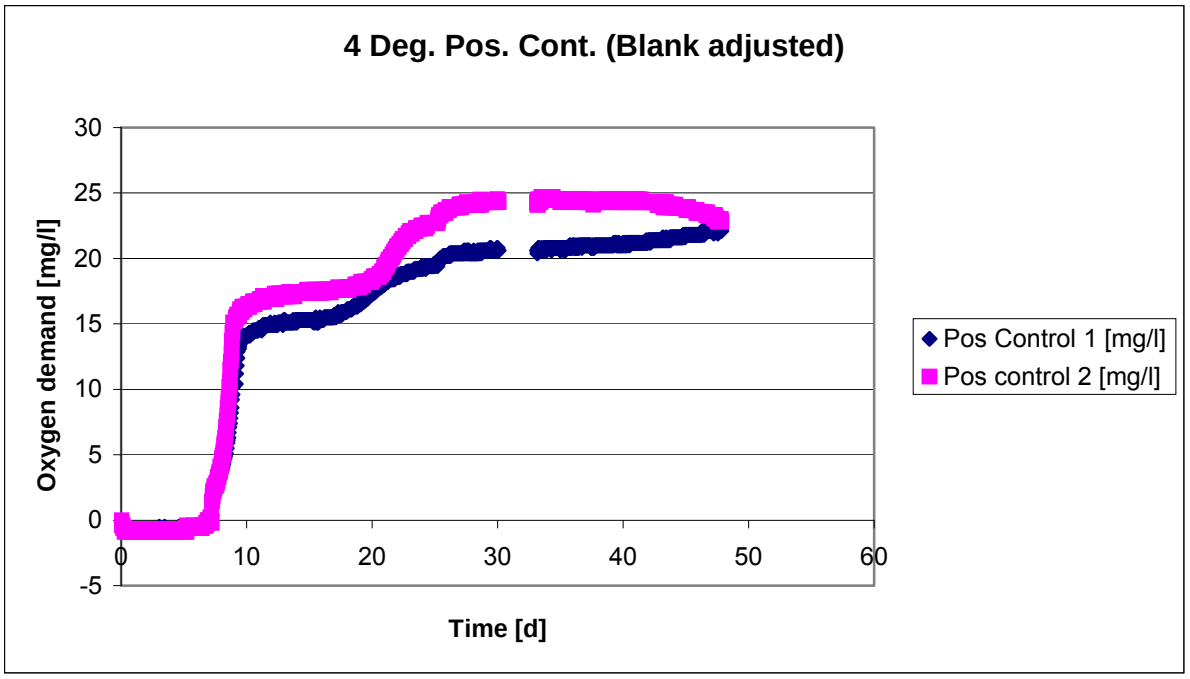


Figure 24: *Results for controls, OxiTop®, 4ºC, blank adjusted.*

Figure 25 presents TOC concentration in control samples at 4ºC. The data are blank adjusted, which is the reason why some concentrations is registered below zero.

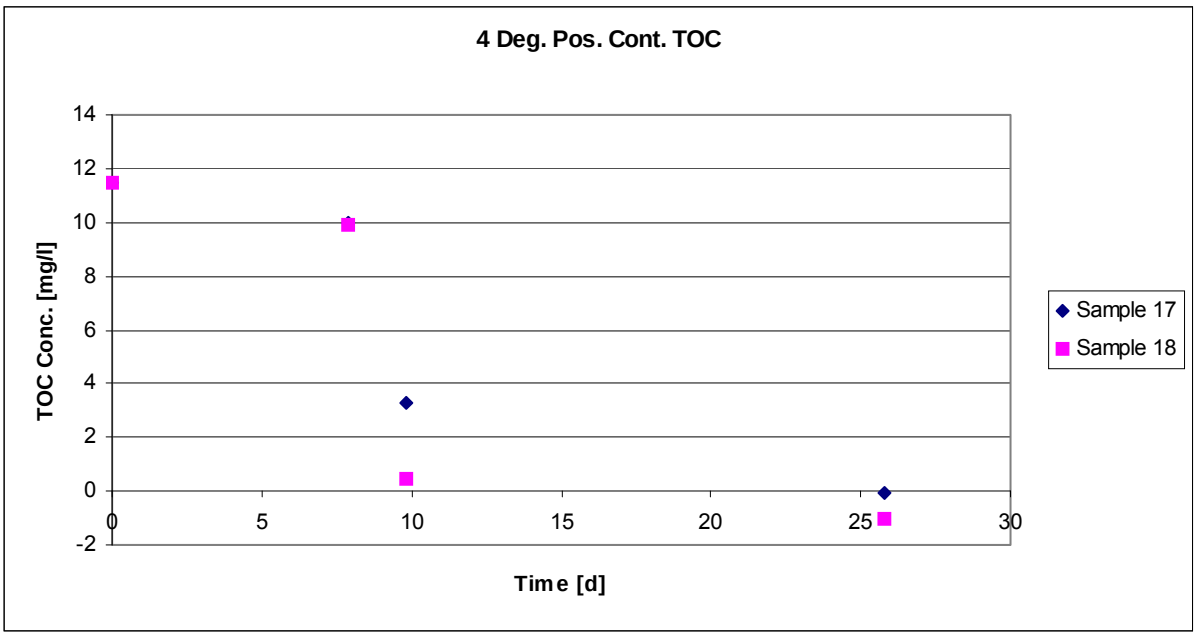


Figure 25: *Results for controls, TOC analysis, 4ºC, blank adjusted.*

Figure 26 presents data from DAPI cell count for control samples at 4ºC.

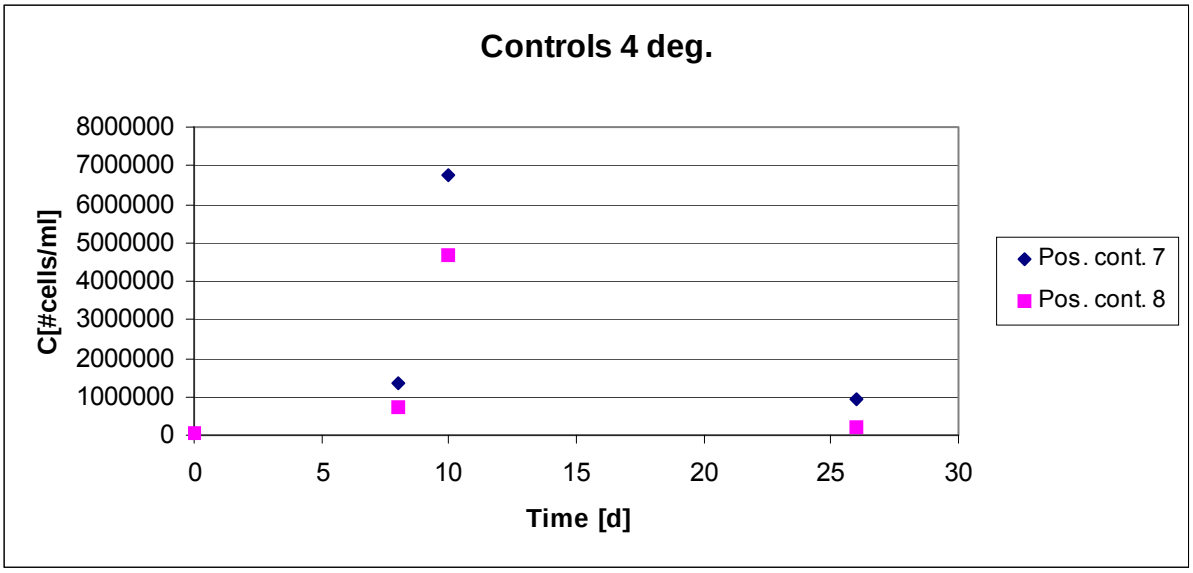


Figure 26: *Results for control samples, DAPI cellconting, 0.5ºC.*

4.3 Results 8ºC

4.3.1 Samples 8ºC

Figure 27 presents the blank adjusted BOD curves for naphthalene samples at 8 ºC.

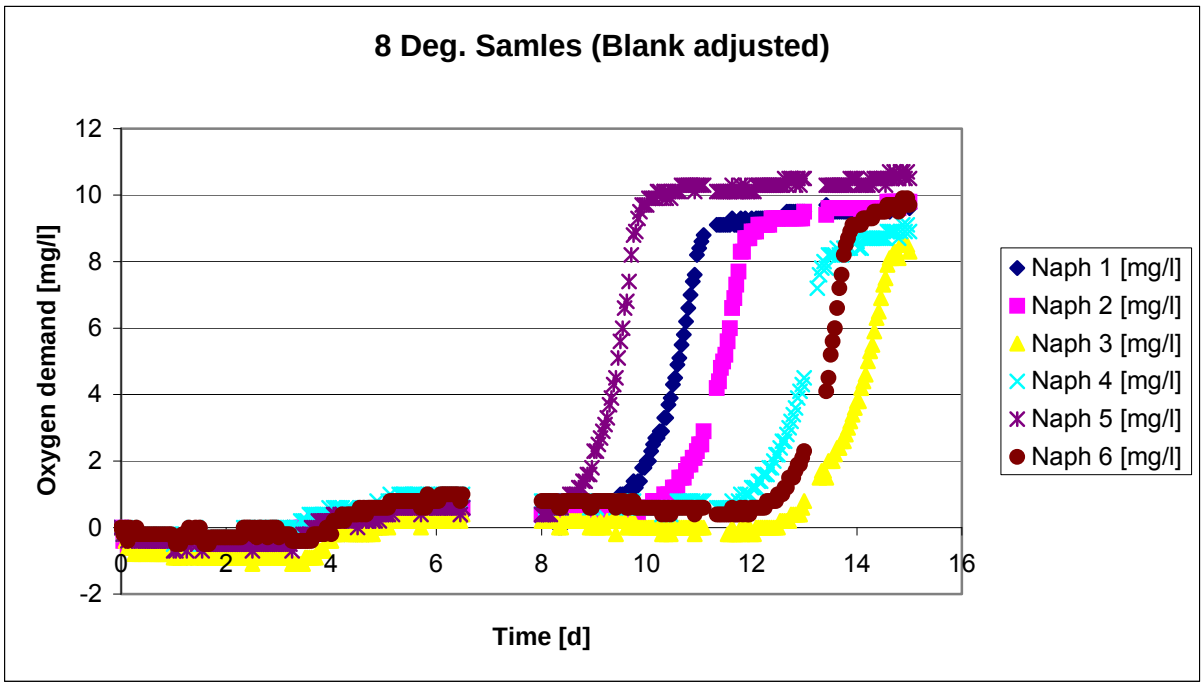


Figure 27: *Results for naphthalene samples, OxiTop®, 8ºC, blank ajusted.*

Figure 28 presents the data from GC analysis for naphthalene samples at 8ºC.

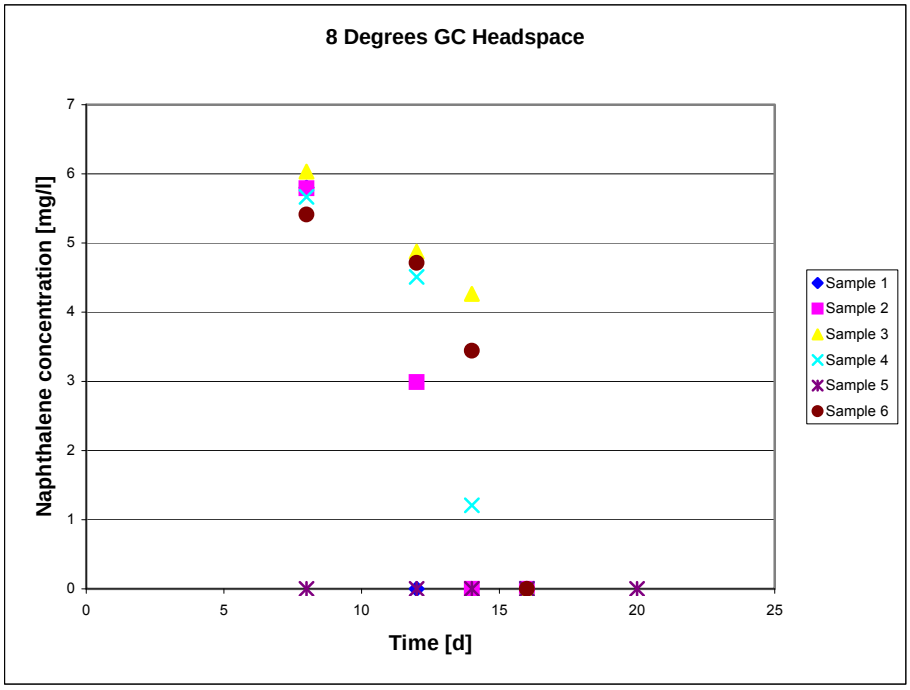


Figure 28: *Results for naphthalene samples, GC headspace, 8ºC.*

The results from DAPI cell counting for naphthalene samples at 8ºC is presented in Figure 29.

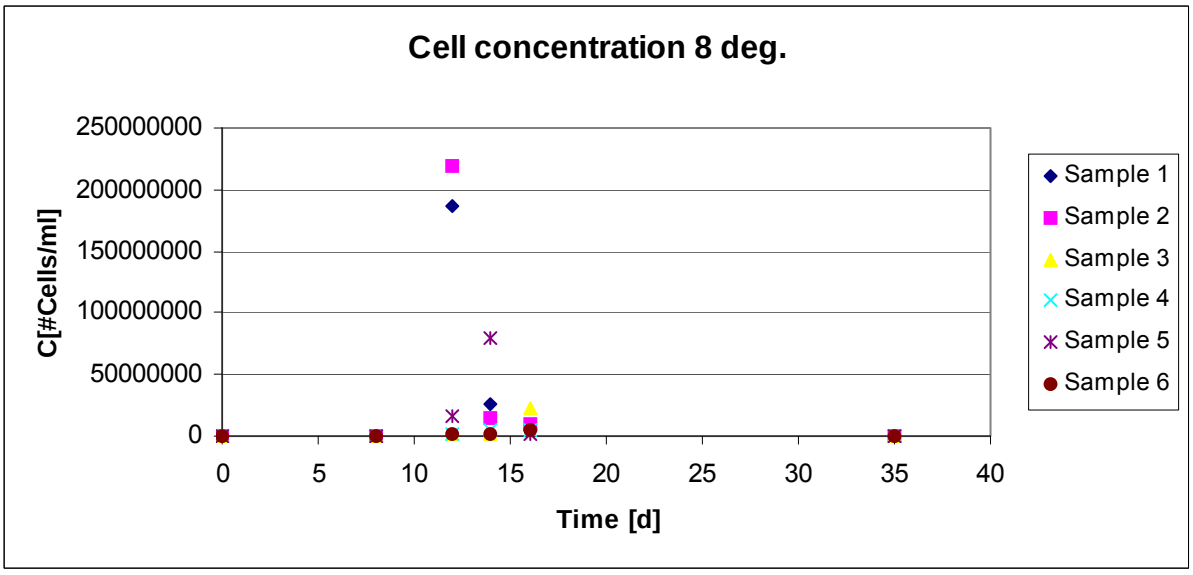


Figure 29: *Results for naphthalene samples, DAPI cellconting, 8ºC.*

Table 3 presents 50% degradation times and degradation constant, k_1, at 8ºC.

Table 3: 50% degradation time and degradation constant, 8ºC

Sample no	**$t_{1/2}$ [d]**	
21	10.58	
22	11.45	
23	14.12	
24	13.04	
25	9.50	
26	15.50	
Mean 8 deg:	12.37	
St. dev.:	2.26	
St. dev. Mean	0.9	
k_1:	0.056	d^{-1}
st. dev. K1	0.004	

4.3.2 Controls 8ºC

Figure 30 and 31 shows the BOD curves for control samples at 8ºC. Fig. 31 is blank adjusted.

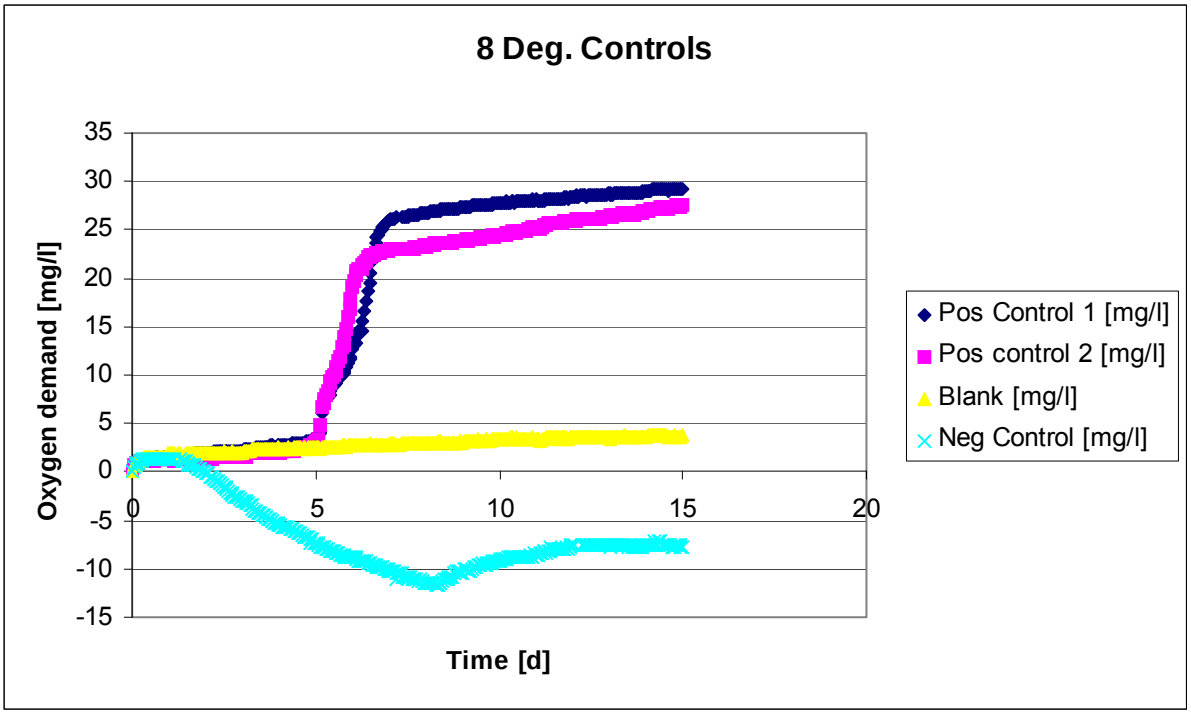


Figure 30: *Results for controls, OxiTop®, 8ºC.*

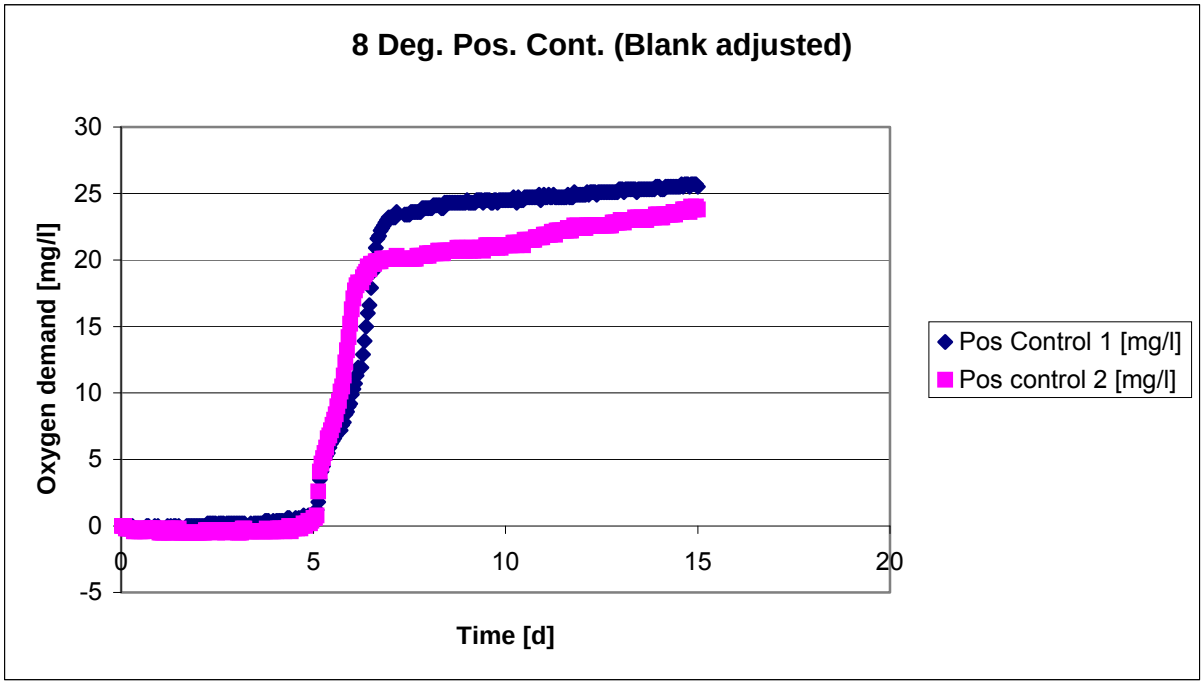


Figure 31: *Results for controls, OxiTop®, 8ºC, blank adjusted.*

Figure 32 presents TOC concentration in control samples at 8ºC. The data are blank adjusted.

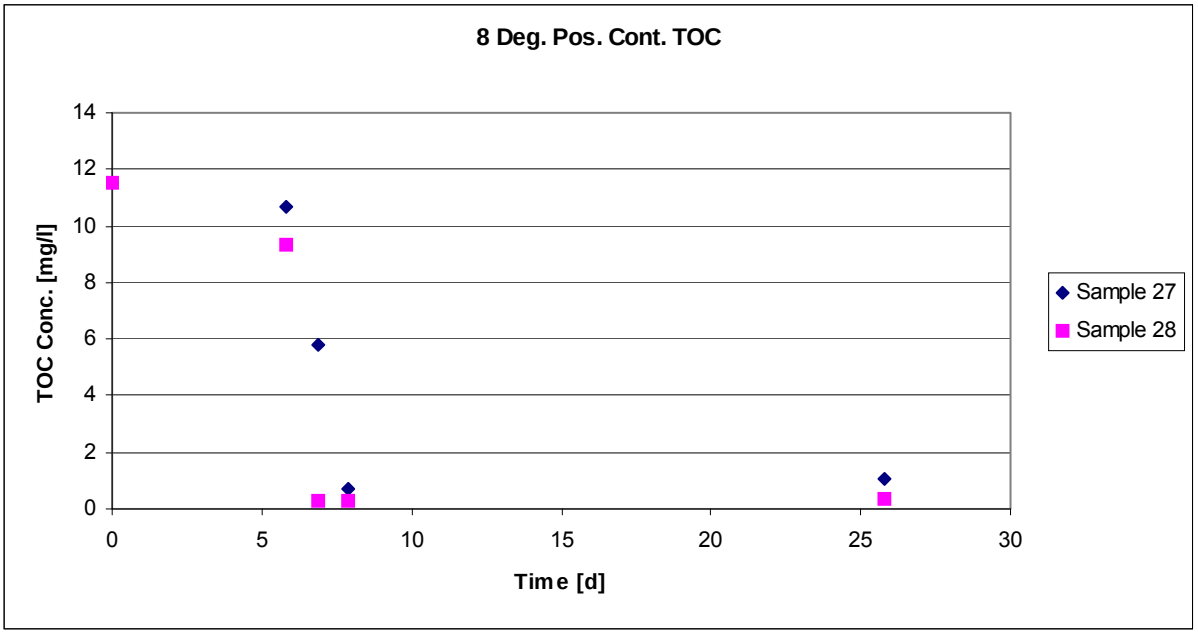


Figure 32: *Results for controls, TOC analysis, 8ºC, blank adjusted.*

Figure 33 presents data from DAPI cell count for control samples at 8ºC.

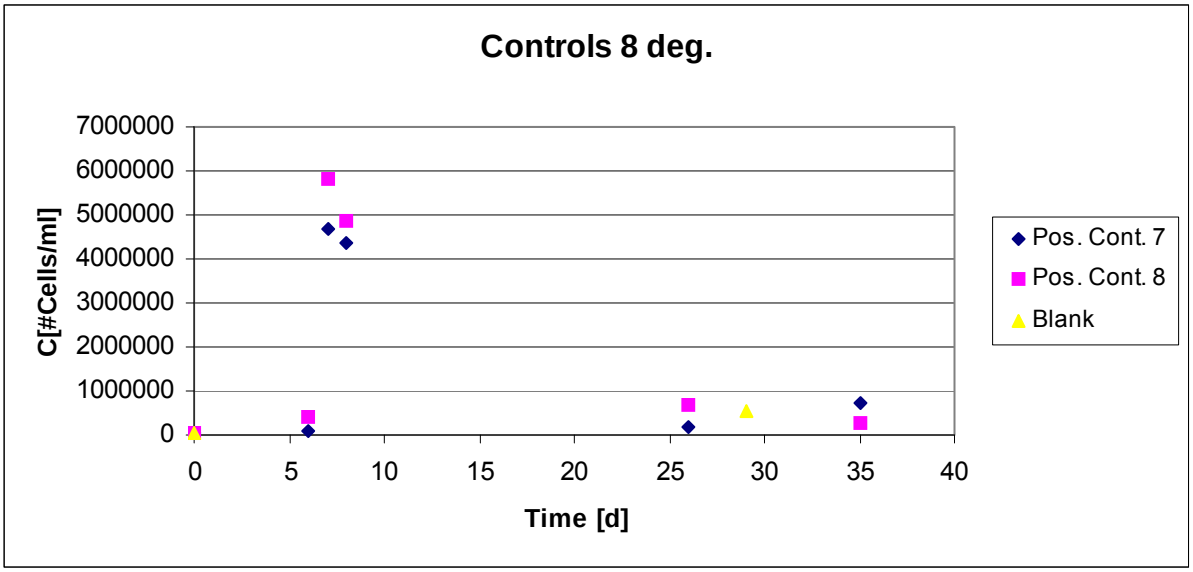


Figure 33: *Results for control samples, DAPI cellconting, 8ºC.*

4.4 Results 15ºC

4.4.1 Samples 15ºC

Figure 34 presents the blank adjusted BOD curves for naphthalene samples at 15 ºC. The reason why the sample number 5 shows such an odd behavior, is that DGGE sample was taken after 5 days.

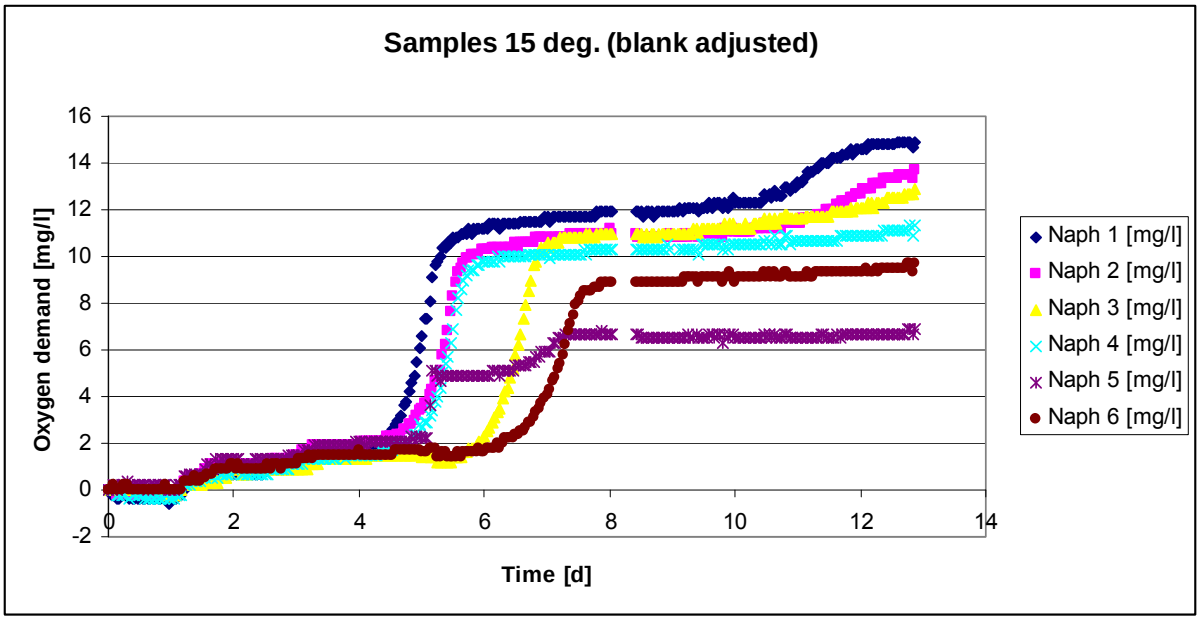


Figure 34: *Results for naphthalene samples, OxiTop®, 15ºC, blank adjusted.*

Figure 35 presents the data from GC analysis for naphthalene samples at 15ºC.

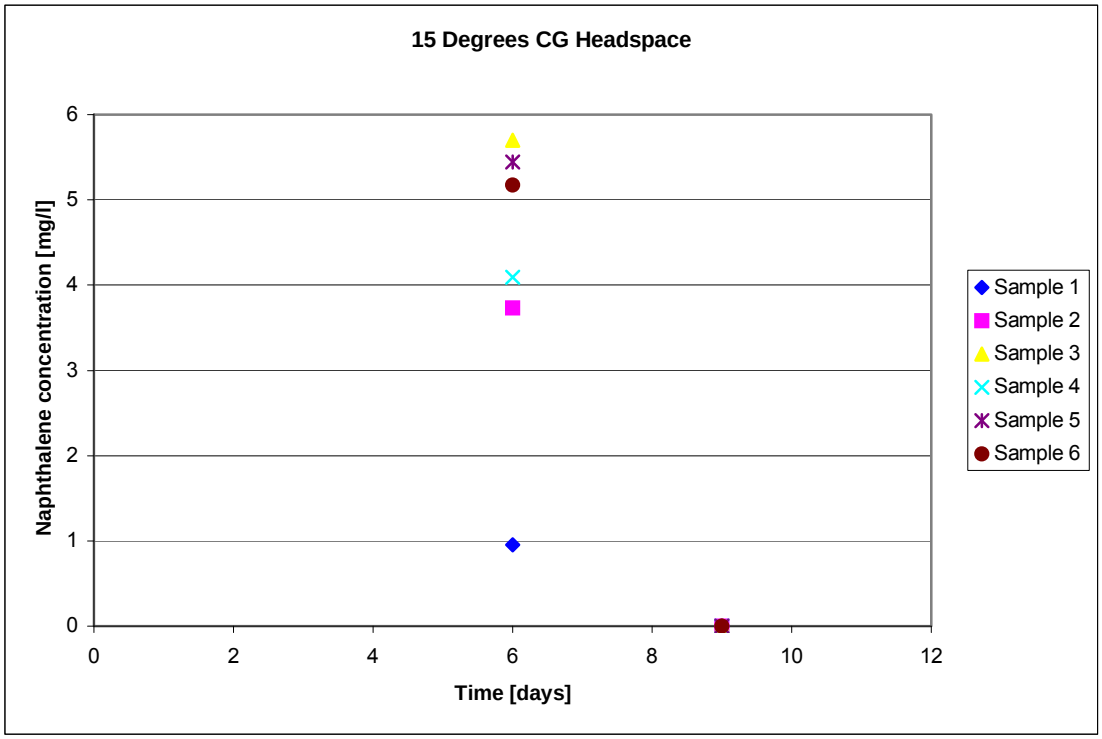


Figure 35: *Results for naphthalene samples, GC headspace, 15ºC.*

The results from DAPI cell counting for naphthalene samples at 15ºC is presented in Figure 36.

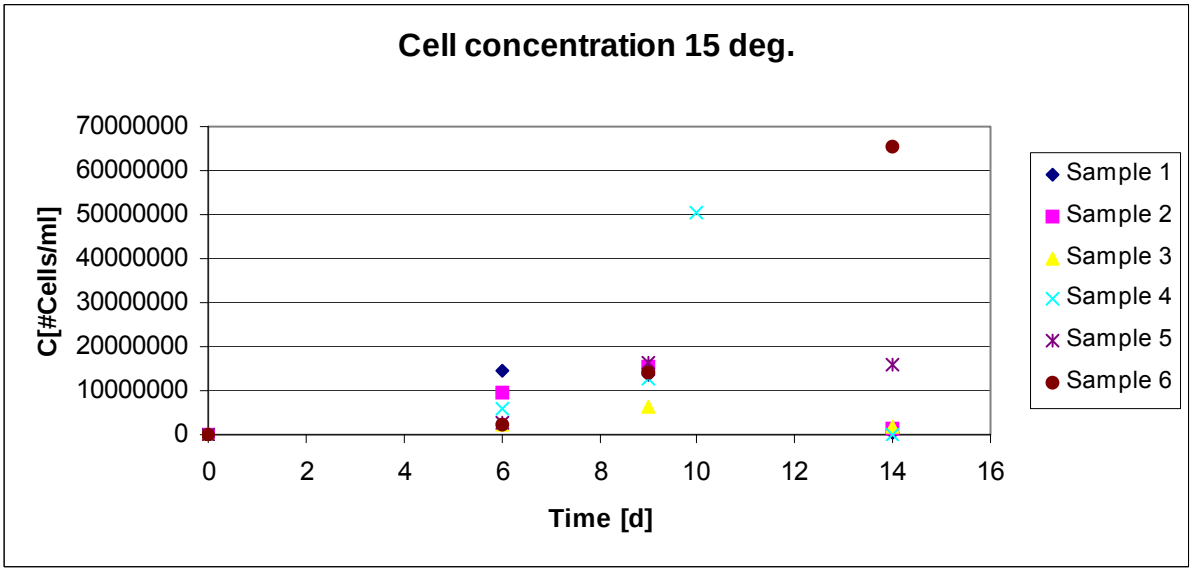


Figure 36: *Results for naphthalene samples, DAPI cellconting, 15ºC.*

Table 4 presents 50% degradation times and degradation constant, k_1, at 15ºC.

Table 4: 50% degradation time and degradation constant, 15ºC

Sample no	$t_{1/2}$ [d]	
31	5.12	
32	5.54	
33	6.58	
34	6.50	
36	7.16	
Mean 15 deg	6.18	
St. dev.:	0.83	
St. dev. Mean	0.4	
k_1:	0.112	d^{-1}
st. dev. K1	0.007	

4.4.2 Controls 15ºC

Figure 37 and 38 shows the BOD curves for control samples at 15ºC. Fig. 38 is blank adjusted.

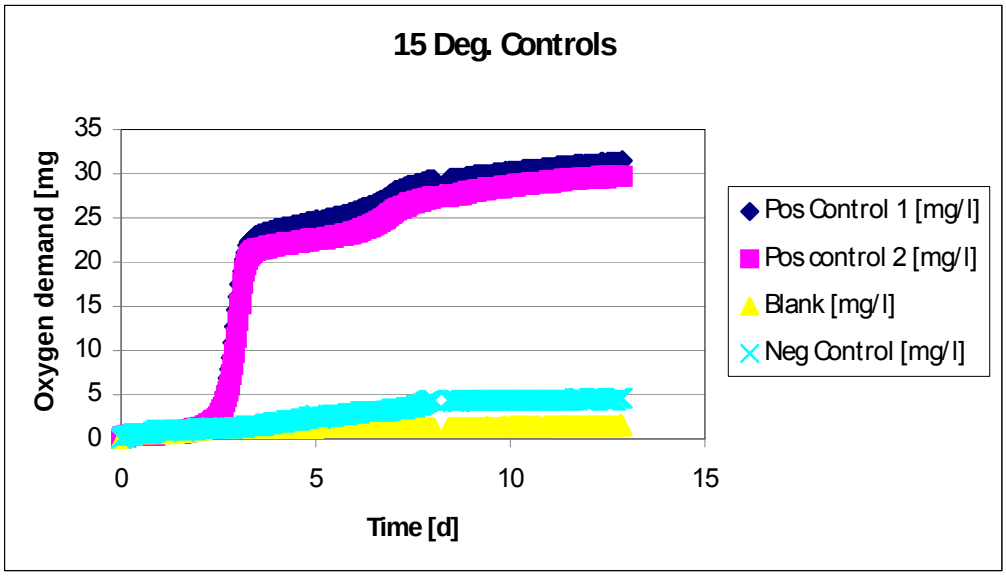


Figure 37: *Results for controls, OxiTop®, 15ºC.*

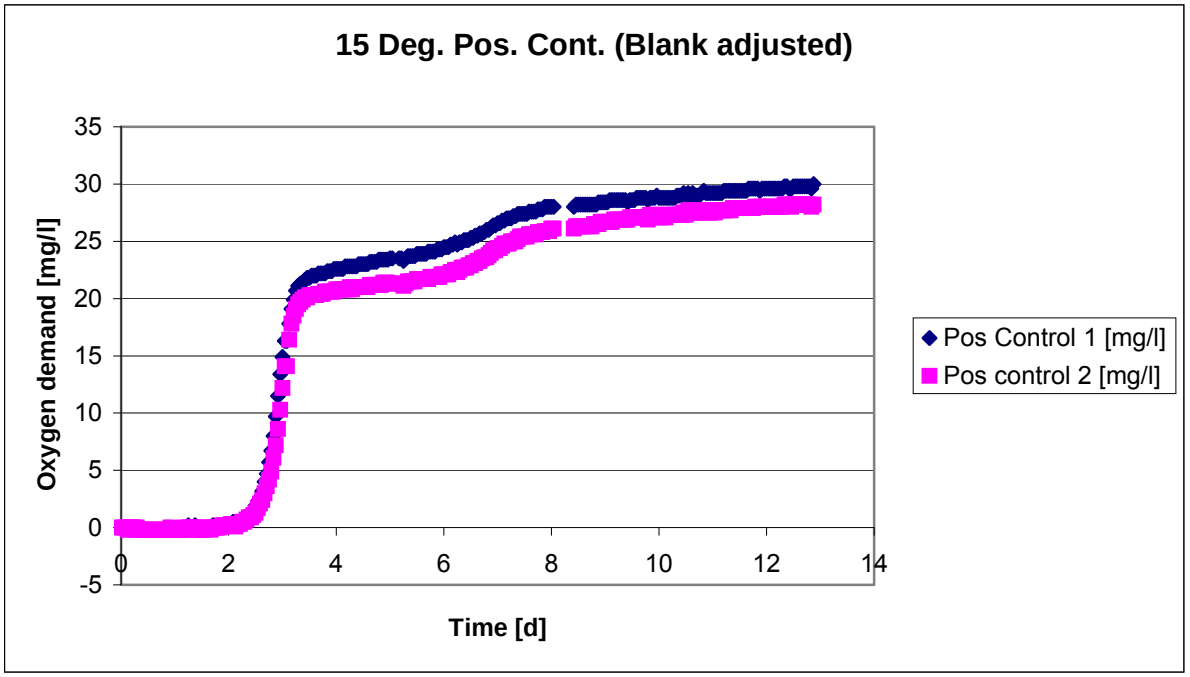


Figure 38: *Results for controls, OxiTop®, 15ºC, blank adjusted.*

Figure 39 presents TOC concentration in control samples at 15ºC. The benzoate seems to have been taken up by microorganisms before the first sampling.

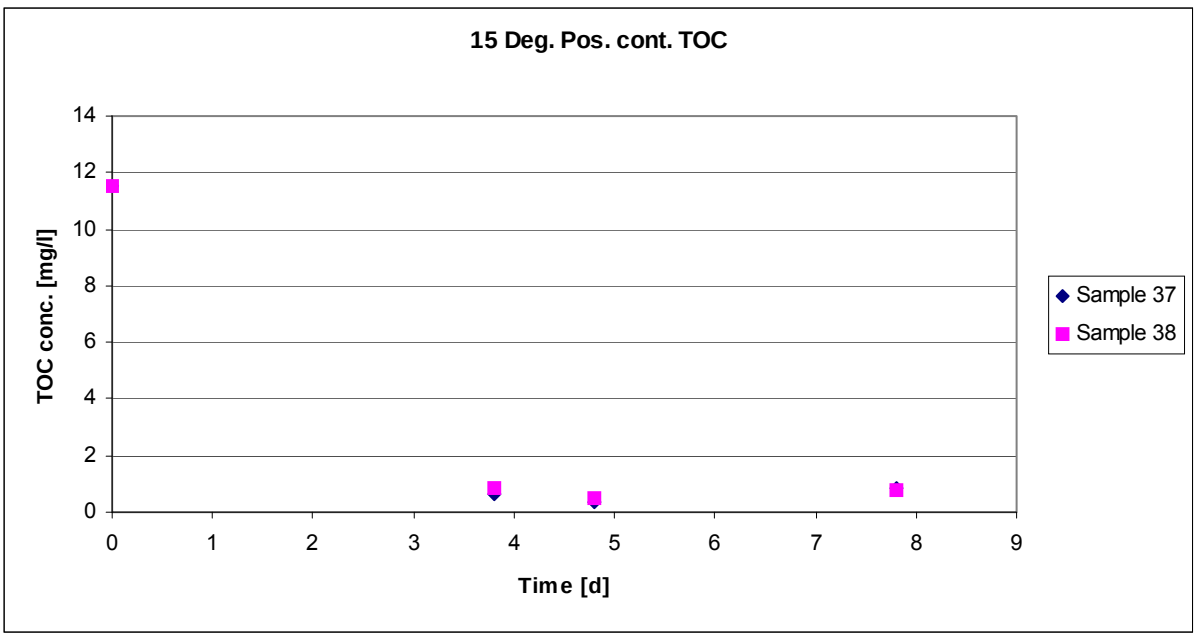


Figure 39: *Results for controls, TOC analysis, 15ºC, blank adjusted.*

Figure 40 presents data from DAPI cell count for control samples at 15ºC.

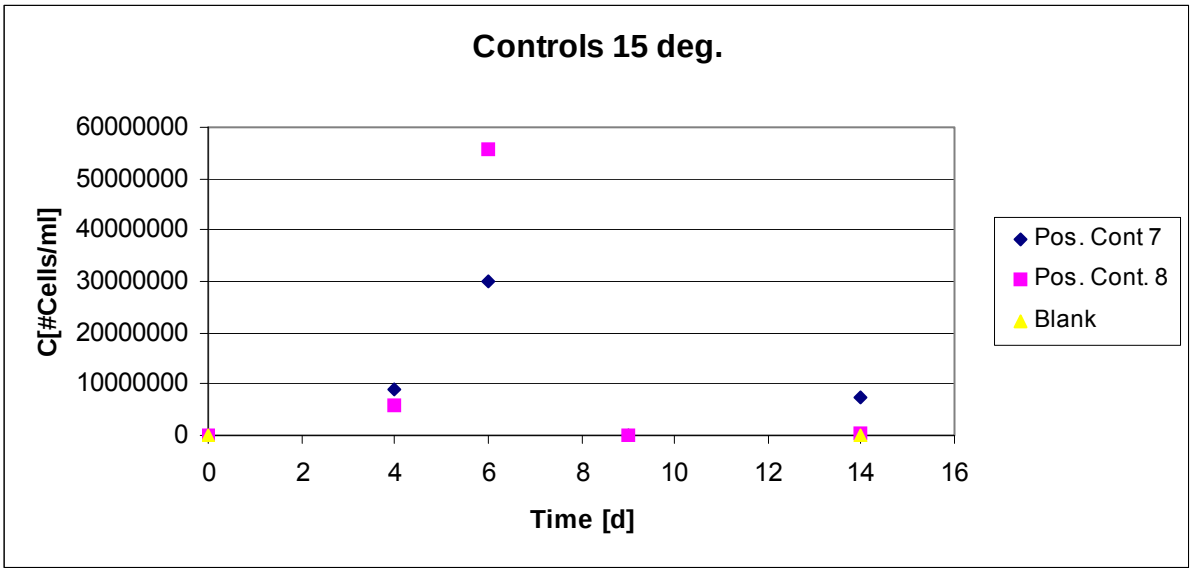


Figure 40: *Results for control samples, DAPI cellconting, 15ºC.*

4.5 Growth coefficients

Table 5 and Figure 41 presents the growth coefficient as a function of temperature. The relationship between k_1 and temperature in the temperature area of the research temperature, assuming first order kinetics, was found to be:

$k_1 = 0.0002t^2 + 0.0029t+0.0196$

The expression was found using polynomic regression in Microsoft Excel.

Table 5: k1 values for different samples, with their calculated standard deviations.

Temperature [Deg. C]	k_1 [d^{-1}]	St. dev., k_1
0.5	0.021	0.002
4	0.035	0.001
8	0.056	0.004
15	0.112	0.007

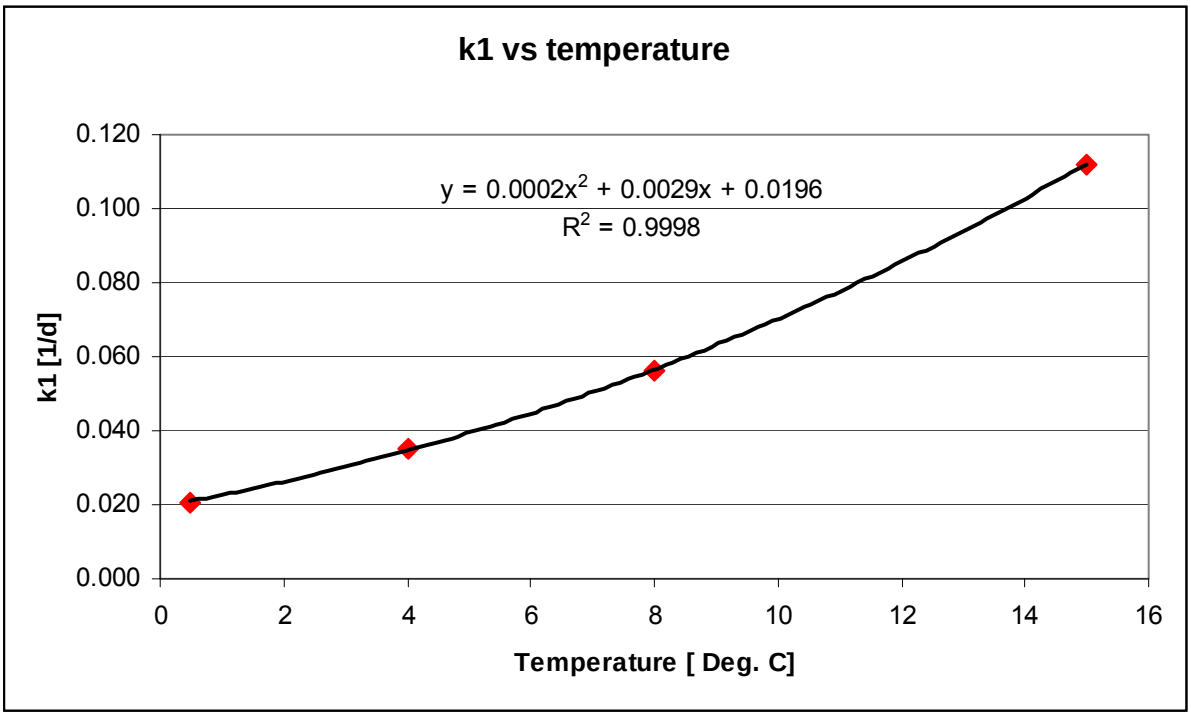


Figure 41: *Growth coefficient as a function of temperature.*

5. Discussion

5.1 Differences between lab scenario and natural environment

This work presents results from a research of biodegradation of naphthalene at four different temperatures relevant for Norwegian climatic conditions. Naphthalene has been chosen as organic substrate, because it is a quantitatively important compound in produced water and also the water soluble fraction of fuel oils.

There are lots of systematic differences between the conditions in the sample bottles of this research and petroleum released to sea. One difference is that the research system has only one available carbon source, naphthalene. When petroleum is released to sea, it is a complex mixture of chemicals that is being released to the marine environment. The degradation of the more complex organic chemicals could be inhibited by the availability of more easily degradable organic compounds. [11]

Another difference between this lab experiment and a natural environment is that in this experiment natural predators are filtered away. This could be a factor that makes biodegradation go faster in the lab experiment compared to in a real marine environment, because the degrading bacteria becomes fewer when many are taken by predators.

There are also larger organisms in the marine environment that compete with the bacteria about the organic substrate, like green algae. This would also affect the bacterial degradation. [26]

It is also a difference from natural marine environment with the abundant availability of nutrients that bacteria in this research have had. But in the sea there is also continuous supply of nutrients from neighboring water. Because of this it can be a good simulation of the real situation, in many cases when readily degradable carbon is not available in so large amounts that nutrients are being depleted in large volumes of water, to add more than sufficient nutrients for full substrate biodegradation.

Still another difference is that the walls of the bottles were made of glass. Glass is a material that organic molecules not so easily will attach to as most surfaces in the marine environment. Hydrocarbons becomes less bioavailable when they attach to for example particles in soil and sediment. [11] But the glass bottle walls combined with continuous stirring, made the sample bottles a good simulation of biodegradation of naphthalene that is dissolved in the water in the pelagic area of the sea.

Weathering is not possible in a closed system. Naphthalene is a very volatile compound, and therefore I would expect that much of the naphthalene would have evaporated to air if the introduced to a natural environment. (This would have been depending on depth of release point, currents, positioning related to surfaces to which it could attach etc.) Weathering is relevant when looking at the fate of contaminants in marine environment, but it is not part of biodegradation. It is the biodegradation of naphthalene that is the focus of the research presented in this Master`s.

There are lots of other differences that may play a role on speed of degradation, but still it seems like this experiment is suitable to give relevant indications regarding effect of temperature on growth rates in marine environment.

5.2 Evaluating lab methods

5.2.1 OxiTop® method

With data points collected every 1 hour, the OxiTop® manometric respirometric method for oxygen demand measurement provided the closest monitoring of biodegradation in the sample bottles of all the analysis methods used in this research. But the method was not used totally without challenges.

Some challenge can be easily seen from the raw data from naphthalene sample bottles at 0.5ºC presented in Figure 56: The curve is not continuous. One thing is the small ups and downs that happens approximately every 24 hours. That is caused by

the de-icing periods that took us by surprise in the refrigerator set to 0.5 degrees (mentioned in chapter 4 Results). The reason for that this shows so clearly on the OxiTop® curves, is that gases have the highest solubility in seawater at low temperatures. Therefore more of the headspace gases gets into the solution when the temperature is at its lowest. Therefore the headspace pressure will fall, and the OxiTop® system will count the lower pressure as oxygen consumption. When the next de-icing period comes, temperature will rise, and dissolved gas will be pushed out from the water phase, leading to increased pressure in bottle headspace. The increased pressure will be counted as negative oxygen consumption by the OxiTop® system. The effect that the de-icing periods had on the graphs for the bottles at 0.5 degrees was to a great extent eliminated by subtracting the values of the blank sample from the other samples. That is done for all temperatures, also the others that do not have the de-icing problem. It is done because what is of interest in this research is the biological oxygen consumption that comes as a result of the substrate degradation, and not any additional oxygen demand that is caused by anything else, either it be physical, chemical or biological. The reason why the subtraction of the blank sample values from the values of the other samples does minimize the effect that the de-icing periods have on the BOD curves, is that the blank sample bottle was subject to the same de-icing periods as the other bottles, and therefore had similar effect on its BOD curve.

The larger “jumps” on the graph in Figure 56 comes as results of opening of sample bottles. When samples for the other analysis methods were to be taken, the sample bottles had to be opened. It can be observed that sometimes the curves for all of the samples seems seems to go down, while other times they are taking a leap upwards in relation with opening of flasks. That is in fact due to changing atmospheric pressure. The 0-value of the graph is related to the atmospheric pressure on the day the OxiTop® BOD logging was started. If the atmospheric pressure is higher at another day when the bottles are opened, it will show on the curves as a negative oxygen consumption.

Also when the bottles are vented, new oxygen are being supplied to the system, and a new equilibrium is being established. Therefore the first few data points after a venting were removed.

The curve pieces were moved up or down so that it fitted as an extension of the previous curve piece. There are uncertainties related to how the pieces are put together, and especially after the hole with missing data the three days after day 30. This hole in data, came as because the OxiTop® system was not restarted earlier after the program it was set to fulfill automaticly stopped at 30 days.

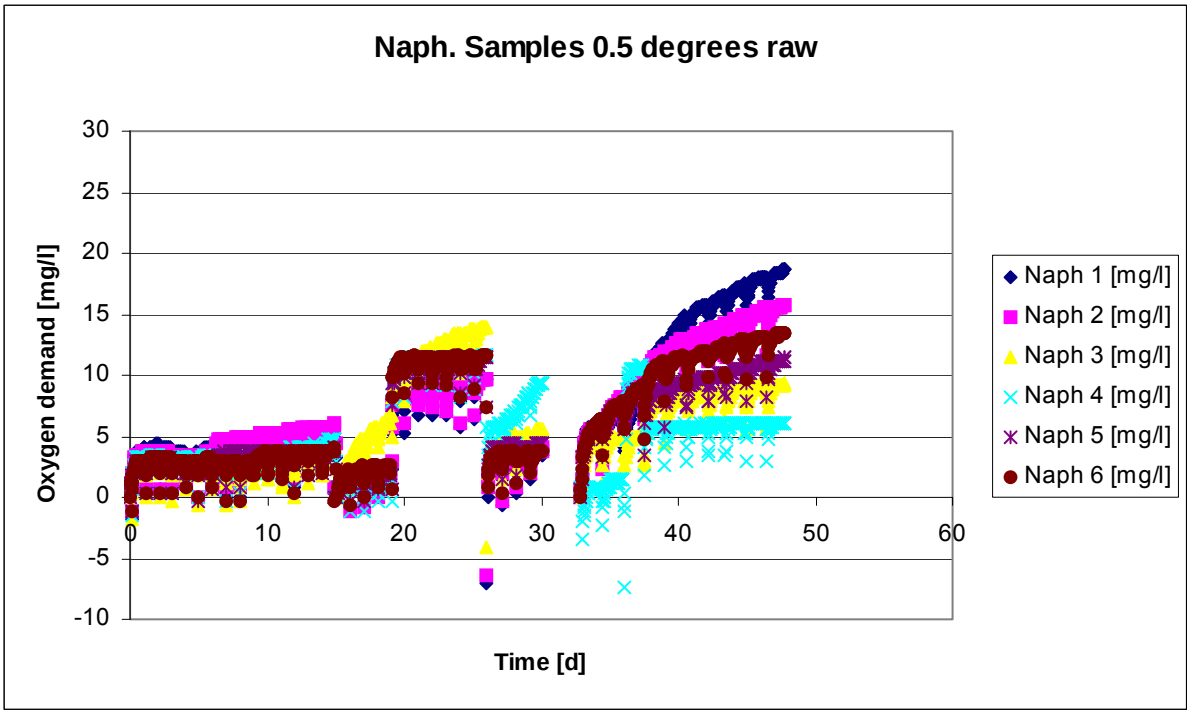


Figure 56: *Raw OxiTop® data from naphthalene sample bottles at 0.5ºC.*

5.2.2 Other methods of analysis

All the manual weighing, pipetting, and transferring, related to both the making of the calibration curves, and the sampling on the lab introduces uncertainties. There is also always a systematic uncertainty in the different instruments. There is not enough time to put numbers on these uncertainties, and some of them are also very difficult to quantify.

The DAPI analysis does also have uncertainties related to the microscope, and to the program used for cell counting. In some cases the counting program do count the

images wrong, like Picture 10 shows us. But it generally gives a good estimation of mean number of cells, because as many as 15 pictures were taken for each sample.

Cell counting exclusively is not a good way to find biomass in an aqueous system, because cells have very different size. A better biomass estimation could have been found if mean bacterial weight had been estimated for each sample. Filtration and weighing could also be used. But that would require large sample volumes to get weighable amounts of solids. One sample bottle could perhaps be sacrificed when the biomass is at is peak. This peak is not necessarily easy to hit, so that would require close monitoring of BOD curve.

5.3 Discussion of research results

The results from the different analytic methods are in many ways giving very consistent results. For example the first sample number at each temperature where the naphthalene concentration starts falling dramatically, is the same sample number where the BOD curve first starts rapid growth. There are a few exceptions from those general trends of consistency, especially among the DAPI results. The results from the DAPI analysis are believed to produce more uncertain results than the chemical analysis and the OxiTop® BOD curves, both because it contains so many steps of manual operations where things can be done inaccurately, and partly because the available microscope is not a very good one, and because the counting program does count number of cells in an image wrong if the images are not good enough. The calculations of biomass and COD based on cell numbers found by DAPI cell counting, have an extra implicit uncertainty attached: The cell size are very different. Some big cells are many times larger than smaller cells. Therefore that kind of biomass estimation has to be regarded as very unprecise. But both the DAPI images and the cell concentration are interesting and valuable pieces to the puzzle that describes biodegradation.

The 50% degradation times that served as data background for the estimated relation between growth rate and temperature (Table 5 and Figure 55), produced a very accurate polynomic regression (almost linear), and the mean 50% degradation

times had very low standard deviations based on spreading of 50% degradation times for single sample bottles.

One thing that is worth mentioning from the results, is that the naphthalene concentration in the samples at 0.5ºC seems to be quite a lot lower at the first sampling date for GC analysis than the naphthalene concentrations at higher temperatures. The first sampling dates for the other temperatures shows concentrations of 5-6 mg/l, while its only 1.5-2 mg/l for the samples at 0.5ºC. That is quite remarkable, since the BOD curve shows that no extensive degradation had started when the first sampling was done. The BOD curves for the different temperatures does also show that the final BOD is not any lower at 0.5ºC than at the higher temperatures. The BOD curves ends at about 15 mg/l at all temperatures. The exception from this are the BOD curves for the samples at 8ºC, where the curves reaches only 10-12 mg/l. But the reason for that is probably that the OxiTop® BOD monitoring was stopped a bit too early at 8ºC. Similar oxygen demands indicates that initial concentration of substrate was also similar.

Some naphthalene may have been lost when the stock solution was transferred to sample bottles, so that the initial naphthalene temperature would be less than 9.5 mg/l, as it was supposed to be, and some crystals were also observed in the stock solution bottle, indicating that not all the naphthalene was dissolved. But it there is nothing in this transfer procedure that would lead to the assumption that there would be less naphthalene in the sample bottles at 0.5ºC. In fact the coldest samples were completed first, and all the time naphthalene evaporation was attempted minimized.

The only difference between the samples at 0.5ºC compared to all the others, except from the temperature was the kind of bottles used for the research. The sample bottles used for at 0.5ºC were the kind with two extra smaller openings in addition to the large one on the top. The big opening on the top, and one of the smaller openings on the side was capped with screw caps. The OxiTop® head was mounted directly on the last opening, without any adapter, only with the rubber containers for NaOH going into the openings. The rubber containers were so long that they reached below the water surface. My theory for the missing naphthalene in the sample bottles at 0.5ºC is that it was attached to the surface of the rubber container. This theory is

backed by the fact PAHs tends to attach to surfaces [11], and that biofilm development was discovered on some of the containers. Biofilm could have developed on the rubber container anyway, but the spot would be extra favourable if the rubber container was covered with substrate.

As a result of this the rubber container for NaOH should be kept out of the water phase to avoid possible attachment of substrate to the rubber, when using the OxiTop® manometric respirometric method for headspace oxygen monitoring.

Several of the OxiTop® BOD curves, and the TOC curves shows negative concentrations. Negative concentrations make no sense. The reason why it has become that way is, for the TOC curves that the values for the filtered seawater blank have been subtracted from the sample values. Uncertainties in all the joints of the procedure are the reason why a blank sample sometimes gets a higher value than a sample where all the organic carbon has been depleted. For the OxiTop® BOD curves, the explaination are both related to the substraction of the blank, and from uncertainties in the manometric respirometric analysis method.

6. Conclusion

Bacterial biodegradation of naphthalene was studied at 0.5, 4, 8 and 15 ºC, under simulated marine conditions. Growth rates were found for each temperature based on 50% degradation times. The growth rates were used to establish a relation between the growth rates (k_1) and temperature.

The found k_1 value at 0.5ºC was $0.021d^{-1}$, at 4ºC it was $0.035d^{-1}$, at 8ºC it was $0.056d^{-1}$ and at 15ºC it was $0.112d^{-1}$. That gives a relation between k_1 and temperature (t) of: $k_1 = 0.0002t^2 + 0.0029t + 0.0196$

Chemical substrate analysis, manometric respirometric method for headspace oxygen monitoring and microscope counting of DAPI stained cells was used to monitor the biodegradation. The results obtained from the different analytic techniques were quite consistent with each other.

7. References

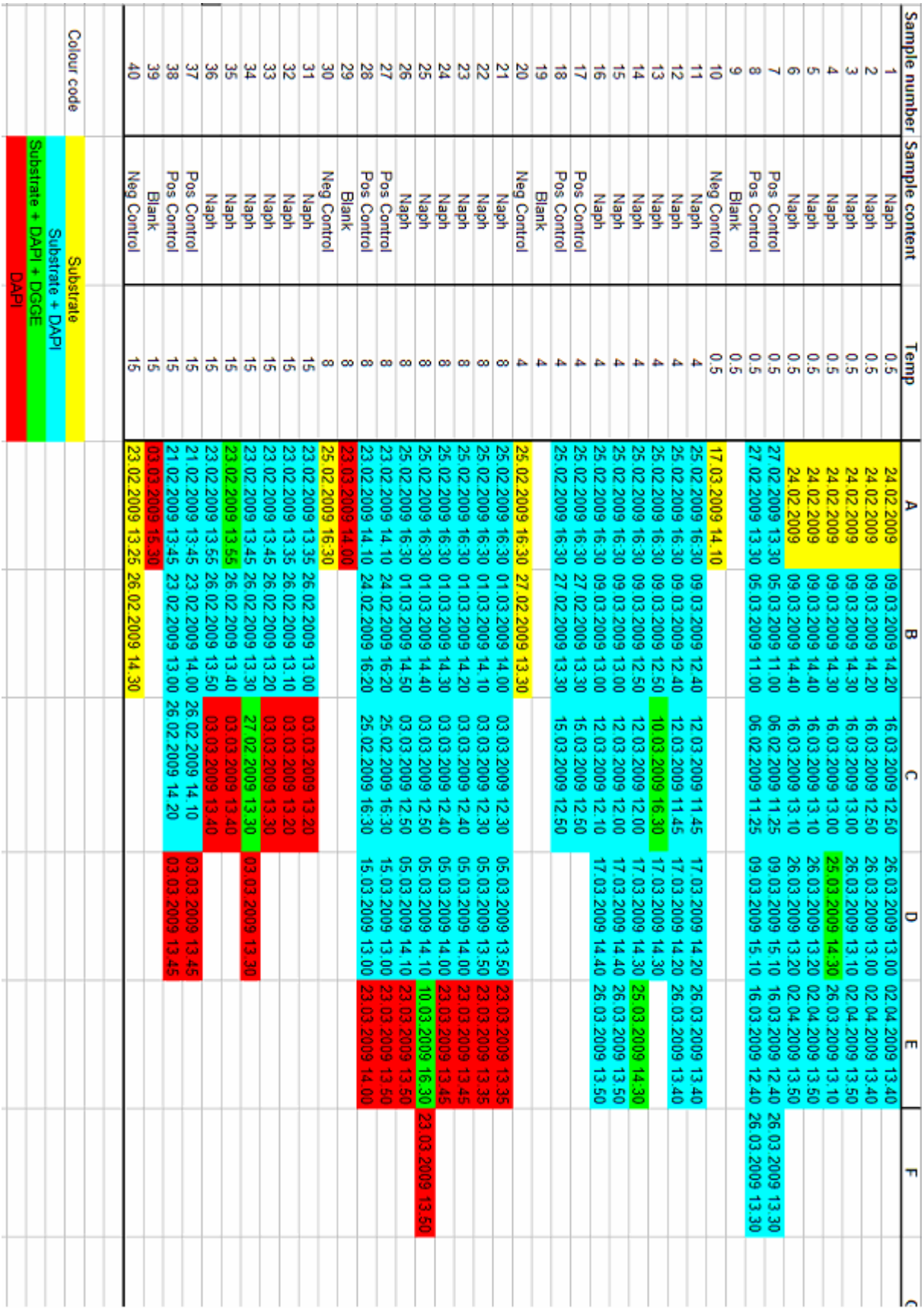
All internet links was valid at June 29, 2009.

1. Atlas, R. M. and Bartha, R., ”Microbial Ecology Fundamentals and applications”, Fourth edition, Benjamin Cummings Publ., New York, 1998, pp. 523-531
2. Hart, H., Craine, E. and Hart, D. J., ”Organic chemistry, a short course”, Elleventh edition, Houghton Mifflin Company, Boston, 2003, pp. 136-140
3. Fused Ring and Bridged Fused Ring Nomenclature (IUPAC Recommendations 1998): http://www.chem.qmul.ac.uk/iupac/fusedring/FR1.html#122
4. Casarett, L. J., Klaassen, C. D., Doull, J, ”Casarett and Doull's toxicology : The Basic Science of Poisons”, sixth edition, McGraw-Hill Professional, USA, 2001 pp. 241-245
5. Marshall A.G. and Rodgers R.P. (2004) “Petroleomics: The next grand challenge for chemical analysis”, Accounts of Chemical Research, 37(1), p. 53-59
6. University of Minnesota, Biocatalysis/Biodegradation Database: http://umbbd.msi.umn.edu/naph/naph_image_map.html
7. Lecture notes, MBI 100 Ecotoxicology
8. MSDS, Naphthalene: http://www.sciencelab.com/xMSDS-Naphthalene-9927671
9. Lloyd, Robert Andrew, (2005) “Pressure effects on marine biodegradation of low molecular weight aromatics” Master Thesis, University of Stavanger
10. Calbet, A., Saiz, E. and Barata, C, (2006) “Lethal and sublethal effects of naphthalene and 1,2-dimetylnaphthalene on marine copepod *Paracartia grani*”, Mar. Biol./Springer, Vol. 151, p. 195-204
11. Peng, R. and Xiong, A et. al., (2008) “Microbial biodegradation of polyaromatic hydrocarbons”, FEMS Microbiol. Rev., Vol. 32 p. 927-955
12. Atlas, R. M. and Bartha, R. (1972) “Biodegradation of petroleum in seawater at low temperatures”, Can. J. Microbiol., Vol. 18, p. 1851-1855
13. Karhu, Kaakinen et al. (2008) “Biodegradation of light fuel oils in water and soil as determined by the manometric respirometric method”, Water air soil pollut/Springer, Vol. 197, p. 3-14

14. Research review, “Effekter av produksjonsvann på egg og larveutvikling samt kjønnsdifferensiering hos torsk”, Havforskningsinstituttet (2001-2003) : http://www.imr.no/__data/page/2090/Produsert_vann_reduserer_veksten_hos_torskeegg_og_-larver.pdf
15. Heggø, A.L., (2005) “Effects of temperature on biodegradation of some low molecular weight aromatics” , Master thesis, University of Stavanger
16. Environmental report, OLF, 2007: http://www.olf.no/getfile.php/Dokumenter/Publikasjoner/Miljørapporter/080805%20OLF%20Enviromental%20report%202007.pdf
17. van Hamme, J. D., Singh, Ajay og Owen, P. W., (2003) “Recent Advances in Petroleum Microbiology”, Microbiology and Molecular Biology Reviews, Vol. 67, No. 4, page 503–549
18. Paul, J.H., “Methods in microbiology – marine microbiology”, Academic Press, San Diego, 2001 pp. 129-160
19. Tchobanoglous, G., Burton, F.L., and Stensel, H. D., “Wastewater Engineering, Treatment and Reuse”, Fourth edition, McGraw Hill/Metcalf & Eddy, New York, 2003, pp. 580-588
20. Si-Zhong, Y., Hui-Jun, J., (2009) ” Bioremediation of Oil Spills in Cold Environments: A Review”, Pedosphere, Vol. 19, Issue 3, p. 371-381
21. Margesin, R. and Schinner, F., (1999) ”Biological decontamination of oil spills in cold environments”, Journal of chemical technology and biotechnology, Vol. 74, Issue 5, p. 381-389
22. Madigan, M. T. and Martinko, J. M. ”Brock, Biology of microorganisms”, Elleventh edition, Pearson Prentice Hall, Upper Saddle River, USA, 2006, pp. 151-157
23. Kommedal, R., Personal communication.
24. OLF, (2004) “Redusert oljeinnhold i produsert vann”, Oljeindustrien, Vol 10, Issue 9,: http://www.olf.no/aktuelt/redusert-oljeinnhold-i-produsert-vann-article1033-198.html
25. Macay, D. and Wania, F., (1995) ”Transport of contaminants to the arctic – partitioning, processes and models”, Science of the total environment, Vol. 161, p. 25-38
26. Ca´ceres, T.P., Megharaj, M. and Naidu, R. (2008) ”Biodegradation of the Pesticide Fenamiphos by Ten Different Species of Green Algae and

Cyanobacteria", Springer Science+Business Media, Published online: http://www.springerlink.com/content/d6t8507524320031/fulltext.pdf

Appendix



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3	Naph	0.5	24.02.2009	09.03.2009 14.30	16.03.2009 13.00	26.03.2009 13.10	02.04.2009 13.50	
4	Naph	0.5	24.02.2009	09.03.2009 14.30	16.03.2009 13.00	25.03.2009 14:30	26.03.2009 13.10	
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8	Pos Control	0.5	27.02.2009 13.30	05.03.2009 11.00	06.02.2009 11.25	09.03.2009 15.10	16.03.2009 12.40	26.03.2009 13.30
9	Blank	0.5						
10	Neg Control	0.5	17.03.2009 14.10					
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39	Blank	15	03.03.2009 15.30					
40	Neg Control	15	23.02.2009 13.25	26.02.2009 14.30				

Colour code
Substrate
Substrate + DAPI
Substrate + DAPI + DGGE
DAPI

App. Fig. 1: *Sample ID file, with all the sampling times and analysis methods.*