Faculty of Science and Technology

MASTER´S THESIS

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Abstract

The oil and gas activities in the northern region have showed an increase over the last years. This year a new field, Skrugard, was discovered in the Barents Sea and the two parliaments in Norway and Russia confirmed the official delimitation line in the former "grey zone" area. As a result of this, the oil and gas activities in this area are only expected to grow.

Chemical dispersants are used in oil spill clean-up processes. It dissolves the oil into smaller particles so it dissolves more easily into the water column. The dispersant makes it easier for the oil to form smaller particles or droplets that are dispersible in the water column. When the oil goes into the water column it is more available to the organisms living there, like fish and mussels. The oil and gas reservoirs in the northern areas like the Barents Sea are located closer to shore than in the southern parts of Norway. If there was an oil spill in this region, the use of chemical dispersant can be necessary in order to prevent it for reaching the shoreline since the oil has a shorter time/distance to be weathered.

In this thesis Atlantic salmon and blue mussels were exposed to oil with and without the present of chemical dispersant agent. The dispersant used in this study was of the same type that were used in the Gulf of Mexico after the Deep Water Horizon accident, Corexit 9500. The objective was to study the effect of the dispersant chemical in relation to uptake and effects of oil in fish and mussels. The result from the exposure time showed that it was a statistically significant difference in the uptake of pollutant in Atlantic salmon in the oil-exposed groups with and without chemical dispersant agent compared to the reference group. However it was not detected a statistically significant difference between the oil-exposed group without chemical dispersant compared to the oil-exposed group with chemical dispersant. In general is seems as the oil-exposed group without chemical dispersant had a higher response than the oil-exposed group with chemical dispersant. The effects study of mussels showed an increasing trend in the groups exposed to oil with and without chemical dispersant compared to the reference group, however this was not statistically proven.

Acknowledgement

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A special thank to my fellow students Marta Velicharova, Suganya Yogarajah and Anton Zubov, which worked together with me as a group on the practical part of this thesis. I would like to thank them all for their contribution and support in the preparation of this thesis work. I would also like to thank Siw Anette Busk for helping us with the sampling.

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1. Introduction

1.1 Oil and gas activities in the Northern Region

The Norwegian oil adventure began in 1969 when Phillips Petroleum found oil and gas in the Ekofisk field, about 250 km west of Stavanger. The production on Ekofisk began in 1971. Today Norway is the world's third largest exporter of oil and gas (Saudi Arabia being the largest and Russia the second largest). Figure 1 shows the annual production of oil and gas on the Norwegian Continental Shelf.

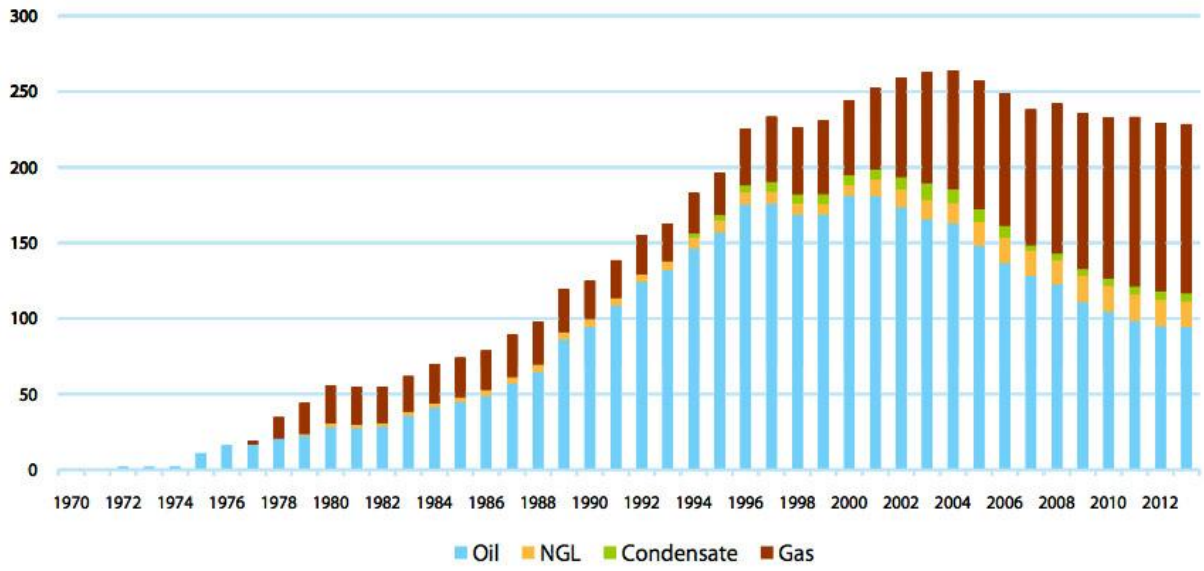

Figure 1. Petroleum production on the Norwegian Self from 1971-2008 with forecast up to 2010. The amount is given in Sm3 o.e. (OLF 2009).

Since the oil peak in 2001 the oil production has been decreasing, whereas the gas production has increased and is expected to be stable for some years to come due to new gas reservoir discoveries. However, the combined amount of oil and gas is decreasing. As a result of the peak oil scenario, oil and gas industries are looking for new reservoirs in areas like Lofoten and the arctic.

More than 60 exploration wells have been drilled in the Barents Sea and a total of 39 production licenses have been awarded since 1980 (OED and NPD 2009). But the Barents Sea is considered an "immature" petroleum area. Many of the fields yield a

series of minor and medium sized gas discoveries. Snøhvit is the only field in production. The gas from Snøhvit is being transported through pipelines to Melkøya, where it is being processed further into liquefied natural gas (LNG) and transported in special designed vessels. Construction of the Goliat field was approved in 2009, and the projected production start will be in 2013 (OED and NPD 2009). Statoil, Eni Norway and Petoro discovered a new oil field in the Barents Sea the 1th of April 2011. The field, Skrugard, is located approximately 100 km north of the Snøhvit field and has an estimated volume of 150-250 million recoverable barrels of oil equivalent (boe) (Statoil 2011).

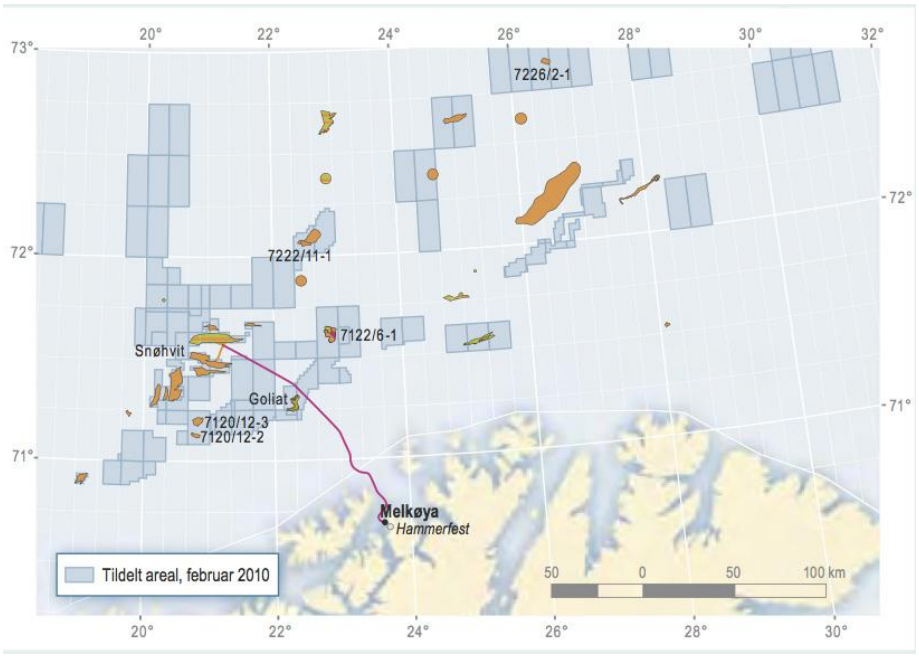

Figure 2. Geographical overview of the different fields and reservoirs in the Barents Sea, not including Skrugard. (OED and NPD 2009).

The Shtokman field is the world's larges subsea gas field. It is on the Russian side of the Barents Sea and is located 555 km northeast of Murmansk. It covers an area of 1400 square kilometre and is estimated to contain 3700 billion standard cubic meters equivalent of gas, and 31 million tonnes of condensate. This is equivalent to the amount of gas found so far on the Norwegian continental shelf. The stakeholders in Shtokman are Gazprom (51 %), Total (25 %) and Statoil (24 %) (Statoil 2008).

1.2 Scope of this work

The aim of this thesis was to study the biological effects of oil exposure in fish and mussels with and without the presence of a chemical dispersant. The dispersion agent that was used, Corexid 9500, is of the same type that was used in the Gulf of Mexico after the Deep-Water Horizon accident in 2010, whereas the oil that was used was an Arctic crude oil. Atlantic salmon (*Salmo salar*) and blue mussel (*Mytilus edulis*) were exposed to a sub-lethal concentration of the crude oil administered in seawater with and without a sub-lethal concentration of dispersion agent. The exposure period was 13 days including two days of sampling.

An important aspect of this study was the performance of the practical work as a group work that included both Russian and Norwegian students. Apart from me, the other students were: Marta Velicharova, Suganya Yogarajah and Anton Zubov. The biomarker methods used where approximately the same for all of the group members. Although the practical work was performed as a group, all students had different approaches to their thesis works.

The results from the different biomarker methods were shared between students in order to have a better overview of the biological effects in the exposed organisms. In the theoretical part, all of the biomarker methods used in this study will be briefly described since all of the biomarker responses will be given in the result. In the methodology part only the biomarker methods preformed by me, or where I participated, will be described.

2. Theoretical Background

2.1 Basic concept in ecotoxicology

The term ecotoxicology was introduced by Truhaut in 1969, he defined it as the science describing toxic effect of various compound on living organisms, especially on population and communities within an ecosystem (Walker 2006). Ecotoxicology concerns all environmental compartment, but aquatic ecosystems like freshwater, estuaries and marine ecotoxicology are much studied. In this thesis it is the marine ecosystem that is being studied.

In this thesis it is important to understand some basics in ecotoxicology. Areas like bioconcentration, fate of organic pollutants in individuals and toxicity tests will thus be described in this sub-chapter.

2.1.1 Bioconcentration

When a chemical is entering the marine ecosystem not all of the pollutant can enter the organism, it can be partitioned between different phases like water, sediment or biota. The bioconcentration factor (BCF) is the internal exposure concentration in an organism, it is also known as body burden. BCF is determined by uptake and elimination processes of chemical in an organism, this process are influenced by different factors such as temperature, ventilation rates, metabolism, type of species and the characteristic of the chemicals.

At equilibrium, the BCF is calculated based on:

$$BCF = \frac{C_b}{C_w}$$

Where C_b is the concentration in biota and C_w is the concentration in water, these BCF are specific for each species and compound (Walker 2006).

2.1.2 Fate of organic pollutants in organisms

The fate of a xenobiotic in an individual organism can be represented as figure 3 shows.

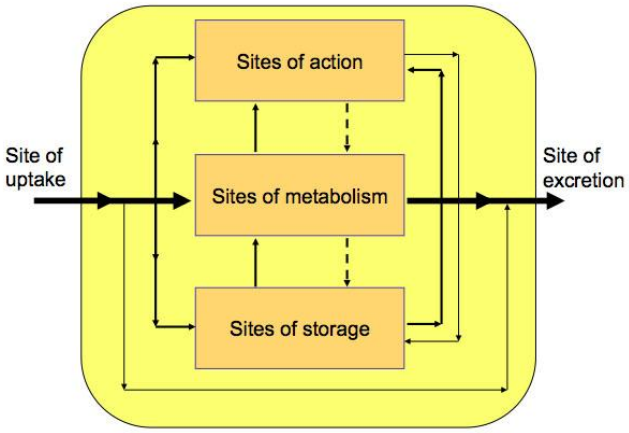

Figure 3. From a conceptual point of view there are five major sites of pollutant interaction in an organism.

Uptake route

Aquatic organisms are exposed directly to many pollutants dissolved or suspended in the water. An important route of entry for many dissolved pollutants are uptake over respiratory surfaces or skin. For fish and mussels the major uptake route is their gills. Uptake from food may also be important, but this is particularly important for terrestrial animals such as birds, mammals and reptiles. It is also important to consider the transfer of pollutants from parent to offspring as a route of uptake (Walker 2006).

Distribution

In vertebrates, absorbed pollutant travels through the bloodstream, and in the lymph (to a lesser extent). If the pollutant is absorbed through the gut, much of the pollutant will initially be taken to the liver by the hepatic portal system. Figure 4 shows the fish circulation system: the heart pumps blood through the gills filaments, where oxygen is being absorbed and carbon dioxide is being expelled. Then the oxygenated blood is spread to all part of the body (Beyer 2010a).

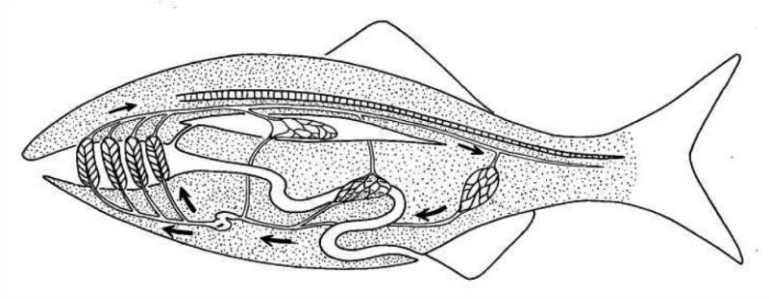

Figure 4. A schematic representation of the circulation system in fish (Mackean 2004)

In invertebrates the movement of organic pollutant is in the haemolymph. Within blood and lymph, organic molecules are distributed between different components according to their solubility properties. Lipophilic compound will be associated with lipoproteins and membranes of blood cells, it will have little tendency to dissolve in blood water. Conversely, more polar components will tend to dissolve more in water an associate less with lipoproteins and membranes of blood cells (Walker 2006).

Storage

Xenobiotic can be located in places where they are not able to interact with their site of action and therefor not subject to metabolism. For lipophilic pollutant preferred lipid rich compartments in the organism. These can be fat deposit and lipid rich tissue, but it can also be circulating lipoproteins micelles and subcellular compartment e.g. fat vacuoles and membrane structures. Some pollutant, especially toxic metals, can sequester into specialized sequester proteins or biochemical inert storage compartments such as bones and teeth.

Metabolism

Enzymic metabolism for most lipophilic pollutants occurs in two phases, see figure 5.

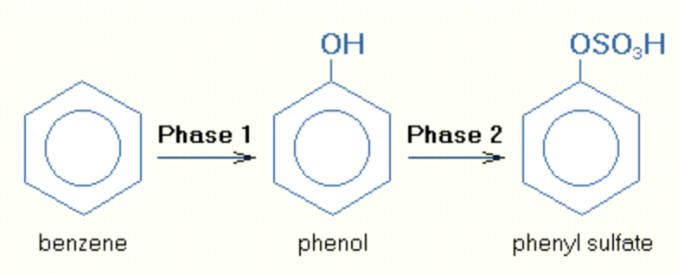

Figure 5. A simplified scheme of phase I and II biotransformation.

Phase I involves oxidation, hydrolysis, hydration or reduction in most cases which leads to production of metabolites that contains hydroxyl groups. The reaction in phase I, is mostly catalysed by enzymes in smooth endoplasmic reticulum (SER). In phase II, which happens in SER and cytosol, there is a conjugation of a substrate with a highly hydrophilic group. These two groups lead to a progressive increase in water solubility, from a lipophilic pollutant to a more polar metabolite an then to an even more polar conjugate (Walker 2006).

Sites of excretion

Major pathways for excretion of xenobiotic pollutant is the renal system (kidney-urine), liver-bile route and across respiratory epithelia (gill and lung). Minor routes of excretion can be through the mother's milk, sweat, saliva, tears and semen.

Elimination of xenobiotic pollutant in the feces occurs from two processes: excretion in bile and direct excretion into the lumen of the gastrointestinal tract. Biliary route is an important mechanism for fecal excretion of xenobiotic, and it is even more important for the excretion of their metabolites (Walker 2006)

2.1.3 Toxicity test

To study the individual organisms response to different chemical pollutions, toxicity tests are used. The test is typically performed on a population exposed to different concentration of a chemical under controlled conditions over a specific period of time. All chemicals are toxic in a sufficient enough amount, but a chemical is only considered toxic if it can induce harm at low concentrations. Rand and Petrocelli (1985) defined toxicity as the "inherent potential or capacity of a material to cause adverse effect in living organisms" (Rand and Petrocelli 1985).

In toxicity tests, the adverse effects of chemical on the organism depend on the dose and the time of exposure. Toxicity can be measured in many ways. Most commonly, the measure (also known as endpoint) is mortality, but there is a growing interest in the use of more sophisticated indices. Biochemical, physiological, reproductive and behaviour effect can all provide measures of toxicity. Most commonly toxicity tests gives an

estimate of the dose or concentration that will cause a toxic response at a level of 50 %, e.g. the median lethal dose that will kill 50 % of a population. Another approach is to establish the highest concentration or dose that will not cause an effect.

In lethal toxicity tests LD_{50} represent the median lethal dose, whereas LC_{50} represent the median lethal concentration. In toxicity tests that determine these values, it is also possible to determine the highest dose or concentration that cause no toxicity, known as No-Observable Effect Dose (NOED) and No-Observable Effect Concentration (NOEC). NOED and NOEC can only be determined in situations where Lowest Observable Effect Dose (LOED) or Lowest Observable Effect Concentration (LOEC) is known. In toxicity tests with endpoints other than mortality, ED_{50} and EC_{50} are determined. Here the dose or concentration producing the effect in 50 % of the population is determined. As with lethal toxicity tests, NOED and NOEC can also be measured following this approach. However values for NOED and NOEC are only meaningful if a higher dose has shown to produce an effect.

With regard to the test duration, toxicity tests can be split into acute- and chronic toxicity tests. Acute toxicity tests are designed to evaluate the relative toxicity of chemicals for selected organisms in short term exposure tests, usually 24, 48 and 96 hours. Endpoints in acute toxicity tests are normally mortality. Chronic toxicity tests allow evaluation of chemical stress under long-term exposure, example of endpoint can be immobility and growth inhibition (Walker 2006; Beyer 2010b).

Results from toxicity tests can be plotted in a dose-response curves, see figure 6. This graph relates the chemical concentration to the percentage of organisms in test groups exhibiting a defined response.

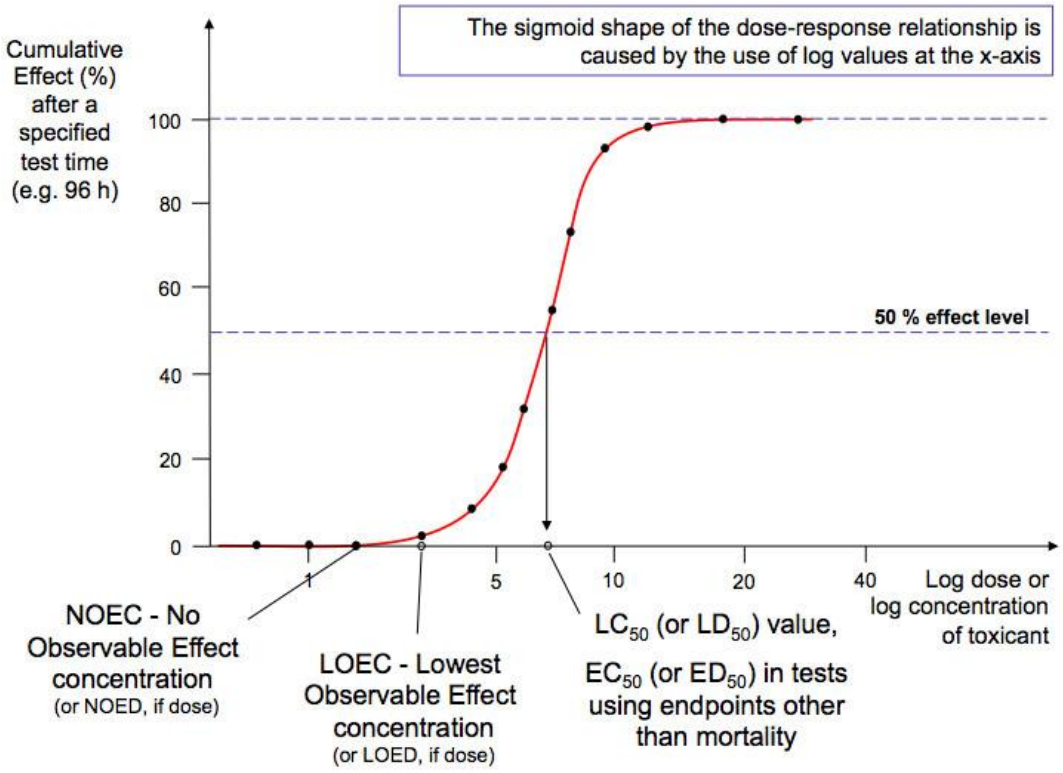

Figure 6. Dose-Response curve (Beyer 2010b).

In order to prevent multiplication of toxicity tests and to improve the validity, comparability and acceptance of these tests, internationally accepted standard testing protocols are organized by Organization for Economic Cooperation and Development (OECD). Some test standardized by OECD e.g. growth inhibition tests of algae, acute toxicity test of zooplankton and acute toxicity test for fish are mandatory tests for toxicity testing of offshore chemicals in Harmonized Offshore Chemical Notification Format (HOCNF) developed by OSPAR (Oslo and Paris commission) (OSPAR 2008; Beyer 2010b).

2.2 Oil contamination of the marine environment

The main sources of oil inputs into the World Ocean are: Natural sources (hydrocarbon oil seeps), offshore oil production (operative discharges and accident), maritime transportation (accidents, illegal discharges), land based discharges and runoff (refinery effluents, municipal waste water, industrial waste water and urban runoff and rivers) and dumping to the sea.

2.2.1 Crude oil

Crude oil is petroleum in its natural state prior to any refining process. The composition of crude oil varies from different geographical areas, but it generally consists of hydrocarbon ranging from smaller, volatile compounds to very large non-volatile compounds. Crude oil can also contain different amounts of sulphur, nitrogen, oxygen, mineral salts and trace metals such as nickel, vanadium and chromium. The hydrocarbon structures in oil can be saturated or unsaturated, and their shape can be characterized as straight, branched or as a ring structure, see figure 7 for some examples. The most commonly found molecules in crude oil are paraffins (alkanes), naphtenes (cycloalkanes), and aromatic hydrocarbons.

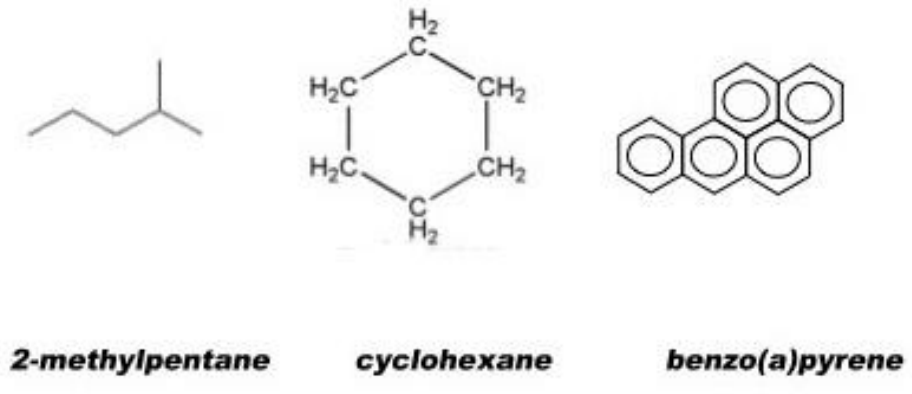

Figure 7 Examples of crude oil components.

Alkanes has the general formula C_nH_{2n+2}, they are saturated and can be either straight or branched. Alkanes with less than four carbon atoms are gaseous at room temperature and are characterised as the petroleum gases. Alkanes with more than 25 carbon atoms are characterised as paraffin wax.

Naphtenes are saturated and have the general formula C_nH_{2n}, they have similar properties to alkanes but with a higher boiling point.

The general formula for aromatic hydrocarbons is C_nH_n, and as the formula implies these are unsaturated compounds. They include at least one benzene ring of six carbons. Three double carbon-to-carbon bonds float around the ring and add stability. As a result of this, benzene rings is very persistent and can have toxic effects on the environment (Brady 2004). Aromatic hydrocarbons are often addressed to as polyaromatic hydrocarbon (PAH) if it consists of more than one aromatic ring.

Polyaromatic hydrocarbons

PAHs are among the most ubiquitous and widespread organic pollutants. Sources to PAHs can be both natural and anthropogenic, e.g. oil spill, oil seeps and incomplete combustion of organic material. Most PAHs are very toxic and are classified as pollutant chemicals of high priority in connection with environmental legislation, investigations and monitoring. Benzo(a)pyre is one example of a PAH, see figure 7. It consists of five benzene rings, and is one of the most studied environmental contaminants. It is mutagenic and highly carcinogenic (Walker 2006).

2.2.2 Oil spill fate in the marine environment

When oil enters the marine environment it undergoes complex processes that disperse and degrade the oil. A collective term for these processes is weathering. Weathering include spreading, evaporation, dispersion, emulsification, dissolution, oxidation (including photo-oxidation), biodegradation, aggregation and sedimentation, see figure 8 (Beyer 2010c; ITOPF 2011)

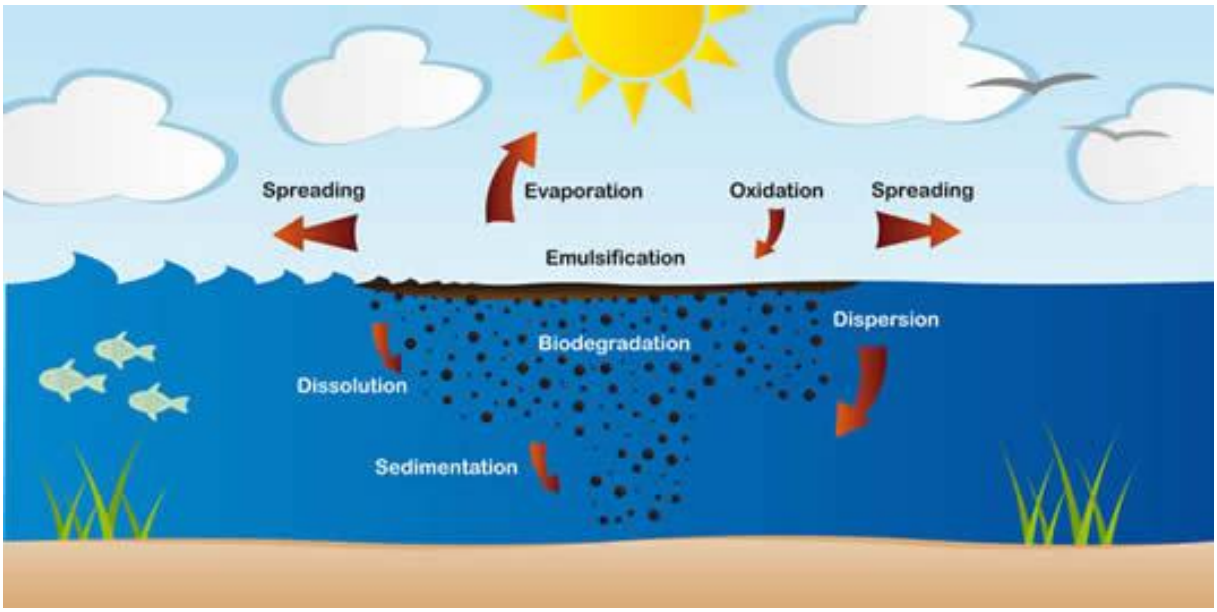

Figure 8. The main weathering processes (ITOPF 2011).

Oil is weathered in different ways, e.g. natural dispersion of oil into the water can cause part of the oil to leave the sea surface, whereas for others like evaporation or formation of water in oil emulsions can cause the oil that remains on the surface and to become more persistent.

The persistent of the oil also influence the way an oil slick breaks up. In light products, like kerosene, the oil is non-persistent and tends to evaporate and dissipate quickly. But if the oil is persistent, such as crude oil tend to be, it breaks up and dissipate more slowly and it usually require a clean-up response. Parameters such as density and viscosity affect the persistence of oil, so basically the higher the values are the longer the break-up time it.

Dissipation of oil does not occur immediately, and the time it takes depends on a series of factors. These factors include the amount and type of oil spilled, weather conditions and whether the oil stays at sea or if it is washed ashore. This process can sometime be quick and on other occasions slow, especially in sheltered and calm areas of water (ITOPF 2011; Patin 2011).

Several models have been developed to predict the trajectory and dispersion of oil spills at sea. The different models will not be described further, but one example is given below.

Figure 9 shows a model based on the properties of different oil types and gives the volume of oil and water-in-oil emulsion remaining on the sea surface, as a percentage of the original volume. Group 3 shows the North Sea crude oils.

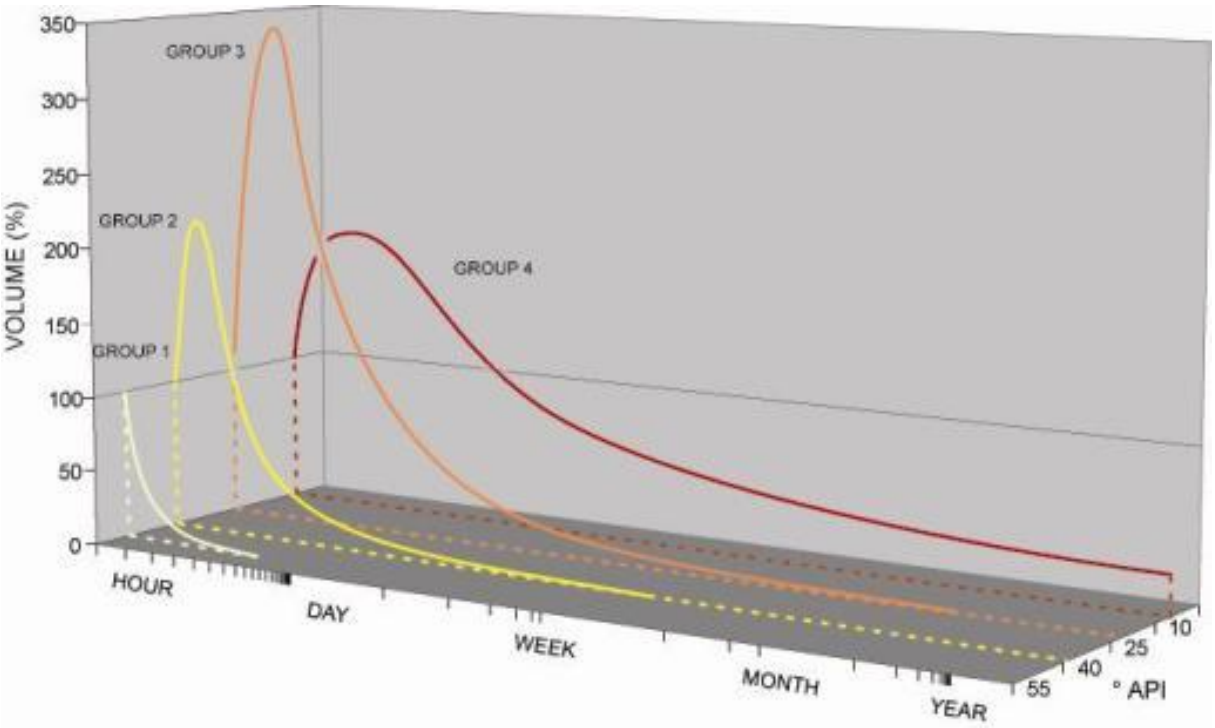

Figure 9. Volume of oil and water in oil emulsion remaining on the sea surface, as a percentage of the original volume. (ITOPF 2011).

Table 1 shows the density of the different groups and some examples.

Table 1. Description of the different groups (ITOPF 2011).

Group:	Density	Examples
Group 1	> 0.8	Gasoline, Kerosene
Group 2	0.8 – 0.85	Gas Oil, Abu Dhabi Crude
Group 3	0.85 – 0.95	Arabian Light Crude, North Sea Crude Oils
Group 4	< 0.95	Heavy Fuel, Venezuelan Crude Oils

2.2.3 Oil spill effects in the marine environment

The consequences of an oil spill depends on different factors like type of oil, amount of oil being spilled, geographical area of the oil spill, season and weather conditions (OED and NPD 2009). The acute risk for seabirds and sea mammals are obvious, a huge number of these animals may come in contact with the oil spill that could lead to their death. However, the effect situation after the acute oil spill is more uncertain. Long term effects of acute oil spills in costal ecosystems have been debated for a long time. There is a disagreement in this area. Some studies at previously heavily oil polluted shore locations have reported the existence of significant long term impact in various organisms for many years after the visual signs of pollution (Golet, Sieser et al. 2002). However, other studies tend to conclude the overall health condition of animal population and communities at heavily oil polluted shore locations to recover more quickly, within a few years (Pinet 2009).

Fish larva seems to be the most vulnerable with regards to oil spills. Given a situation with a little fish larva stock and a small central area (like the situation observed for spawning herring in 1970s), an oil spill in this region would be very unfortunate. This situation can affect a whole spawning area and in worst case eliminate a whole age group. The probability for this to happened is very low, but Havforskningsinstituttet

believes that a extreme low fish stock could appear in periods with a higher fishing pressure, like in the 1970s, and even in periods with little human impact, like observed in the beginning of the 1990s. As a result of this they advise against all kinds of offshore activities in areas where the fish is spawning. Lofoten is an example for such an area (Olsen 2009).

To increase the knowledge about the long-term effect of the petroleum industry The Norwegian Ministry of Petroleum and Energy, Ministry of the Environment and the petroleum industry helped financing the research program "PROOF" (long term effects of petroleum discharge to sea) under the Norwegian research council. PROOF was developed in 2002, and the program is being pursued as a subprogram, PROOFNy, under the research program "the sea and the coast" PROOFNy includes e.g. the effects in the water column and the long-term effect of acute discharges and cutting discharges (OED and NPD 2009). Laboratory study developed by NTNU in Trondheim financed by PROOFNy, indicated that *Calanus finmarchicus* (zooplankton specie) was more robust towards oil pollution than previous thought. Since this study is not verified throughout field studies, the result must be applied with caution (Olsen 2005-2009). Another study financed by PROOFNy, indicated that fish exposed to low levels of produced water over time gave changes to their DNA, the fish matured later and it showed indication of growth inhibition. This study was preformed by NIVA (Norsk Institutt for Vannforskning) and the University of Oslo, biological department (Hylland 2005-2009).

2.2.4 Chemical dispersion of oil in seawater

Chemical dispersant is a common tool to use in oil clean-up processes. It dissolves the oil into smaller particles so that the oil is being dissolved more easily into the water column. After the accident in the Gulf of Mexico, tons of the chemical dispersion agent Corexit 9500 was sprayed onto the oil spill. The same dispersion agent was used in this study.

Natural dispersion is when waves cause an oil slick to break up and move into the water column. When a braking wave (> 5 m/s) is passing through an oil slick at sea, the oil slick is temporarily broken into smaller oil droplets. Most of the oil droplets are large, 0.1 mm to several mm in diameter, and they will quickly rise back to the sea surface where they again will form an oil film after the wave has passed. It is only the smallest droplets that will become dispersed into the water column. Chemical dispersant is added to the oil slick to accelerate the natural process and rapidly convert a much larger proportion of the oil slick into very small oil droplets.

Dispersants are made of surfactants (surface active agents) dissolved in one or more solvents. The surfactants reduce the interfacial tension between oil and water, and easily make small oil droplets (typically 10-50 μm in diameter), even under low turbulence conditions. Surfactant molecules possess hydrophilic (water-seeking) head-groups that associate with water molecules, and lipophilic (in this case oil –seeking) tails that associate with oil. The oil droplets are thus surrounded by surfactant molecules and stabilized, which helps promote rapid dilution by water movements. Figure 10 gives an illustration of the mechanism when applying dispersant (IPIECA 2001; Sintef and Lewis 2001).

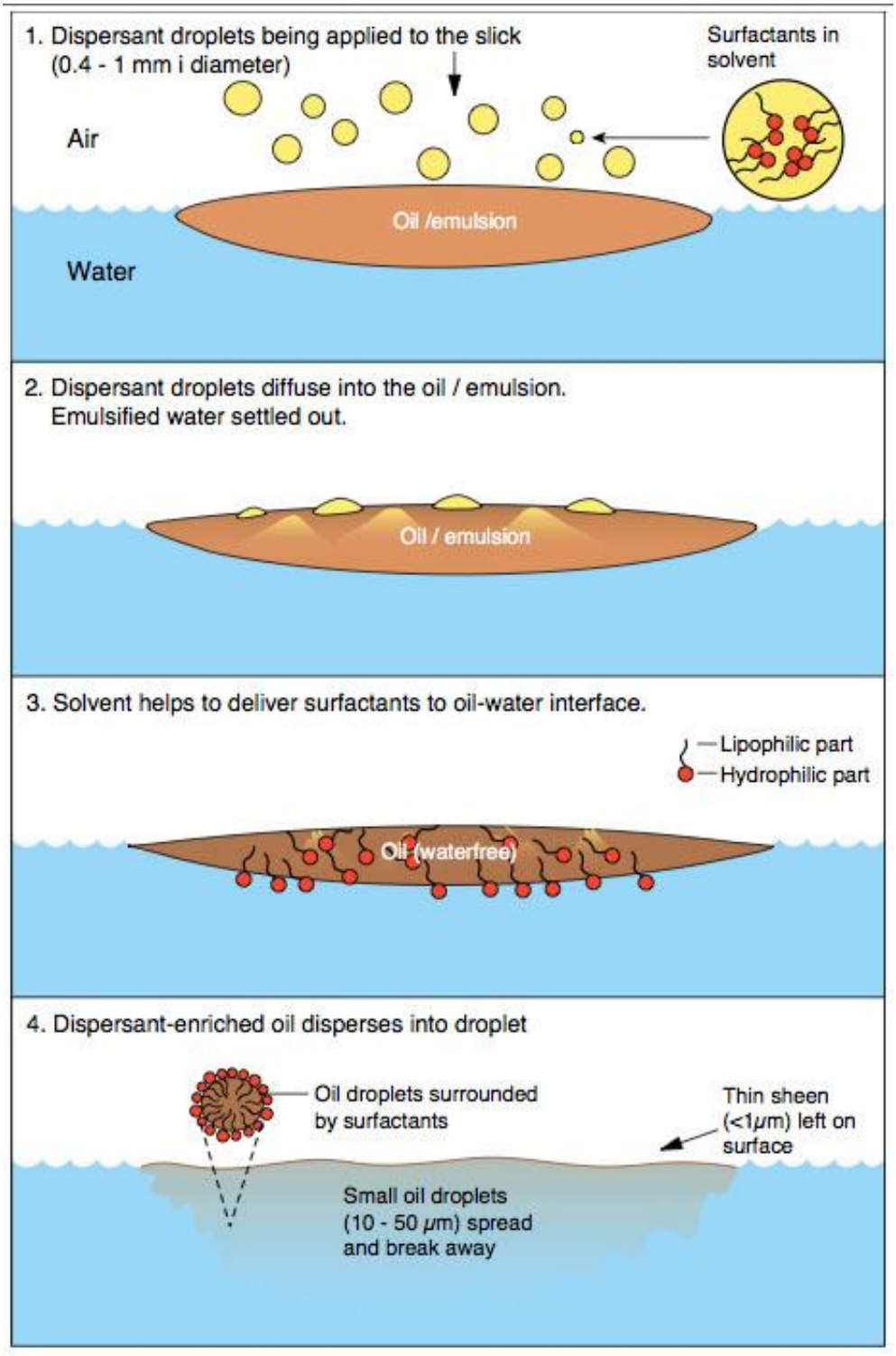

Figure 10. Mechanism of dispersion applied to an oil slick (Sintef and Lewis 2001).

Many of the first dispersants used in the 70s and 80s showed a high toxicity to marine organisms. However, today the laboratory data indicates that modern dispersant exhibit relatively low toxicity in itself to marine organisms (ITOPF 2010).

Since chemical dispersant make the oil more available in the water column, it is a risk that this can lead to harmful effects upon the organisms living there. Different assessment has to be made regarding the use of chemical dispersant. Will the oil on the water surface harm mammals and birds, and will it reach the shoreline? Or, will the dispersed oil be harmful for the organisms living in the water column, like fish, eggs and larva?

In the northern part of Norway, like the Barents Sea, the oil and gas reservoir are closer to shore than in the southern parts of Norway (Petroleumstilsynet 2011). If there was an oil spill in the northern region, the use of chemical dispersant agents can be necessary in order to prevent it for reaching the shoreline.

2.2.5 Vulnerability of the northern region to marine oil spills

The northern region includes the Lofoten-Barents Sea region. This region has lower species richness (biodiversity), than more southern regions. Species diversity tends to decrease with both decreasing ocean temperature and higher latitude. A lower biodiversity is associated with lower resilience. Also, this area is the home of many valuable and vulnerable organisms. For example, the Lofoten-Barents Sea hosts large seabird colonies and contains the nursery area of important fishes. In addition, there are conflicts of interest between petroleum activities and other activities in this area, for example important fisheries and nature conservation.

In higher latitude seas, oil degradation is likely to be slower than in temperate region due to lower temperature, less light (in winter) and the presence of ice. These are condition that together with limited infrastructure makes clean-up operations in case of an accident more difficult.

In both the Norwegian Sea and the Barents Sea there are important commercial fish stocks. The Norwegian Sea is characterised by migratory pelagic species like Norwegian

spring-spawning (NSS) herring and blue whiting (*Micromesistius poutassou*). The Barents Sea has both important pelagic species (e.g. capelin, immature NSS herring and polar cod) and demersal species (e.g. North-East Arctic (NEA) cod, and haddock). Both cod and herring have important spawning areas in the Lofoten and Vesterålen area. Figure 11 shows the fishes spawning location and advection routes of eggs and larvae of North-East Arctic cod, Norwegian spring-spawning herring and Barents Sea capelin.

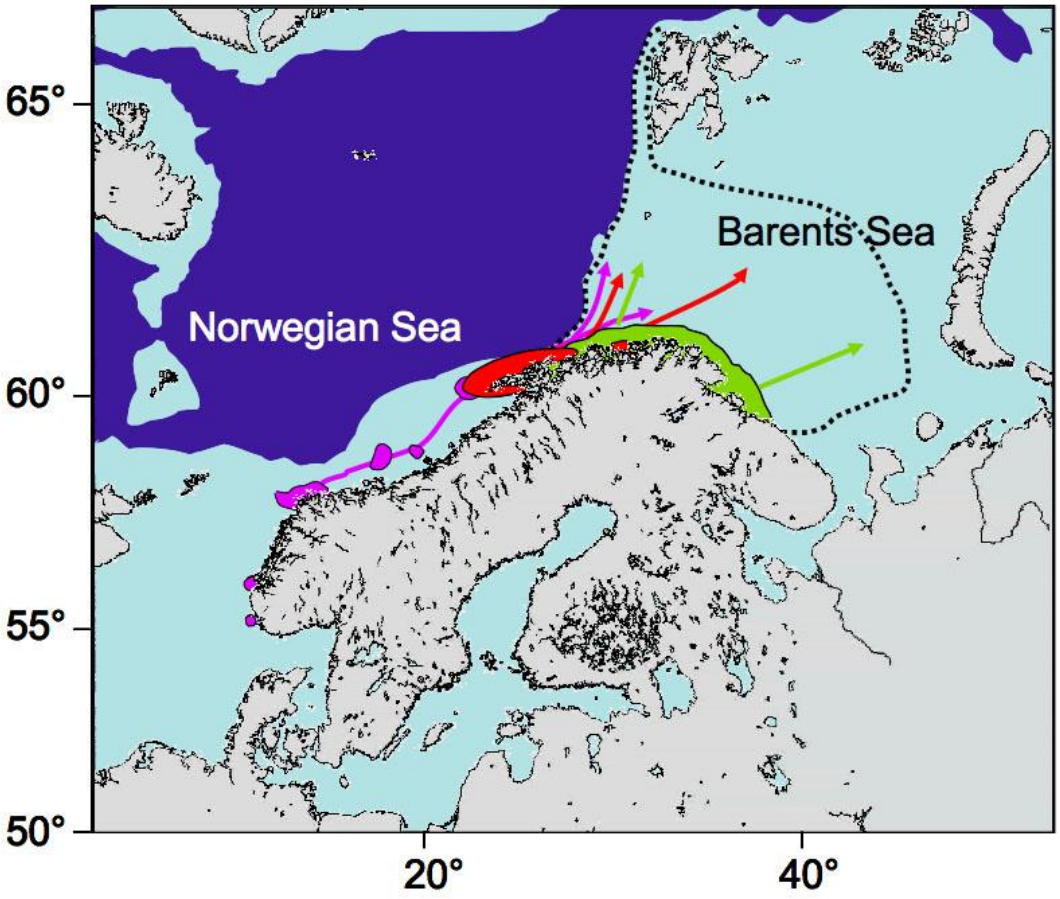

Figure 11. Map showing the spawning location and the advection routes of eggs and larvae for three fish stocks: North-East Arctic Cod in red, Norwegian spring-spawning herring in purple and Barents Sea capelin in green. The dotted line indicates maximum extension of these species in the Barents Sea in the first summer after spawning. Light blue: continental shelf (<250 m), dark blue: deep sea (Forsgren, Dalsgaard et al. 2009).

Cod and herring are close to their climatic limit in the Lofoten-Barents Sea, and they have a short, intensive spawning season and localised spawning areas like figure 11 shows. This makes them more vulnerable to perturbations like oil spills. But on the other hand, commercial fish stocks in the Barents Sea seem to be relatively well

managed and not over fished to the same extent as in the North Sea, which should make them less susceptible to other stressors.

The consequence of an oil spill in the Lofoten-Barents Sea depends on a number of oceanographic (e.g. wind, current) and ecological (e.g. spawning sites, natural mortality) factors. Moreover, we need more knowledge on both the direct and long-term toxicological effects of oil-related stressors. Our current knowledge on effect of oil spill and petroleum activities on marine ecosystems at high latitudes is based on studies following the Exxon-Valdez oil spill in sub-arctic Alaska. The result of these studies showed many cases of unforeseen long-term negative effects, and several of the affected populations have not yet recovered.

The Lofoten-Barents Sea has a significant important marine life. In addition to hold some of the worlds most commercial important fish stocks, it is home to a wide range of valuable marine species like cold-water coral reefs, seabird colonies and polar bears. The ecosystem faces extreme variation in light over the year, which highly affects the production. There is also large variation in production between areas within the region. There can be large concentrations of fish larva in certain areas due to advection, e.g. capelin shows extreme fluctuations in population size between different years. The consequence of an oil spill in this region very much depends on when and where it happens. If an oil spill occurs at the “worst” place and at the “worst” time the impact could be very severe (Forsgren, Dalsgaard et al. 2009).

A literature study about the vulnerability of the northern marine ecosystem compared to the southern marine ecosystem was preformed by NINA in 2009. This study indicated that there are several aspects of the Lofoten-Barents Sea region suggesting that oil spills in this region are likely to make more damage to the environment than further south in the Norwegian Sea. Some of their indications are listed in table 2.

Table 2. Aspects of the Lofoten-Barents Sea area, based on NINAs report regarding if the marine ecosystem in the Lofoten-Barents Sea is more vulnerable to oil pollution than the ecosystem further south in the Norwegian Sea and in the North Sea (Forsgren, Dalsgaard et al. 2009).

	Lofoten-Barents Sea	Comments
Oil activities:		
Oil degradation	Slower	Low temperature, darkness, ice
Cleaning up	More difficult	Infrastructure, darkness, temperature, ice
Vulnerability:		
Benthic organisms		
-Soft bottom benthos	More vulnerable	Stronger response to oil (experiment)
-Sponges and corals	More vulnerable	More sponges, worlds largest CWC reef
Fish	More vulnerable	Important keystone species Important nursery areas Largest remaining stock of Atlantic cod
Seabirds	More vulnerable	Larger and more aggregated populations Pelagic, diving, low fecundity species
"Whole ecosystem"	More vulnerable	Fewer species (less resilience) "Hot spot" areas and animal aggregations
Conflicts of interest:	Higher	Important fisheries tourism (e.g. Lofoten, Svalbard) Nature Conservation

2.2.6 Collaboration Norway – Russia on marine environment issues

Norway and at the Soviet Union signed the first governmental agreement on cooperation in the conservation area as early as 1988. On a political level the collaboration operates though the Norwegian-Russian environmental commission and meets once a year alternately in Norway and Russia.

Joint project is lead by the ministry of Environment on the Norwegian behalf, and carried out mainly by directorates. Some of the participating agencies are: the Norwegian Polar Institute, the Directorate of Nature Management, the Climate and Pollution Agency, the Directorate for Culture Heritage, the County Governor of Finnmark, the Norwegian Radiation protection Authority and the Norwegian Institute of Agriculture and Environmental Research (Bioforsk). Geographically the collaboration involves the Barents region and its coastal waters. It is made emphasis on regulatory cooperation and on competence building in Russian conservation management and the industrial sector as well as specific environmental projects.

Since the Norwegian and Russian sector of the Barents Sea has a unified ecosystem, it is important that management of resources in the Barents Sea by both countries are carried out in a sustainable way from an ecosystem-based approach. Hence the management of the Barents Sea must be based on a scientific foundation, and strict environmental standards must be imposed in accordance with the vulnerable nature of the area. The purpose of the joint projects concerning the marine environment is to get a good knowledge base for preserving the clean, rich ecosystem in the Barents Sea.

A Norwegian-Russian working group on marine environmental cooperation lead by the Norwegian Ministry of Environment and the Russian Ministry of Nature Resources was established in 2005. In 2009 they presented a joint Norwegian-Russian environmental status report for the entire Barents Sea. The report addressed that the environmental situation of the Barents Sea was generally satisfactory. However, there was concerns about the effects of climate change, continuing spreading of foreign species, damage caused by trawling and low levels of some commercial fish stocks as a result of overfishing. Increased petroleum and shipping activities was also highlighted as significant challenges.

As result of the increased petroleum activity in the Barents Sea, one focus of the marine environmental group will be on projects concerning e.g. comparison of Norwegian and Russian legislating and practices for petroleum-related activities in the Arctic, exchange of experience relating to supervision and control and harmonization of methods for environmental monitoring (Regjeringen 2011).

Norway and Russia has been negotiating the so-called "grey zone" (disputed area) for over 40 years. This area covers 170 000 km^2 and is now split in two in a 50/50 deal. The agreement was signed 15th of September 2010, and ratified by the two parliaments in February/March 2011. The agreement includes the continuation of the Norwegian-Russian fisheries cooperation, as well as provision concerning cooperation on the exploration of any petroleum deposits that extend across the delimitation line. Figure 12 shows the former disputed area and the official dividing line (Regjeringen 2010; Sagex 2011).

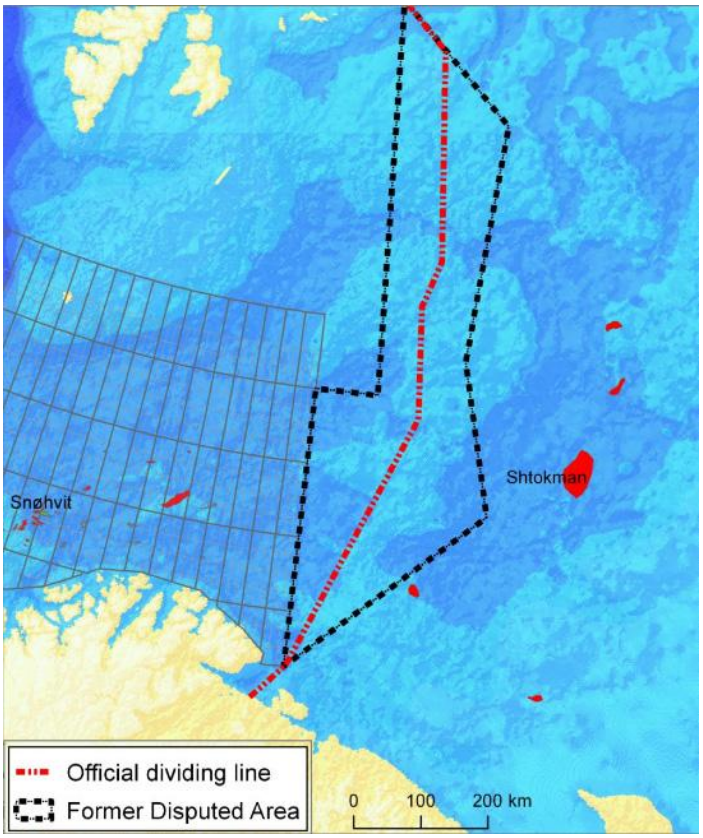

Figure 12. New borderline and former disputed area (Sagex 2011).

2.3 Biomarkers

2.3.1 Introduction

The marine environment is the ultimate sink for chemical pollutants, either from direct discharge or from land sources and via the atmosphere. The ability of pollutant to accumulate, transform and degrade complicates the study of pollutant exposure to marine ecosystem. The harmful effects of pollutant become apparent after longer periods of exposure, at this point they may have gone beyond the point where it can be reversed. Therefore, it is important to study the biological markers that could reflect the early-warning signals. In the past, ecological risk assessment and analysis of adverse effect have been based on measured physical effects and chemical parameters, with limited ability to determine the biological effects of exposure (Walker 2006; Kjersem 2007).

Biomarkers or biological markers can be defined as any response that can be detected within a living organism (or within a biotic system) that can be linked to the presence and/or toxic action of a pollutant chemical (or an adverse stressor) (Peakall 1994). A biomarker typically deals with assessments of pollution stress situation measured in biota, normally measured at the level of individual or lower. Figure 13 shows the different biomarker levels. The importance of data and level of uncertainty increases with an increase in biotic level, whereas the present knowledge and ease of obtaining data decreases.

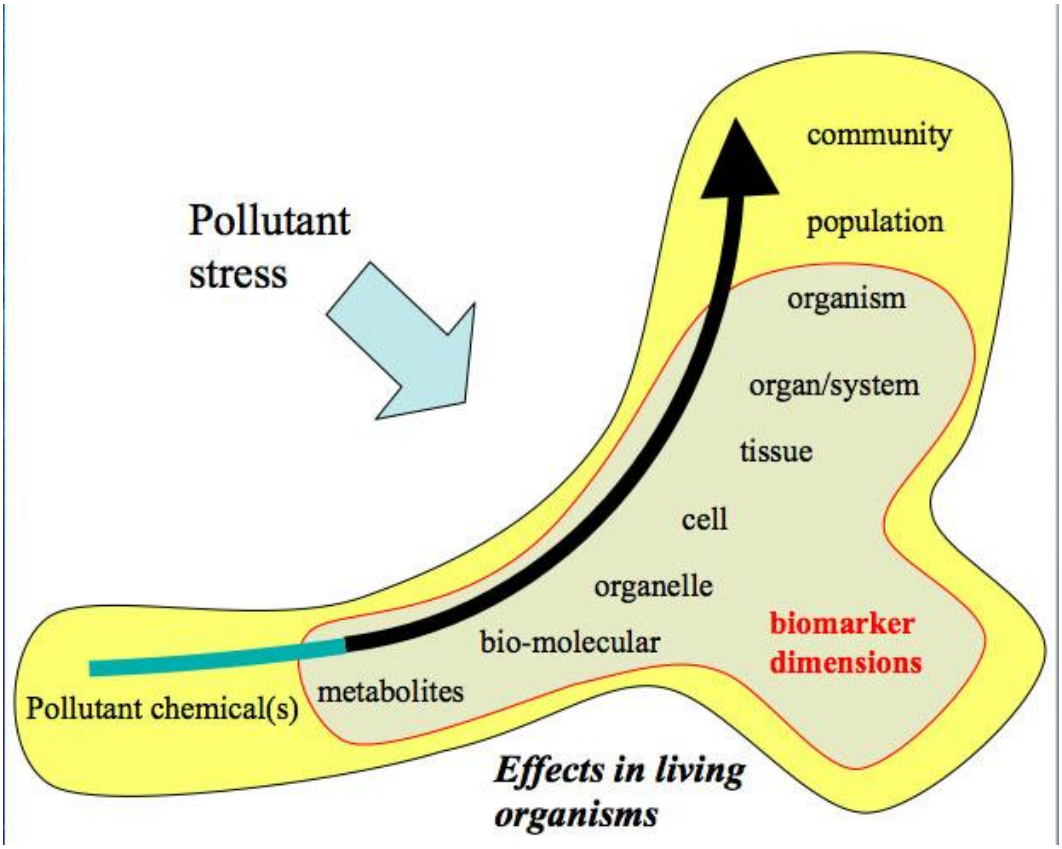

Figure 13. Illustrates how an adverse effect of pollutant exposure can be described as a sequential and hierarchical row of disorders within the biological system (Beyer 2010d).

2.3.2 Different types of biomarkers

The biomarkers response can be considered as exposure or effect indicators. Biomarkers of exposure can be used to confirm and assess the exposure of species to a particular substance and therefore provide the relationship between external exposure and internal dose. PAH metabolites in bile are one example of a biomarker of exposure. Biomarkers of effects include measurable biochemical, physiological or other alteration within tissue or body fluid of an organism that can be associated to external exposure of a chemical. DNA damage and lysosomal membrane stability alteration are biomarkers of effect, but they also serve as indicators of exposure (Oost, Beyer et al. 2002).

Some examples of different biomarkers are listed in table 3.

Table 3. Examples of some biomarkers (Walker 2006).

Biomarker	Biological system or function involved	Pollutant stressor
Inhibition of aminolevulinate dehydratase (ALAD)	Enzyme function, synthesis of porphyrins and heme	Lead
Inhibition of acetylcholine esterase (AChE)	Enzyme function, nerve-muscle signal transmission	Organophosphate and carbamate pesticides
Induction of cytochrome P450 1A1 monooxygenase	Detoxification of hydrophobic pollutant	Dioxins, toxic PCBs, carcinogenic PAHs
PAH-DNA adduct	Gene integrity	Carcinogenic PAHs
Eggshell thinning	Bird reproduction	DDT, DDE
Vitellogenin induction in male fish	Gamete development (reproduction)	Xenoestrogenic compounds
Salmon homing	Reproductive behaviour	Organophosphate pesticides

2.3.3 Biomarkers used in this study

PAH-metabolites in bile

The presence of PAH metabolites in fish bile is the final stage of the biotransformation process whereby lipophilic compounds are transformed to a more soluble form and then passes from the organism in bile or urine. Bile is stored in the gall bladder before it is released to play a role in the digestive process. This period of storage permits a degree of accumulation of metabolites and therefore increases their concentration. The fact that the metabolites are in the bile and about to leave the organisms when sampled underlines the fact that this is a biomarker of exposure and not effect.

Fluorescence analysis provides a sensitive rapid, semi-quantitative and cost efficient estimation of PAH metabolite concentration in bile. This measurement can be made either by certain fixed excitation and emission wavelengths or by scanning over a range of wavelengths.

The bile pigment biliverdin (bile protein) concentration should always be measured as part of the analytical procedure. The bile density should not be significant different between samples in order to achieve optimal interpretation condition. If the concentration is significant different it has to be taken into consideration in the data interpretation (Beyer and Bamber 2004).

Ethoxyresorufin-*O*-Deethylase (EROD)

Cytochrome P450 (CYP) is a group of membrane bound enzymes essential in the metabolism of both endogenous and exogenous compounds in cells. PAH has shown to be potent inducers of CYP1A in several fish species. CYP1A induction can for instant be measured by ethoxyresorufin-o-deethylase (EROD) activity. EROD is a biomarker of exposure to planar organic pollutants, among them PAHs. Since ethyoxyresorufin is a substrate for CYP1A the formation of resorufin is therefore an indirect measure of CYP1A activity. The individual variability in EROD is not larger than normal, but it is important to include factors like sex, age and temperature when interpreting of the result, since they play a role in regulating the CYP1A level.

EROD is based on the CYP1A catalytic formation of the product resorufin from the substrate ethoxyresorufin. Resorufin can be detected by fluorescence (Børseth, Aarab et al. 2005).

Lysosomal membrane stability

Lysosomal membrane stability is considered to be a general measurement of stress. Theoretically, membrane stability decreases in response to stress as membrane permeability increases. The mechanism may involve direct effects of chemical or the increased frequency of secondary lysosomes in toxicant-stressed cells.

Lysosomal membrane stability in macrophages is used as a measure of pollutant in invertebrates. It is measured by means of Neutral Red Retention Time (NRRT) assay. Neutral Red accumulates in the lysosomal compartment of the cells. A reduction in membrane integrity causes the dye to leak into the cytosol. This effect can be quantified since the cells from animal exposed to environmental pollutants will exhibit reduced retention time compared to cells of animal from clean sites. Figure 14 shows healthy and dead mussel haemolymph cells (Brooks, Sundt et al. 2009). Lysosomal membrane stability is most commonly used with circulating cells, e.g. haemocytes in blue mussels, but it exist a similar method on tissue.

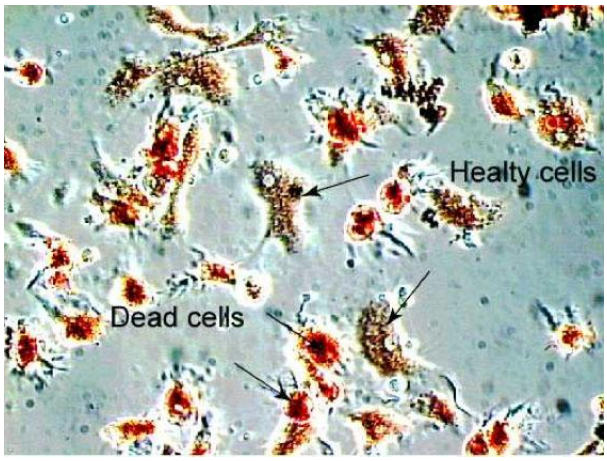

Figure 14. Microscope view (400x magnification) of mussels haemocytes used in neutral red retention assay, showing both live and dead cells (Brooks, Sundt et al. 2009).

Histopathology

Histopathology is the study of adverse biological condition of tissue by use of microscopy techniques. This biomarker can be employed both for fish and invertebrate study organisms.

After the tissue has been collected it is placed on cassets and stored in a fixative in order to stabilize the tissue and to prevent decay. The tissue can stay in the fixative for quite some time, and the most common fixative is formaldehyde. The next step is chemical fixation, the cassets are immersed in multiple baths of progressively more concentrated ethanol in order to dehydrate the tissue. After ethanol the cassets are fixated with toluene or xylene before treated with extremely hot liquid, usually paraffin. Paraffin replaces the water in the tissue and turns soft tissue into a sample miscible with paraffin. The next step is embedding where additional paraffin is added to create a block, which is attached to the outside of the cassette. The tissue can now be cut into thin sections with a microtome (2-7 μm) and placed on a glass slide for staining. Staining is the final stage before interpretation in the microscope. Here the section is stained with one or more pigments in order to see the different cellular components. To see contrast, counterstains are used. The most commonly used stain is hematoxylin and eosin, hematoxylin stains nuclei blue and eosin stains cytoplasm and the extracellular connective tissue matrix pink. The slides are now ready for interpretation in a microscope (Kumar, Abbas et al. 2007).

3. Method

An experiment where two species were exposed to oil with and without dispersion was conducted in order to see if there was an additional biological effect in the organisms exposed to oil with chemical dispersion. The experiment including the laboratory analysis was mainly performed at Akvamiljø AS, but some analysis and sample preparations were also done at Stavanger Hospital and the University of Stavanger.

This chapter is divided into six parts: organisms used in this experiment, experimental design, experimental monitoring, sampling, biomarker methods and statistic method.

3.1 Organisms used in this experiment

In order to use fish as research organisms an application to Forskningsdyrutvalget was sent and approved before the experiment was carried out. For mussels this is not necessary.

3.1.1 Atlantic salmon

Atlantic salmon, *Salmo salar*, is of the fish family Salmonidae. It is found in the northern Atlantic Ocean and in rivers that flows into the north Atlantic. Wild Atlantic salmon live in freshwater from one to five years, according to river location, before they go into the ocean (Shearer 1992). The first phase in freshwater is the alevin stage, here the fish stay in breeding ground and use the remaining nutrition in their yolk sac. In the development stage, their gills develop and they become active hunters. Next phase is the fry stage, the fish grow and subsequently leave the breeding ground in their food search and moves to areas with higher prey concentration. Their last stage in freshwater is when they develop into sparr and prepares to go into the Atlantic Ocean. When sparr develops into smolt, they leave the river and go into the ocean. This normally happens between March and June. In this development stage they experience a period of rapid growth during their first years in saltwater. When the Atlantic salmon is large enough it changes into the grilse phase and becomes ready to return to the place they were born. In aquaculture fry are kept in large freshwater tanks for 12 to 20 months until they develops into the smolt phase and are taken out to sea where they are held for up to two years (Heen, Monahan et al. 1993).

Smolts where purchased from a local fish farm and transported to Akvamiljø in a tank filled with fresh seawater, a small amount of oxygen was given from a oxygen container. The fish were put in the reference tank over night in order to reduce stress from transportation before the exposure period started.

Figure 15. Atlantic salmon, smolt.

3.1.2 Blue mussel

Blue mussel, *Mytilus edulis*, is from the family Mytilidae and can be found in temperate and polar waters around the world, e.g. in the coast of Norway. They live in intertidal areas attached to rocks and other hard substances by their byssal threads (thread-like structure). The two shell valves are equal in shape and held tightly closed by a posterior adductor muscle when they are exposed to air. The mussel feed and breads though their gills. A selective process transports the particles trapped onto the cilia on the gills and carries it to the mouth. There are three main methods of culturing mussels: bottom cultivation, bouchot culture and suspended rope method. These methods will not be further described but in general aquaculture involves placing small ("seeds") mussels in an area where growing conditions are optimum. Mussels can be grown to marketable size in 12 to 18 months (Beaumont, Gjedrem et al. 2007).

Blue mussels where purchased from Helgø, a high quality grocery store. Helgø gets fresh mussels every day (this was checked in advance). The mussels were transported on ice

and placed in the same reference tanks as the fish for one day before the exposure experiment started.

Figure 16. Blue mussels, (Zubov 2011).

3.2 Experimental design

The setup is illustrated in figure 17. Four tanks were filled with fresh seawater and as figure 17 shows tank 1, 2 and 3 were also filled with oil, oil and dispersion and just dispersion, respectively. The concentration in each tank is given in table 4 and general parameters are listed in table 5. The header tanks had continuously magnetic stirring. Akvamiljø has a direct access to seawater from the sea outside their facilities and seawater from Mekjarvik was thus used in this experiment.

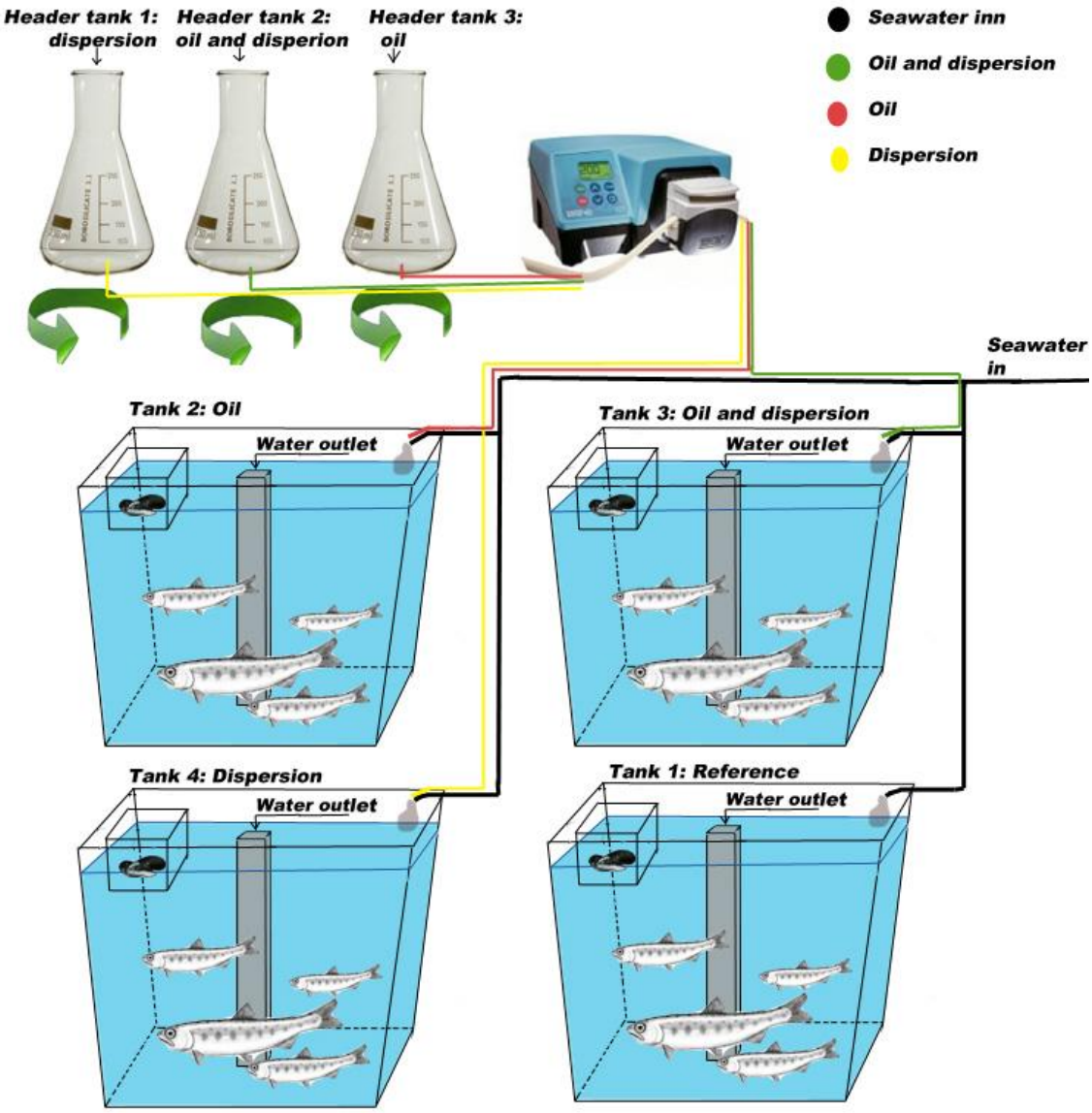

Figure 17. Schematic illustration of the exposure setup used for exposure of fish and blue mussels to oil and dispersant agent. The green rotating arrows illustrates magnetic stirring in the header tanks.

Figure 18. Picture of the exposure setup shows the four grey tanks used for the current study. Two of the grey tanks in the picture were not used, and the two green tanks in the front of the picture were used by Anton Zubov in his study (Zubov 2011).

Table 4. Tank concentration.

Tank/header nr	Concentration:
Header-tank 1	0.04 ppm dispersant
Header-tank 2	2 ppm oil and 0.04 ppm dispersant
Header-tank 3	2 ppm oil
Tank 1	Reference group (seawater only)
Tank 2	1 ppm oil
Tank 3	1 ppm oil and 0,02 ppm dispersant
Tank 4	0,02 ppm dispersant

Table 5. General parameters.

***Two days of sampling.**

Parameter	Type/unit/Value
Duration of experiment:	12/13 days*
Oil:	Arctic crude oil
Dispersion:	Corexit 9500
Tank volume:	About 300 l
Nr of fishes in each tank:	10
Nr of mussels in each tank:	20
Temperature:	6-8 °C
Water flow:	2 l/min
Pump flow:	1 ml/min

3.3 Experimental monitoring

On a daily basis (with some exceptions), water temperature and water flow was measured. Excretion was removed from the tanks and the header tanks were refilled when necessary. The general fitness of the organisms was observed and any mortality was noted, all these datas can be found in appendix A.

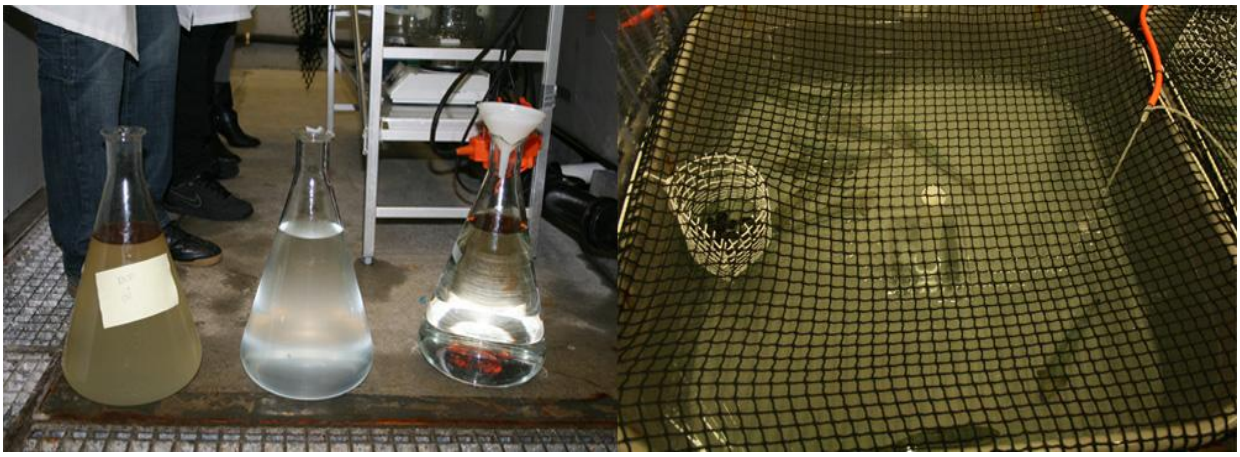

Figure 19. Refilling of header tank and daily observation (Zubov 2011).

3.4 Sampling

In order to prevent the fish for unnecessary suffering it was placed in a bucket with anaesthetic before its aorta was cut and its spine broken. The height, weight and sex of the fish were recorded and it was examined for external features such as wounds. In each individual the bile and liver was operated out. The bile fluid was drained in a capillary tube and stored on ice and the livers weight was noted before it was being processed any further, see figure 20. For mussels only the length for each individual was measured before the haemolymph were drained and their shell cut open for further analysis.

Figure 20. Sampling, Atlantic salmon (Zubov 2011).

3.5 Biomarker methods

All the sampling and analyses were prepared according to standard operating procedure (SOP) developed by Akvamiljø AS, these SOPs can be found in appendix B.

3.5.1 Lysosomal Stability

In each of the tanks 15 mussels were analysed. 7 mussels from each tank were analysed the first day of sampling and 8 mussels the second day of sampling.

At the beginning of both days a working solution, neutral red, was made out of a premade neutral red stock solution. The stock solution was prepared by dissolving 20 mg of dye in 1 ml of dimethyl sulfoxide (DMSO), it was stored in the freezer at -4°C. The working solutions were made by taking 5 µl of the stock solution and dilute it in 995 µl filtered seawater. Both these solutions were stored in lightproof bottles.

The procedure described below is carried out on 15 mussels at a time:
A knife was gently pressed into the mussel in order to have a clearing of a few mm, seawater was drained out of the mussel and 0.3 ml haemolymph was tapped out (using a needle with size 0.6 x 25 mm). The highlighted area in figure 21 is the adductor muscle where the haemolymph is obtained from. The haemolymph was mixed firmly but gently in an eppendorf tube with 0.3 ml filtered seawater. 30 µl of the cell suspension was transferred from the eppendorf and onto the centre of a microscope slide. The slides were placed in a lightproof humidity chamber for 15 minutes, before the excess suspension was gently tapped off. 30 µl working solution was added to the cell layer and a cover slip (22 x 22 mm) was placed on the slide. An additional 15 minutes of incubation followed.

Figure 21. Blue mussel, placement of the adductor muscle is highlighted.

The slides were analysed in a microscope at x40/100 magnification. The light was kept at a minimal tolerable level and the examination time per slide was approximately 1 minute. After the first examination additional 15 minutes incubation followed, then the incubation time was 30 minute until there was 50 % dye loss from the lysosomes to the cytosol (mean retention time, NRRT).

3.5.2 Histology

Two baker buffer solutions were made, one for fish and one for mussel. The baker solution for fish was prepared by mixing 100 ml formaldehyde (37 %) with 10 g $CaCl_2$ in a 1000 ml volumetric flask, the mixture was diluted up to the 1000 ml mark and stored in a plastic bottle at 4 °C. The procedure for the mussel baker solution was the same, but in addition to $CaCl_2$, 25 g of NaCl was added.

After the haemolymph had been taken out of the mussel for lysosomal stability analysis, the mussel was cut open. By using a scalpel a cut section of the mussel's gills, gonads and digestive gland were made. The cut sections was placed on pre-labelled cassettes and put into the mussel baker solution. For fish, a cut section of their gills on both sides were made by a sharp scissor. The cut sections were placed on pre-labelled cassettes and put into the fish baker solution. Both baker solutions with cassettes were stored at 4°C.

The cassettes were embedded and waxed at Stavanger Hospital. The cutting was preformed at Akvamiljø with a cut thickness of 5 µm. After a cut had been made, it was carefully placed in water (40°C) and transported to an objective glass and heated at 40 °C. The samples were stained at Stavanger Hospital before they were analysed in the microscope.

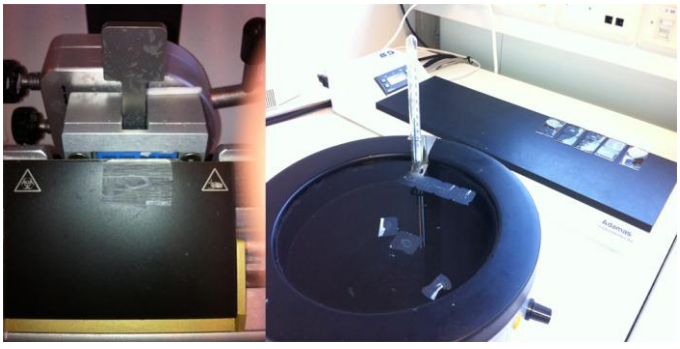

Figure 22. Histology cutting.

3.5.3 Fixed fluorescence and biliverdin test

PAH metabolites in bile are measured semi quantitatively by fluorescence detection. The data for general concentration of the bile fluid is required since bile fluorescence detection is used for assessing PAH exposure. This information is retrieved from spectrophotometric analysis of biliverdin. The fixed fluorescence (FF) test was carried out at the University of Stavanger, whereas the biliverdin analysis was preformed at Akvamiljø.

The bile samples (15 µl) were diluted in a 1:1 methanol: water mixture (1485 µl) and centrifuged (5000 G, 5 min). The supernatant (187.5 µl) was then diluted a second time with the 1:1 methanol: water mixture (2810 µl). Slit widths were set at 2.5 nm for both excitation and emission wavelength and the diluted samples were analysed by FF at the wavelength pairs 290/335, 341/383 and 380/430 nm, which is optimised for detection of 2-3 ring, 4-ring and 5-ring PAH metabolites, respectively.

The supernatant from the first dilution was used to measure the biliverdin content in the fish bile. For this purpose a spectrophotometer with an absorbance of 660 nm was used.

3.5 Statistic method

Data from fixed fluorescence and EROD analysis was tested statistically in order to see if there was a significant difference between the exposed groups compared to the reference group. In comparison to a Student T test, which only can compare to groups, a Dunnetts´ test can be used. A Dunnett´s test compares multiple groups with respect to a reference group.

The statistic was calculated in a program called JMP. The data was tested for normality, and the variance was tested in order to see if there were any differences in the four groups. Some of the data gave a poor statistic result with respect to normality (probability factor <0.05), so the normality was tested on log-transformed data´s. This gave a much better result, also for the group variance (>>0.05). The statistic calculation was thus made on log-transformed data´s.

The groups that showed a statistically significant difference are marked in the box plot diagram in chapter 4 with a star. The box plots are made from non-transformed data, and these datas can be found in appendix A.

4. Result

4.1 Experimental data

Water flow and temperature measurements were performed approximately everyday. The tanks were also checked for any fish mortality. Sampling started the 24th of January and had a duration of two days, making the exposure time 13 days.

Table 6. Water flow (l/min) in the different tanks under the exposure period.
***Higher water flow due to higher biomass.**

01.2011	13.01.2011	14.01.2011	15.01.2011	16.01.2011	17.01.2011	18.01.2011	19.01.2011	20.01.2011	21.01.2011	22.01.2011	23.01.2011	24.01.2011	Average
Tank 1	5.0*	5.0*	6.0*	-	5.2*	5.0*	5.5*	2.0	1.9	2.0	-	-	5,3*/2,0
Tank 2	2.0	2.1	2.0	-	2.0	2.0	2.0	2.0	2.0	2.0	-	-	2.0
Tank 3	2.0	2.0	2.0	-	2.0	2.0	2.1	2.0	2.0	2.0	-	-	2.0
Tank 4	2.0	1.9	1.9	-	2.0	2.0	2.0	2.0	2.0	2.0	-	-	2.0
Average	2.00	2.00	1.97		2.00	2.00	2.03	2.00	1.98	2.00			

The water flow in the different tanks during the exposure time is given in table 6. The water flow was stable throughout the experiment. Since it was a higher biomass in the reference tank the first half of the exposure, the water flow was thus higher in this period.

Table 7. Water temperature (°C) in the different tanks under the exposure period.
*** Not included in the calculation.**

01.2011	13.01.2011	14.01.2011	15.01.2011	16.01.2011	17.01.2011	18.01.2011	19.01.2011	20.01.2011	21.01.2011	22.01.2011	23.01.2011	24.01.2011	Average
Tank 1	-	7.6	7.3	-	6.0	7.6	8.3	8.4	8.5	8.1	-	-	7.7
Tank 2	-	7.8	7.4	-	6.2	7.3	3.2	8.3	8.6	7.8	-	-	7.6
Tank 3	-	7.6	7.8	-	6.5	7.0	8.0	8.1	8.3	8.3	-	-	7.7
Tank 4	-	7.8	7.7	-	6.0	7.8	8.6	8.6	8.5	8.2	-	-	7.9
Average		7.7	7.55		6.175	7.425	8.3	8.35	8.475	8.1			

The temperatures in the different tanks are given in table 7. With exception to the temperature data for tank 2 (oil exposure) retrieved on the 19th of January, the temperature appeared to be stable. It is most likely that the temperature deviation in tank 2 is a writing or reading mistake from the log, since all the four tanks had the same

water source. If it was a lower temperature in the seawater from the ocean this day it would be so in all the four tanks, not only in tank 2.

Table 8. Fish mortalities during the exposure time.

01.2011	13.01.2011	14.01.2011	15.01.2011	16.01.2011	17.01.2011	18.01.2011	19.01.2011	20.01.2011	21.01.2011	22.01.2011	23.01.2011	24.01.2011	Sum
Tank 1					1F								1F
Tank 2						1M							1M
Tank 3						1M							1M
Tank 4							1F					1F	2F
Total													**3F and 2 M**

Table 8 shows the observed fish mortalities. In total there were a mortality of three female fishes and two male fishes. It is difficult to explain why these individuals died, but one reason could be stress due to transport, or stress due to change in their environment.

These parameters are listed in order to show that the experiment went according to plan and that there were no obvious differences in the organism's environment, and no differences between the four tanks. Since the water flow and temperature were stable there is no reason to believe that it had any influence on the different biomarker responses.

4.2 General biological observation

Below general biological observations for Atlantic salmon are listed.

Table 9. Physical parameters: Size, weight, length, LSI and conditional factor.

	Reference		Oil		Oil and Dispersant		Dispersant	
	Average	STDEV	Average	STDEV	Average	STDEV	Average	STDEV
Size (cm)	29.57	1.51	29.71	1.60	28.14	1.21	28.00	1.41
Weight (g)	233.37	37.54	246.71	32.43	194.80	15.23	206.67	40.59
LSI (%)	2.19	0.33	1.96	0.48	1.94	0.43	1.92	0.33
Conditional factor	0.90	0.07	0.94	0.05	0.88	0.08	0.93	0.06

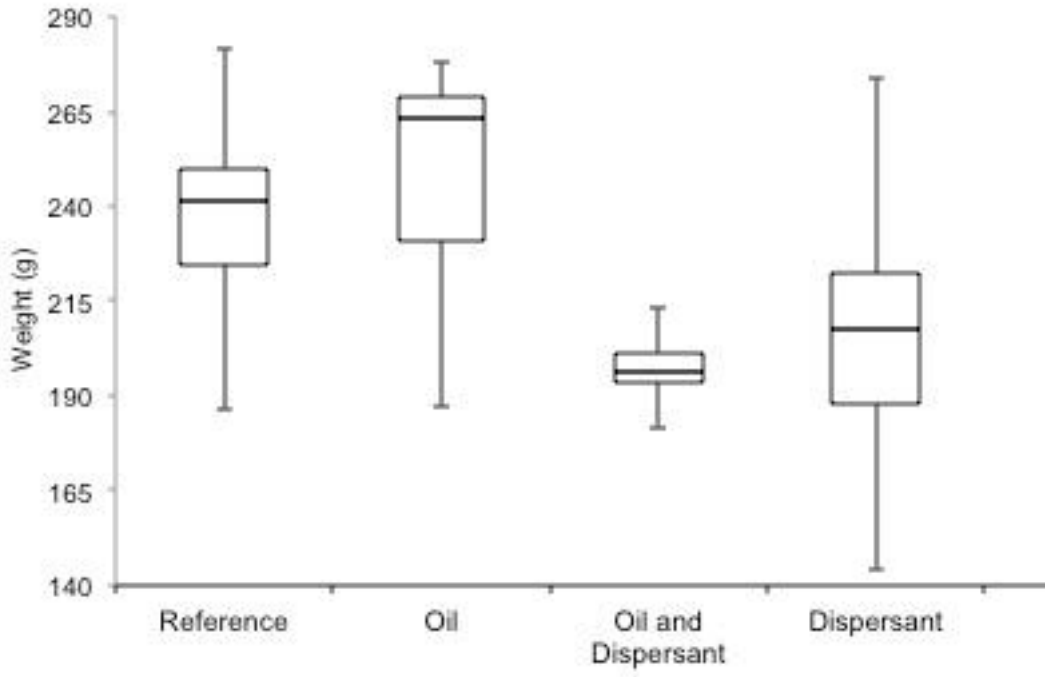

Figure 23. Fish weigh (gram). The figure shows median and quartiles (box) and 1.5 IQR (whiskers).

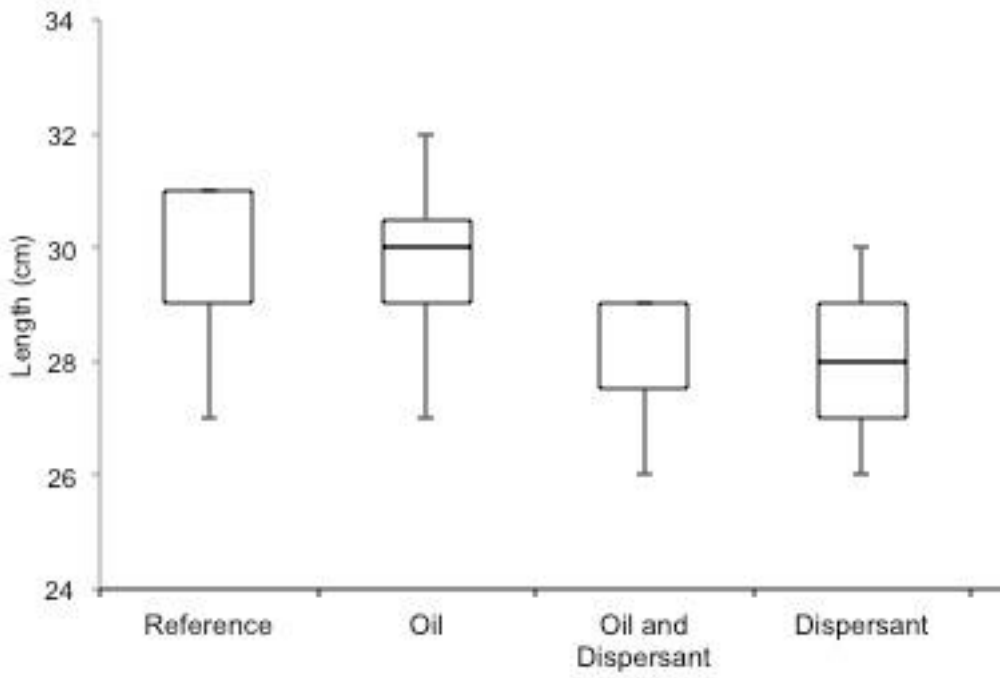

Figure 24. Fish length (cm). The figure shows median and quartiles (box) and 1.5 IQR (whiskers).

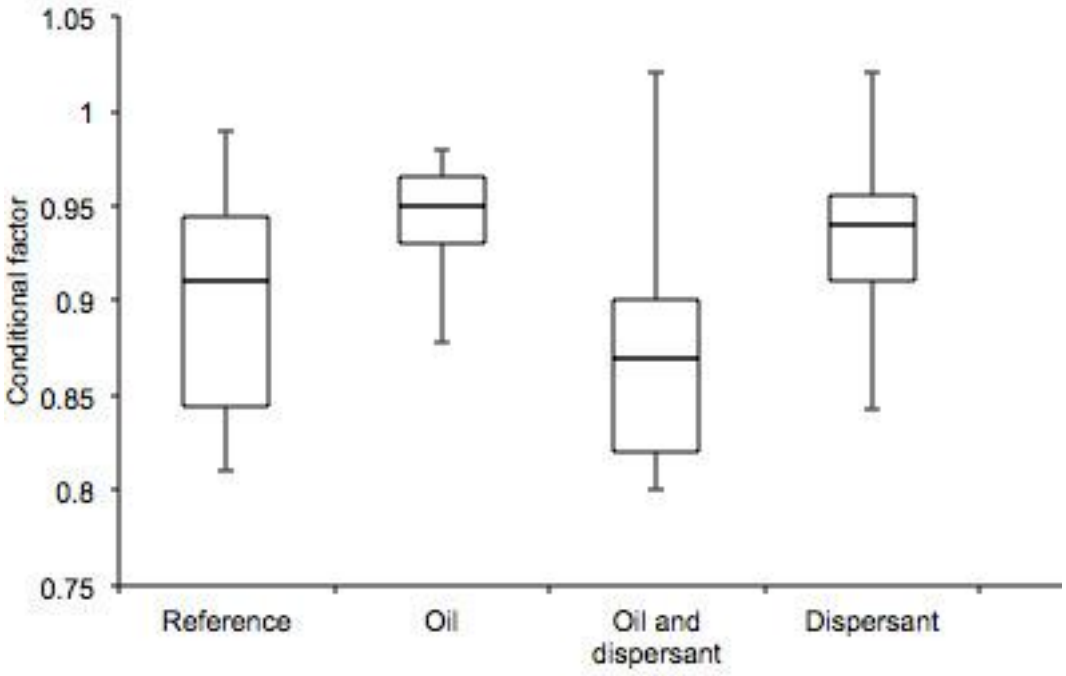

Figure 25. Conditional factors. Calculated from $K = \frac{Weight\,[g]}{(Length\,[cm])^3} \times 100$. The figure shows median and quartiles (box) and 1.5 IQR (whiskers).

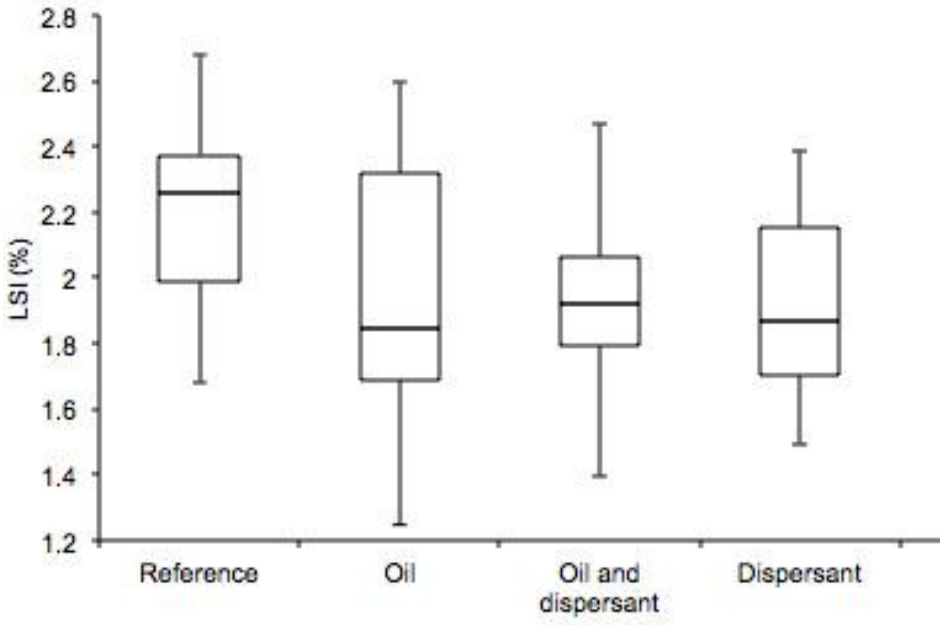

Figure 26. LSI-Liver Somatic Index $\left(\frac{Liver\ weight\ [g]}{Total\ fish\ weigth\ [g]} \times 100\right)$. The figure shows median and quartiles (box) and 1.5 IQR (whiskers).

In table 9 the physical parameters are listed. The whole sampling sheet can be found in appendix A. The fish's weight, length, conditional factor and liver somatic index (LSI) are illustrated in box plots in figure 23, 24, 25 and 26, respectively.

From the box plots one can be see that there are no major differences in the length and weight of the different groups. The oil with dispersant group and the dispersant exposed group seems to be slightly smaller than the reference group and the oil-exposed group. This is statistically proven not to be the case, since there were not discovered any statistically significant differences between the groups.

The proportion between the fish weight and length is given by the conditional factor. In other words it shows the fish fat-state and gives an indication on the general health condition of the individuals. A too low conditional factor indicates that the fish is not healthy. It is important to keep in mind other factors that could increase or decrease this factor. Smolt normally experiences a decrease in their conditional factor, which indicates that important metabolic processes within the fish are complete. From the conditional

factor the fishes are regarded as healthy and there were not proven any statistically significant differences between the groups compared to the reference group.

Figure 26 shows the LSI (liver somatic index), which is the fishes liver weight in comparison to its total weight given in percentage. Short time exposure of xenobiotic can lead to an increase in the fishes liver weight. Long time exposure on the other hand can lead to shrinkage of the fish liver, since the fish will use more energy in order to keep their natural processes going as usual. If there were any differences between the groups it would have been expected to see an increase in the fish liver weight in the exposed groups compared to the reference group, since this experiment was a short time exposure. But it was not detected any statistically significant differences in the LSI between the different groups compared to the reference group.

The ratio between the fish sex seems to be almost a 50/50 split in all of the four tanks, see appendix A. This is good in order to exclude factors that are more sex bound. E.g. if the EROD activity was higher in a tank with just female fishes it is difficult to compare it to other groups where the ratio were equal between male and female. Since this factor could be a variation due to sex (female fishes has proven to have a higher EROD activity when they become sexually mature), and not an indication of a higher EROD activity due to oil exposure.

4.3 Biomarkers in Atlantic salmon

4.3.1 Fixed fluorescence and biliverdin test

Table 10. Fixed fluorescence and biliverdin result

	Reference		Oil		Oil and Dispersant		Dispersant	
	Average	STDEV	Average	STDEV	Average	STDEV	Average	STDEV
Biliverdin	0.07	0.03	0.09	0.06	0.08	0.04	0.10	0.05
FF - 290/335	21.50	4.94	57.74	13.60	46.85	13.61	16.80	3.06
FF - 341/383	3.19	0.46	6.80	0.16	5.80	1.30	2.93	0.26
FF - 380/430	1.06	0.26	1.05	0.16	1.25	0.17	0.89	0.10

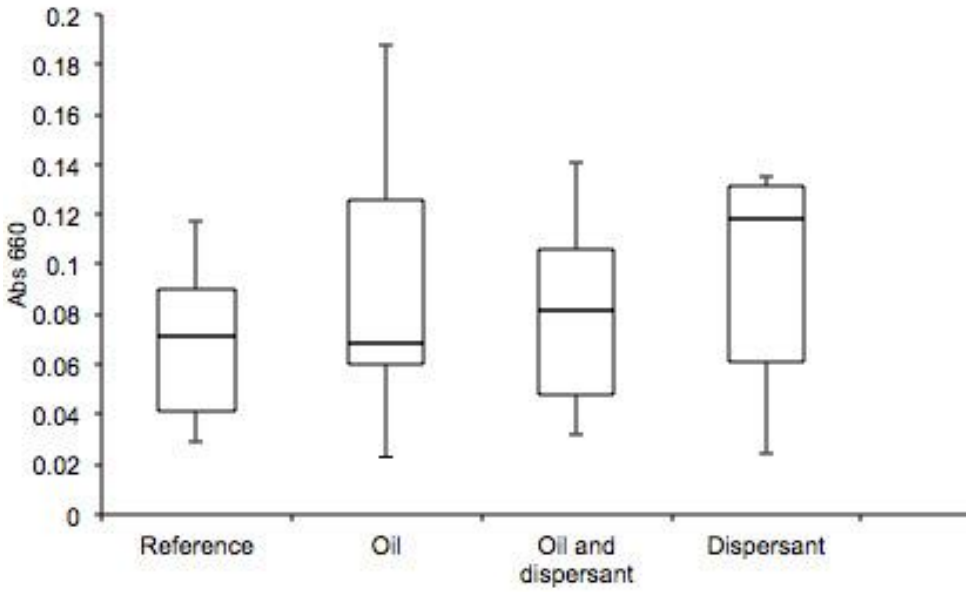

Figure 27. Biliverdin, abs 660 nm. The figure shows median and quartiles (box) and 1.5IQR (whiskers).

In order to make a good interpretation of the result from the fixed fluorescence (FF) test, the protein concentration of biliverdin in fish bile should not vary too much in the different groups. As shown in figure 27, the biliverdin concentration does not tend to vary from the different exposure groups, which is good. This was also proven statistically. This means that any variation in the protein concentration of biliverdin do not have to be accounted for in the FF test results.

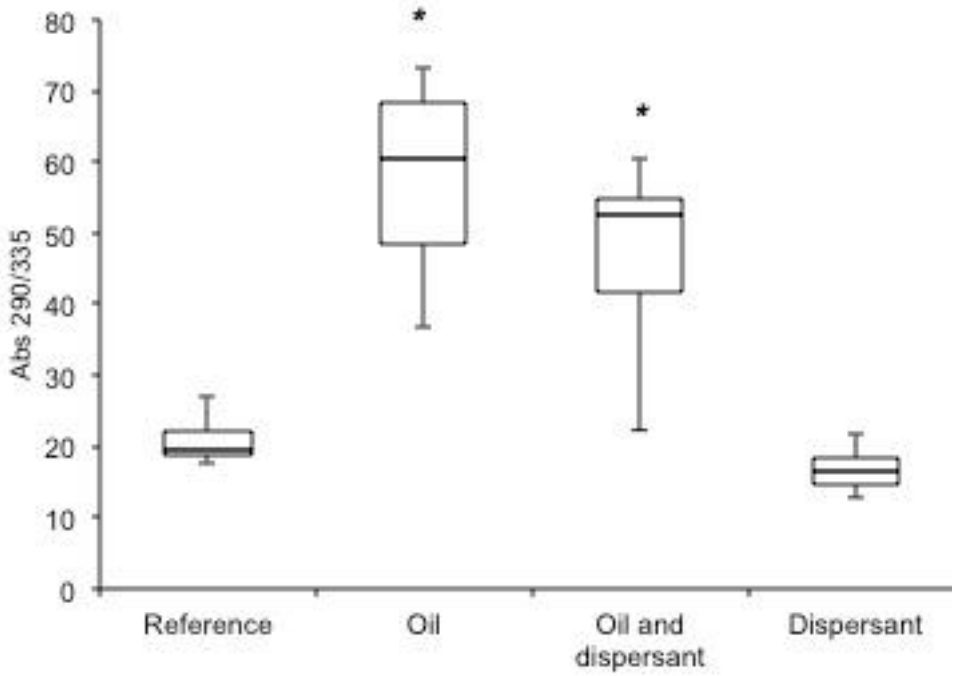

Figure 28. FF test, abs 290/335 nm. The figure shows median and quartiles (box) and 1.5IQR (whiskers). * Statistically significant different from reference group (Dunnett´s test).

Figure 28 shows the result of the wavelength pair 290/335 nm. It is a statistically significant difference in the groups exposed to oil with and without dispersant compared to the reference group. This was also expected since the wavelength pair 290/335 detects the smallest PAH compounds (2-3 ring components-naphthalene type of metabolites), and is most easily taken up in the biota. It was not detected a statistically difference in the fishes exposed to just dispersant compared to the reference group, this is also obvious from the box plot.

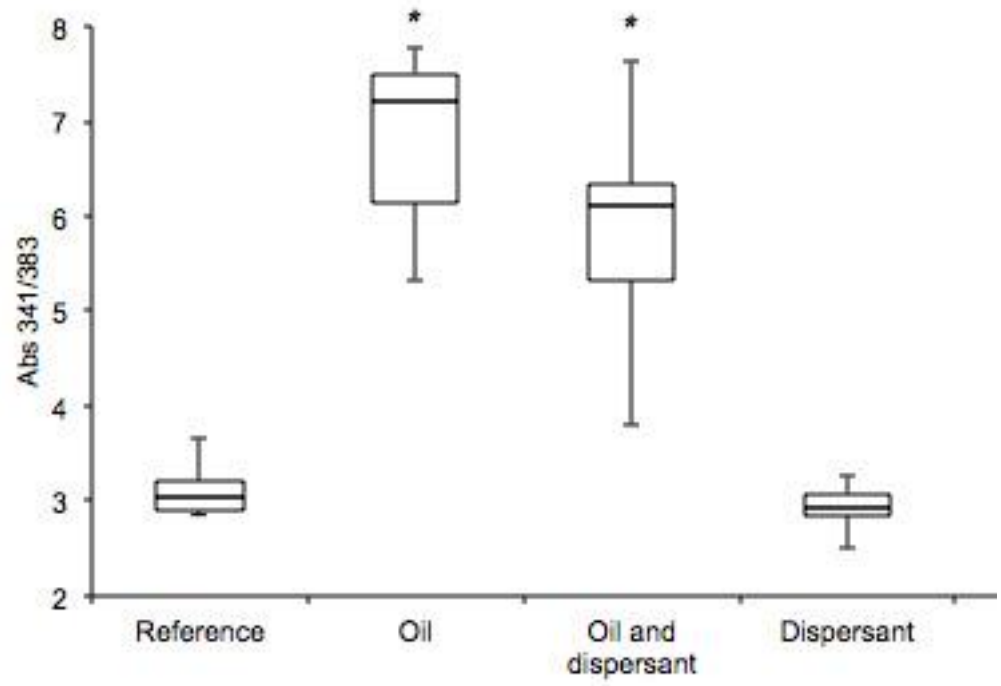

Figure 29. FF test, abs 341/383 nm. The figure shows median and quartiles (box) and 1.5IQR (whiskers). * Statistically significant different from reference group (Dunnett´s test).

The wavelength pair 341/383 detects pyrene and metabolites of pyrene. The result from this wavelength pair is given in table 29. Also here it was detected a statistically significant difference in the groups exposed to oil with and without dispersant compared to the reference group. This wavelength pair also shows the same trend as 290/335, the response is higher in the group exposed just to oil. Also, the dispersant group is not statistically different from the reference group.

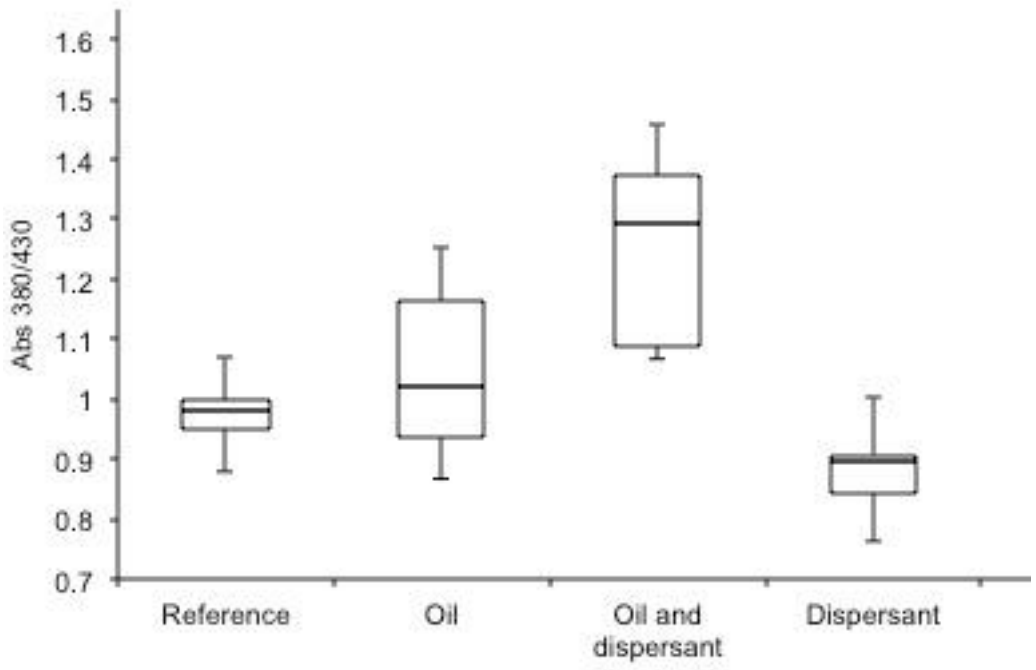

Figure 30. FF test, abs 380/430 nm. The figure shows median and quartiles (box) and 1.5IQR (whiskers).

Benzo(a)pyrene and its metabolites can be detected by using the wavelength pair 380/430, the result is given in figure 30. Also here it was detected a higher response in the group exposed to oil with and without dispersant compared to the reference group. But the detected response was not large enough to prove a statistically significant difference.

This wavelength pair was the only one giving a higher response in the fishes exposed to oil with dispersant compared to the fishes exposed to oil without dispersant. But it is not proven to be a statistical significant difference between these groups. If the fishes exposed to oil with dispersant were compared towards the fishes exposed just to dispersant if would however be a statistically significant difference. But in this thesis all the different groups are compared toward the reference group.

4.3.2 EROD

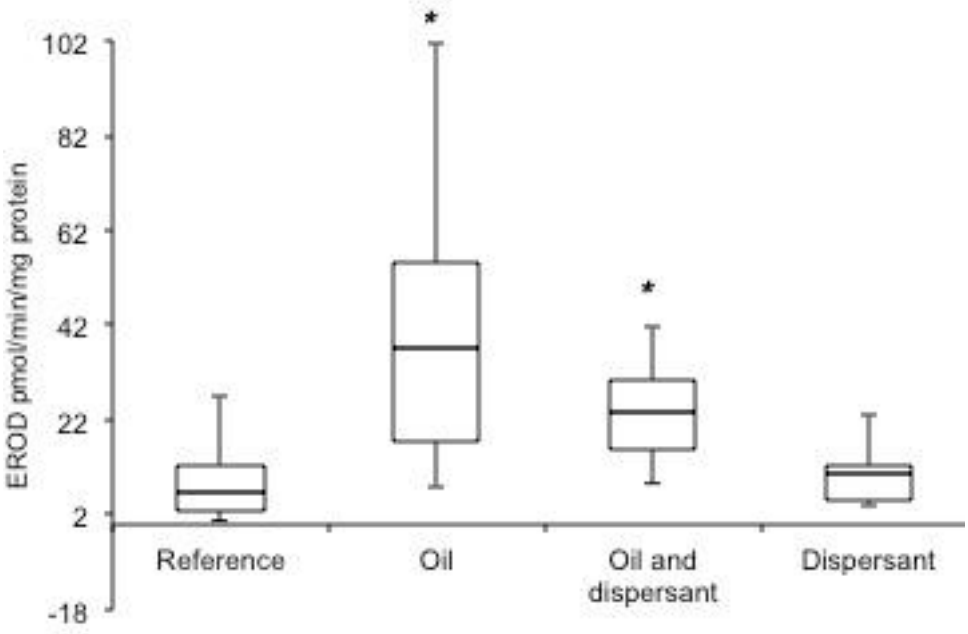

Figure 31. EROD activity measured in pmol/min/mg protein at 535/580 nm. The figure shows median and quartiles (box) and 1.5IQR (whiskers).
*** Statistically significant different from reference group (Dunnett´s test).**

The EROD activity was measured fluorimetric with a wavelength pair of 535/580 nm. The result from this test is given in figure 31. As for the FF result also here the oil exposed groups with and without chemical dispersant agent present had a statistical significant difference compared to the reference group. One individual showed a negative value, see appendix A, this individual is not included in Figure 31 and in the statistical interpretation.

4.3.3 Histopathology

Table 11. Histology result fish

yyyy.mm.dd - ind.-no	Species	Treatment	Size (cm)	Weight (g)	Sex	Clubbing	Fusion	Aeroism
20110120 - 210	Salmo salar	Reference	31	281.90	F	4	0	0
20110120 - 211	Salmo salar	Reference	27	161.40	M			
20110120 - 212	Salmo salar	Reference	31	257.70	F			
20110120 - 213	Salmo salar	Reference	31	242.20	M			
20110120 - 214	Salmo salar	Reference	29	220.80	F	3	0	0
20110120 - 215	Salmo salar	Reference	29	241.40	M	3	0	0
20110120 - 216	Salmo salar	Reference	29	228.20	F	2	0	0
20110120 - 217	Salmo salar	Dispersant	30	275.60	F	1	0	0
20110120 - 218	Salmo salar	Dispersant	28	207.30	F			
20110120 - 219	Salmo salar	Dispersant	29	220.20	F	0	3	0
20110120 - 220	Salmo salar	Dispersant	29	224.00	M	2	0	0
20110120 - 221	Salmo salar	Dispersant	27	188.50	M			
20110120 - 222	Salmo salar	Dispersant	26	143.90	F			
20110120 - 223	Salmo salar	Dispersant	27	187.20	M	3	0	0
20110120 - 224	Salmo salar	Oil	31	277.80	M	2	3	0
20110120 - 225	Salmo salar	Oil	30	265.30	F	4	2	0
20110120 - 226	Salmo salar	Oil	32	272.10	F			
20110120 - 227	Salmo salar	Oil	29	226.60	F			
20110120 - 228	Salmo salar	Oil	29	234.90	F	2	0	0
20110120 - 229	Salmo salar	Oil	30	263.10	M	4	0	0
20110120 - 230	Salmo salar	Oil	27	187.20	F			
20110120 - 231	Salmo salar	Oil+Dispersant	27	201.50	F			
20110120 - 232	Salmo salar	Oil+Dispersant	26	164.00	M	2	2	0
20110120 - 233	Salmo salar	Oil+Dispersant	29	201.40	F			
20110120 - 234	Salmo salar	Oil+Dispersant	29	195.50	M	0	4	0
20110120 - 235	Salmo salar	Oil+Dispersant	29	196.60	M	4	0	0
20110120 - 236	Salmo salar	Oil+Dispersant	29	213.20	F			
20110120 - 237	Salmo salar	Oil+Dispersant	28	191.40	F	4	4	0

Abnormalities studied on fish gill tissue was clubbing, fusion and aeroism. All these parameters are indication of unhealthy condition, meaning that there was something in the water that the fish is reacting to. Table 11 list the histology result in colour codes and numbers. Green (0) indicates that there was no visual harm in the tissue examined and red (4) indicates 50 % or more damage in the tissue (illustrated on the bottom of the table).

Clubbing was present in almost all of the slides in some degree. From table 11 it seems as fusion has a slight increase in the oil-exposed groups with and without dispersant compared to the reference group. It also seems as the oil with dispersant group has a higher occurrence of fusion than the group just exposed to oil. On the basis of this it could seem as the fishes exposed to oil with dispersion suffered more than the fishes exposed to undispersed oil. However this is only assumption since the amount of data retrieved is too low to have any statistically validity, also the quality of the gill tissue was too poor in order to give an exactly histopathology description. Figure 32 shows fish gills with signs of clubbing and fusion. It also shows the difference between a slide that has the right thickness and a slide that is too thick.

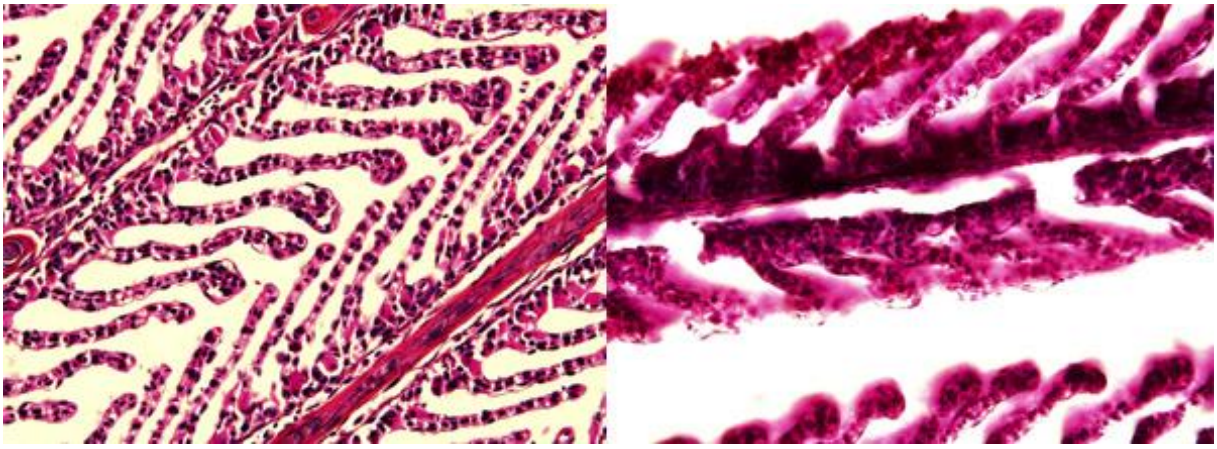

Figure 32. Fish gills showing signs of clubbing (to the left) and fusion (to the right). 400X magnification.

4.4 Biomarkers in Blue mussel

Table 12. Lysosomal stability and histology result

						Size (cm)		NRRT (min)		Histology			
yyyy.mm.dd - ind.-no	Species	Treatment	Size (cm)	NRRT (min)	Comments	Average	STDEV	Average	STDEV	Apoptosis	Atresia	Atrophic	Vacule
20110120 - 001	Mytilus edulis	1. Reference	6		no cells	5.60	0.74	95.00	30.90			0	1
20110120 - 002	Mytilus edulis	1. Reference	5		no cells					1	2	1	0
20110120 - 003	Mytilus edulis	1. Reference	5		no cells								
20110120 - 004	Mytilus edulis	1. Reference	6	120									
20110120 - 005	Mytilus edulis	1. Reference	5	150								0	0
20110120 - 006	Mytilus edulis	1. Reference	7	120									
20110120 - 007	Mytilus edulis	1. Reference	6	120									
20110120 - 043	Mytilus edulis	1. Reference	5	60						1	4	1	0
20110120 - 044	Mytilus edulis	1. Reference	7	60						3	3	1	0
20110120 - 045	Mytilus edulis	1. Reference	5	60								1	3
20110120 - 046	Mytilus edulis	1. Reference	5	90								0	0
20110120 - 047	Mytilus edulis	1. Reference	5	90								0	0
20110120 - 048	Mytilus edulis	1. Reference	6	120									
20110120 - 049	Mytilus edulis	1. Reference	5	60									
20110120 - 050	Mytilus edulis	1. Reference	6	90									
20110120 - 008	Mytilus edulis	2. Oil	5	120		5.40	0.51	87.00	52.64				
20110120 - 009	Mytilus edulis	2. Oil	6	150						1	4	3	0
20110120 - 010	Mytilus edulis	2. Oil	5	150									
20110120 - 011	Mytilus edulis	2. Oil	5	150									
20110120 - 012	Mytilus edulis	2. Oil	5	120								1	4
20110120 - 013	Mytilus edulis	2. Oil	6	120									
20110120 - 014	Mytilus edulis	2. Oil	5	150									
20110120 - 059	Mytilus edulis	2. Oil	5	30						2	2	3	1
20110120 - 060	Mytilus edulis	2. Oil	5	90								3	3
20110120 - 061	Mytilus edulis	2. Oil	6	15						2	2	2	1
20110120 - 062	Mytilus edulis	2. Oil	5	30								1	2
20110120 - 063	Mytilus edulis	2. Oil	6	30						2	3	1	3
20110120 - 064	Mytilus edulis	2. Oil	6	30								2	1
20110120 - 065	Mytilus edulis	2. Oil	5	60								4	1
20110120 - 066	Mytilus edulis	2. Oil	6	60									
20110120 - 015	Mytilus edulis	3. Oil+Dispersant	6	150	little colour	5.47	0.52	105.00	42.02			0	3
20110120 - 016	Mytilus edulis	3. Oil+Dispersant	6	150	little colour								
20110120 - 017	Mytilus edulis	3. Oil+Dispersant	6	150	little colour								
20110120 - 018	Mytilus edulis	3. Oil+Dispersant	6	150								3	1
20110120 - 019	Mytilus edulis	3. Oil+Dispersant	6	150						1	4	1	1
20110120 - 020	Mytilus edulis	3. Oil+Dispersant	5	150						3	1		
20110120 - 021	Mytilus edulis	3. Oil+Dispersant	6		no cells								
20110120 - 067	Mytilus edulis	3. Oil+Dispersant	6	60								0	0
20110120 - 068	Mytilus edulis	3. Oil+Dispersant	5	90						2	1	3	2
20110120 - 069	Mytilus edulis	3. Oil+Dispersant	5	60									
20110120 - 070	Mytilus edulis	3. Oil+Dispersant	5	90								2	2
20110120 - 071	Mytilus edulis	3. Oil+Dispersant	5	90						2	4	1	4
20110120 - 072	Mytilus edulis	3. Oil+Dispersant	5	60								4	0
20110120 - 073	Mytilus edulis	3. Oil+Dispersant	5	60								4	1
20110120 - 074	Mytilus edulis	3. Oil+Dispersant	5	60								3	0
20110120 - 022	Mytilus edulis	4. Dispersant	6	150		5.67	0.62	117.86	39.84				
20110120 - 023	Mytilus edulis	4. Dispersant	7	150									
20110120 - 024	Mytilus edulis	4. Dispersant	6	150									
20110120 - 025	Mytilus edulis	4. Dispersant	5	150									
20110120 - 026	Mytilus edulis	4. Dispersant	6	150									
20110120 - 027	Mytilus edulis	4. Dispersant	6	180									
20110120 - 028	Mytilus edulis	4. Dispersant	5	150									
20110120 - 051	Mytilus edulis	4. Dispersant	6	60									
20110120 - 052	Mytilus edulis	4. Dispersant	6	90						3	4	1	1
20110120 - 053	Mytilus edulis	4. Dispersant	6	90									
20110120 - 054	Mytilus edulis	4. Dispersant	5		no cells								
20110120 - 055	Mytilus edulis	4. Dispersant	6	60						1	3	0	0
20110120 - 056	Mytilus edulis	4. Dispersant	5	90								4	4
20110120 - 057	Mytilus edulis	4. Dispersant	5	90									
20110120 - 058	Mytilus edulis	4. Dispersant	5	90									

4.4.1 Lysosomal stability

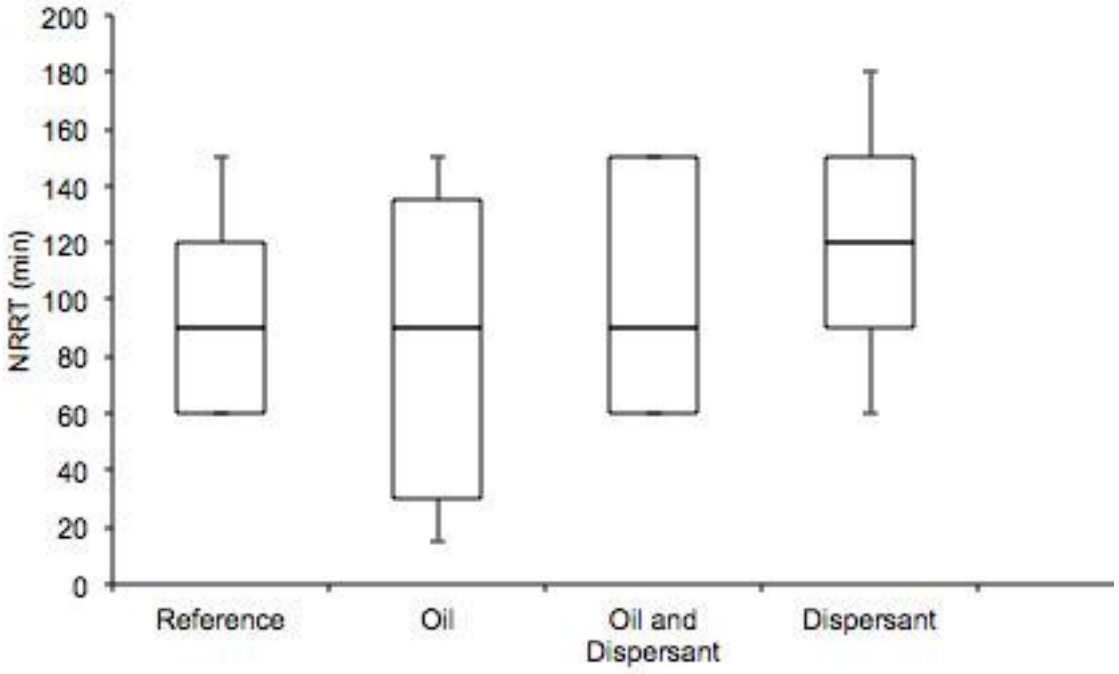

Figure 33. NRRT (min). The figure shows median and quartiles (box) and 1.5IQR (whiskers).

Figure 33 shows the neutral red retention time (NRRT) for blue mussels in the four different tanks and Table 12 lists the NRRT for all the individuals. The NRRT for the reference tank and the different exposure tanks makes little sense and there is also a major difference within the same tank during the two sampling days. It was not detected a statistically significant difference in the different exposure groups compared to the reference group.

4.4.2 Histopathology

Table 12 gives a good visualisation of the histology result with colour codes. Green (0) meaning no visual harm in the tissue examined and red (4) meaning 50 % or more damage in the tissue (illustrated on the bottom of the table). The individuals with no colours in table 12 were not analysed. This was due to no tissue present on the slide or that the tissue present was destroyed during cutting, leading to a too poor quality of the tissue for interpretation.

For male mussels only the digestive gland was examined for abnormalities, whereas for females also their gonads were examined. This is due to the difficulty of analysing the male gonads, extra preparation has to be made. Unfortunately the quality of the gill tissue was too low for any interpretation, also only a few slides had presence of gill tissue.

When looking at the digestive gland for both sexes Table 12 shows that there is an increase in the exposed groups compared to the reference group. In the reference group most of the individuals had colour code 0 or 1 for vacuoles and atrophic in their digestive gland. The oil exposed groups with and without chemical dispersant shows a clearly increase in colour codes 2 and above. In the group with just chemical dispersant the number of individuals good enough for further analysing was too low to form a good basis for interpretation

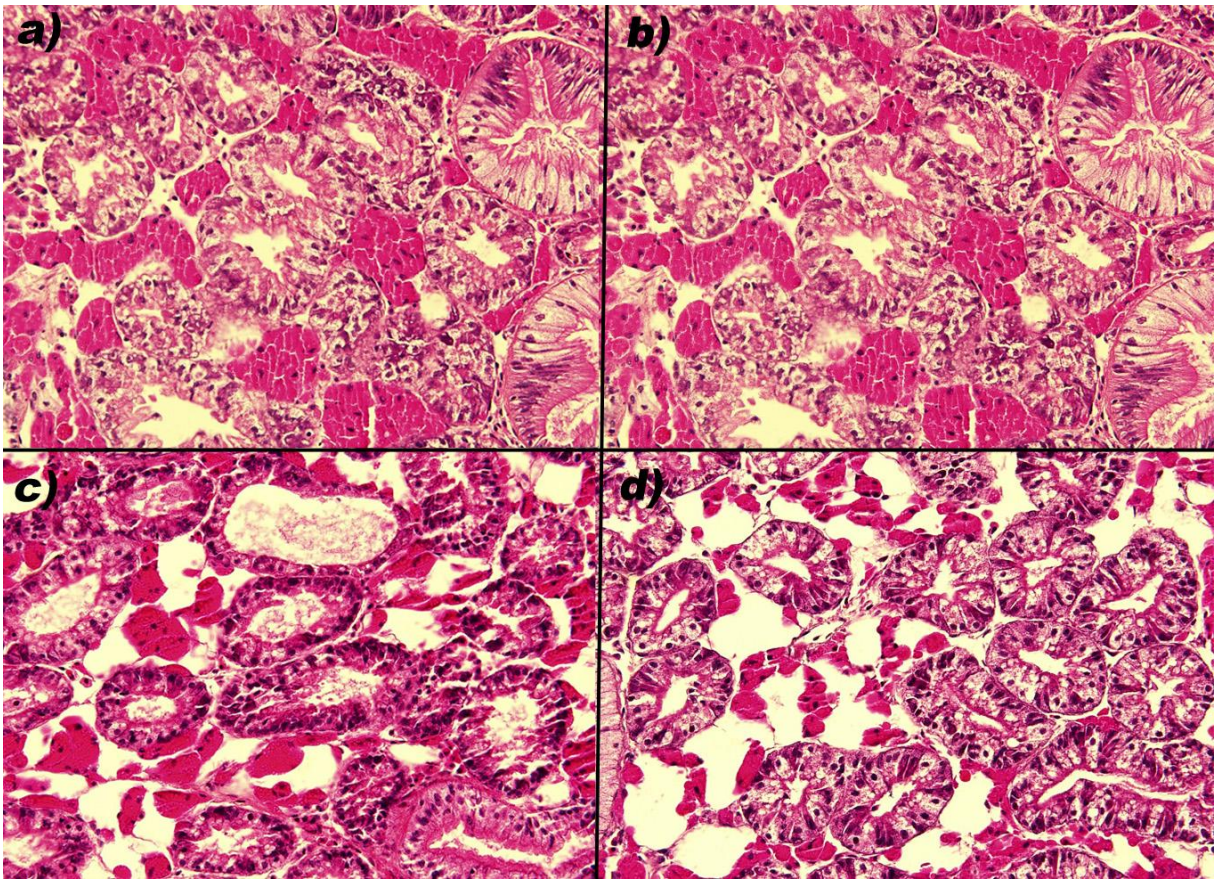

Figure 34. Digestive gland of blue mussels, 400X magnification. Picture a) and b) shows healthy cells. Picture c) shows cells suffering from atrophic and picture d) shows some cells with the present of vacuoles. Picture a, b, c and d are from the reference, dispersant, oil with dispersant and oil without dispersant tanks, respectively.

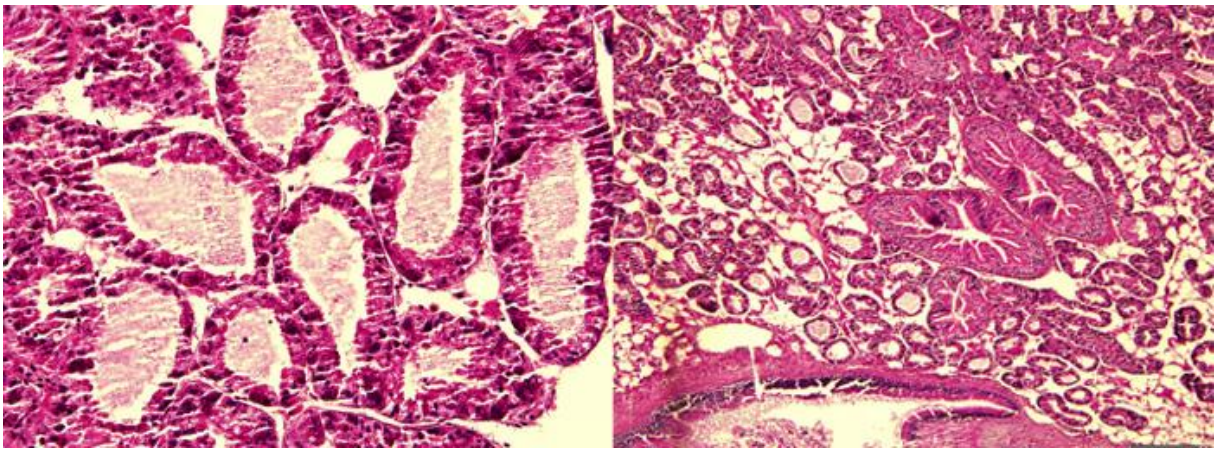

Figure 35. Digestive gland of mussel. 400X magnification to the left and 100X magnification to the right (individual from the oil exposed group).

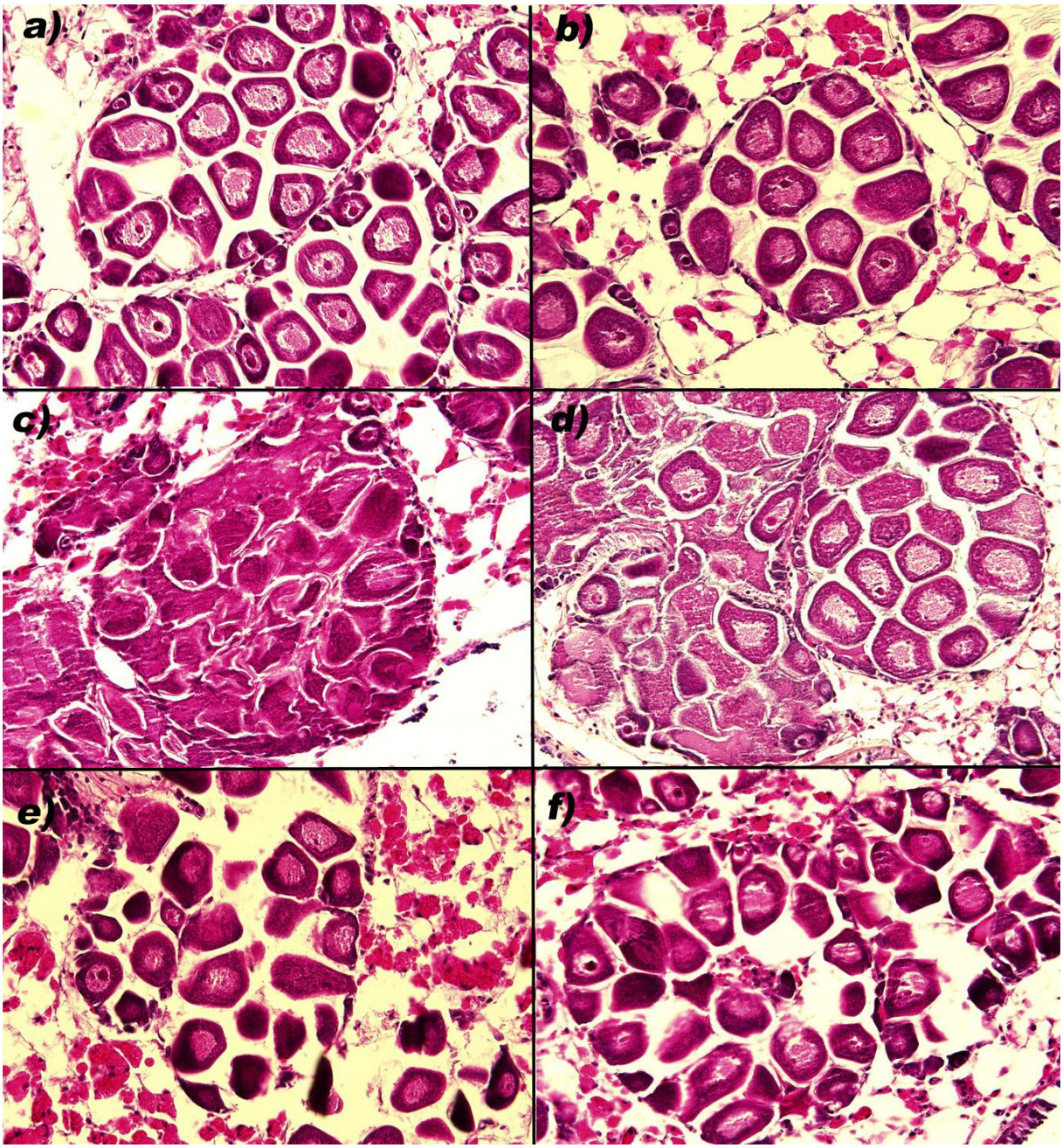

Figure 36. Female gonads from blue mussels showing different effect responses. Picture a) and b) shows somewhat healthy cells. Picture c) and d) shows cells suffering from atresia and picture e) and f) shows cells suffering from apoptosis. Picture a is from the reference tank and the rest is from the oil with dispersant tank.

Figure 34 and Figure 36 shows tissue at different effect stages in digestive gland and female gonads, respectively. Figure 35 shows cells from digestive gland at 400X and 100X magnification, and gives an overview of how many cells that were suffering from atrophic.

5. Discussion

5.1 Experimental factors

Atlantic salmon was used as the fish type for several reasons. The tank had a volume of approximately 300 l, and juvenile fishes were thus required in order to have enough fishes for a good statistical interpretation, but still have enough space in the tank for the fishes. Juvenile fishes also have the advantage that the variance in the individuals due to sex is not so present as if the fish was sexually matured. This is in particular true for EROD activity. Previous studies have shown that the detected P450 level has a tendency to increase in female fishes when they become sexually matured. In the beginning of the planning phase Arctic cod was set as the fish species, but since juvenile cod were not available at the time, Atlantic salmon in their smolt stage were used.

It is easy to get a hold of fresh blue mussels, and blue mussels were thus used in order to have more than one test organism. If both the test organisms showed that they were affected by the oil spill with and without chemical dispersant agent the validity of the test result would be better than if just one organism showed signs of exposure and effects.

It was observed wounds on some individuals, these individuals also showed signs of reduced function. The fishes were observed daily for general observation, but extra attention was given in order to see if the wounds were getting worse or if it was spreading to other fishes in the tank. Fortunately the wounds did not seem to get worse or spread to other fishes. In total six individuals had wounds on their skin. The fishes exposed to oil without dispersant had three individuals with wounds, being the group with the highest number of affected individuals. The oil exposure is not considered as a reason for this, since the fishes exposed to oil with dispersant had the same concentration of oil and only one individual was affected in that tank. One possible cause can be that this group was more exposed to external factors like traffic since it was closest to the area where people worked in the test hall, and that the wounds were a result of stress. But it can also be a coincident that the oil-exposed group had most

individuals with wounds. List of which individuals that were affected is given in appendix A.

5.2 Biomarker responses in Atlantic salmon

Fixed fluorescence and EROD are both biomarkers of exposure, and both of these tests showed that there were an uptake of hydrocarbons in the fish with oil exposure. For FF the wavelength pair 290/335 nm and 341/383 nm were proven to have a statistical significant difference compared to the reference group in the oil exposed groups. Whereas the wavelength pair 380/430 nm did not show any statistically significant difference. The EROD activity test also showed a statistically significant difference in the oil-exposed groups compared to the reference group. The FF and EROD test result shows that the experiment was successful in the way that it was proven to be an uptake of oil components in the fishes exposed to oil with and without chemical dispersant agent. Another aspect of this thesis was to see if there was a higher uptake in the fishes exposed to oil with chemical dispersant compared to the fishes exposed to oil without chemical dispersant. Only the wavelength pair 380/430 in the FF test detected a higher response in the oil with chemical dispersant, but as mention above this was not proven statistically. This aspect of the thesis is discussed further in subchapter 5.4.

One biomarker method of effect was preformed on the fishes, histopathology. Unfortunately almost all of the gill tissue showed damage due to cutting. Cutting on hard tissue like fish gills are very challenging since it is difficult to get thin enough slides for interpretation. All of the slides were cut with a thickness of 5 μm, but for some reason some of the slides were thicker than the others. The few slides good enough for interpretation were not in perfect condition and only a small area of the gill tissue present on the slides were used. As a result of this the amount of data for interpretation of abnormalities were too low for statistically analysing it any further.

5.3 Biomarker responses in blue mussels

Lysosomal membrane stability is a biomarker of effect and is considered to be a general measurement of stress. The NRRT for healthy cells are expected to be around 120-180, which is not the case in this study, see Table 12. Only one individual in the reference group had a NRRT of 150. The first half of the reference group (individuals 001-007) was the first to be analysed, due to lack of experience regarding haemolymph draining this lead to no haemolymph cells in the first three individuals. Seven individuals were analysed the first day of sampling, and 8 the following day. One the second sampling day all of the fish samplings were done, therefore another person performed the draining of haemolymph this day. When comparing these two days it is an obvious difference in NRRT, the first 7 individuals in all four groups had a much higher NRRT compared to the remaining 8 individuals in each groups. Reasons for this is again lack of experience when it comes to haemolymph draining, an additional cause could be different draining techniques.

Draining of haemolymph was difficult since it is impossible to see the adductor muscle where the blood is drained from, you only knew that it was in the area highlighted in Figure 21, chapter 3. When penetrating the adductor muscle you could feel a small resistance, which indicated that the needle was inside the muscle and you could start draining haemolymph. But since the adductor muscle is very small it is easy to press the needle all the way trough, leading to draining of seawater (leftovers after tapping) and sometimes other compartments of the mussels. When pressing the needle multiple times into the adductor muscle it stresses the haemolymph cells leading to a lower NRRT time. Pressing air bubbles out of the needle once the haemolymph have been drained and too harsh mixing of the cell suspension in the eppendorf tube could be additional stressors to the cells. Light is also an additional stressor, and the cells were exposed to light in the preparation of the samples and in the microscope.

The data retrieved from lysosomal stability makes little sense, especially since the reference group seems to come out as the worst group. The result from this test is clearly suffering from a lack of experience when it comes to sample preparation, especially draining of haemolymph. Unfortunately these data's can therefor not be used any further.

Histopathology is also a biomarker of effects. When looking at the digestive gland for both sexes, Table 12 shows that there is an increase in the exposed groups compared to the reference group. In the reference group most of the individuals had colour code 0 or 1 for vacuoles and atrophic in their digestive gland. The oil exposed groups with and without chemical dispersant shows a clearly increase in colour codes 2 and above. In the group with just chemical dispersant the number of individuals good enough for further analysing were too low to form a good basis for interpretation. Data from the group exposed to just chemical dispersant will therefor not be discussed any further. When looking at the female gonads, see Table 12, the apoptosis and atresia had no remarkable differences between the different groups. But since the gonads for the male mussels were not analysed the amount of data retrieved is therefor too low to identify any differences between the groups. However, it seemed as the gonads suffered more from atresia than apoptosis in all four groups.

Atresia, apoptosis, atrophic and presence of vacuoles are all health parameters and gives an indication if the mussels have been suffering as a result of pollutant exposure. The result from the digestive gland interpretation indicates that the mussels exposed to oil with and without chemical dispersant have been suffering more than the mussels in the reference group.

5.4 Effects of dispersant on uptake and effect of oil

Similar studies have been performed earlier by using the same dispersant type, Corexit 9500. One study performed at the School of Environmental Studies at Queen's University in Canada, used EROD activity in trout to see if the fish had a higher PAH uptake in the fishes exposed to oil with chemical dispersant compared to the fishes exposed to oil without chemical dispersant. In their study they concluded that the use of oil dispersant will increase the exposure of fish to hydrocarbons in crude oil (Ramachandran, Hodson et al. 2003). Another study conducted at the CNRS-University in France tested the effect of dispersed oil exposure on the bioaccumulation of PAH and mortality of juvenile *Liza ramada.* Also this study concluded that the fishes exposed to oil with chemical dispersant showed a higher bioconcentration of PAH compared to the fishes exposed to oil without chemical dispersant. They also concluded that there were a higher mortality in the fishes exposed to oil with chemical dispersant (Millinkovitch, Kanan et al. 2010).

The conclusions of these earlier studies also make the result from this master thesis more questionable. As expected from the theory fishes exposed to chemical dispersed oil should show a higher detected response in the FF and EROD activity test compared to the fishes exposed to oil without chemical dispersant. This is because chemical dispersant dissolves the oil droplets into smaller particles so it goes into the water column more easily than undispersed oil. As a result of this the oil is more available for the organisms living in the water column. Why this was not the case in this study is difficult to answer. Before the oil with and without chemical dispersant went into the different tanks it was mixed in the header tanks with seawater under continuous magnetic stirring, see Figure 17 in chapter 3. This could lead to physically dispersed oil making the oil more available in the water column for both of the oil-exposed groups. As mention in chapter 5.1 the seawater outlet was from the top, making it more difficult for the oil to accumulate at the water surface. It was expected to have a lower response in the oil-exposed group without chemical dispersant because of this, since the oil was expected to be more on the surface than for the chemical dispersed oil. One reason for a lower response in the group exposed to chemical dispersed oil could be that the chemical dispersant agents physically changes the oil components, but this was not tested.

From the histopathology result of mussels, it seems as the present of atrophic in the blue mussels digestive gland showed an increased trend in the chemical dispersed oil group compared to the other groups. This is in accordance to what we would imagine from the theory. But however this is only assumption and it is not proven to be a difference in the oil-exposed group with dispersant compared to the oil-exposed group without dispersant.

5.5 Implication for oil spill management in the North

If the results from this study are correct it is great for the oil companies that wants to use chemical dispersant as an oil spill management tool. If there was an oil spill in the north the use of chemical dispersant can be crucial in order to prevent the oil spill from reaching the shoreline. And if the chemical dispersed oil does not give an additional biological effect this is good. However as mentioned in subchapter 5.4 other studies have found that the same chemical dispersant agent as used in this study gives a higher PAH response in the organisms living in the water column when it is used on oil spills.

The decision to use chemical dispersant in oil spill clean up processes in the north is a decision of which area or ecosystem that is most vulnerable. Dispersant applied on the oil spill before it reaches the shoreline potentially decreases the exposure for surface dwelling organisms and intertidal species, while it increases the exposure for water column and benthic species. The decision is normally made regarding the total environmental impact. Would it be worst if the oil is being dispersed into the water column or will the net environmental impact be more crucial if the oil reaches the shoreline and the habitant living there? Each oil spill represents a unique situation and it is therefor no standard answer to this question. Also it is difficult to extrapolate from published research data into field prediction. This is in particular true for long-term exposure.

5.6 Collaboration between Norway and Russia

The need of a collaboration between Norway and Russia when it comes to environmental issues is increasing. Especially now that the disputed area in the Barents Sea has been divided into an official delimitation line. As a result of the official delimitation line and new field discoveries in the area, like Skrugard, the oil and gas activity in the Barents Sea are only expected to grow. Any relation that can be linked to this area with regard to environmental aspect is of value for both countries.

In this thesis and also this master program one of the objective was to link connection among Norwegian and Russian students in the field of environmental science, and to make some kind of relation. This kind of social network is of value for both parts when it comes to sharing information and experience. The collaboration between Norwegian and Russian students like the one preformed in this study is interesting in many ways. One thing is to build relation across the borders, but another thing is that we also get to know how the different countries operate and think in different settings regarding environmental issues. This is something I feel have been well accomplished and it is gained a better appreciation for each other.

5. 7 Future aspects

It would be interesting to see if oil exposure with and without chemical dispersant agent present would result in any abnormalities in the fishes and mussels offsprings. So one idea could be to preform a long time exposure experiment. Also making a more realistic artificial environment, e.g. a larger tanks and reducing external factors like noise and traffic.

6. Conclusion

The fishes exposed to oil with and without chemical dispersant agent showed a statistically significant difference compared to the reference group in the fixed fluorescence test for the wavelength pair 290/335 and 341/383, and for EROD activity.

For mussels the result from Table 12, with regards to histopathology, seems to show an increasing trend in the groups exposed to oil with and without chemical dispersant agent compared to the mussels in the reference tank.

It was not detected any differences between fish and mussels exposed to oil with and without chemical dispersant agent.

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8. Appendix

A – Sampling data

B – SOP

Appendix A

Sampling sheet for Atlantic salmon:

yyyy.mm.dd - ind.-no	Species	Treatment	Size (cm)	Weight (g)	Conditional factor	Sex	Comments	Liver weight (g)
20110120 - 210	Salmo salar	Reference	31	281.90	0.95	F		5.53
20110120 - 211	Salmo salar	Reference	27	161.40	0.82	M	wounds	4.33
20110120 - 212	Salmo salar	Reference	31	257.70	0.87	F		5.83
20110120 - 213	Salmo salar	Reference	31	242.20	0.81	M		5.78
20110120 - 214	Salmo salar	Reference	29	220.80	0.91	F		4.44
20110120 - 215	Salmo salar	Reference	29	241.40	0.99	M		5.69
20110120 - 216	Salmo salar	Reference	29	228.20	0.94	F		3.83
20110120 - 217	Salmo salar	Dispersant	30	275.60	1.02	F		5.04
20110120 - 218	Salmo salar	Dispersant	28	207.30	0.94	F		4.25
20110120 - 219	Salmo salar	Dispersant	29	220.20	0.90	F		4.12
20110120 - 220	Salmo salar	Dispersant	29	224.00	0.92	M		5.35
20110120 - 221	Salmo salar	Dispersant	27	188.50	0.96	M	wounds	4.25
20110120 - 222	Salmo salar	Dispersant	26	143.90	0.82	F		2.26
20110120 - 223	Salmo salar	Dispersant	27	187.20	0.95	M		2.79
20110120 - 224	Salmo salar	Oil	31	277.80	0.93	M	wounds	5.13
20110120 - 225	Salmo salar	Oil	30	265.30	0.98	F		3.31
20110120 - 226	Salmo salar	Oil	32	272.10	0.83	F	wounds	7.06
20110120 - 227	Salmo salar	Oil	29	226.60	0.93	F		3.97
20110120 - 228	Salmo salar	Oil	29	234.90	0.96	F		5.2
20110120 - 229	Salmo salar	Oil	30	263.10	0.97	M	wounds	6.37
20110120 - 230	Salmo salar	Oil	27	187.20	0.95	F		3.04
20110120 - 231	Salmo salar	Oil+Dispersant	27	201.50	1.02	F		3.45
20110120 - 232	Salmo salar	Oil+Dispersant	26	164.00	0.93	M		3.51
20110120 - 233	Salmo salar	Oil+Dispersant	29	201.40	0.83	F	one blind eye	3.79
20110120 - 234	Salmo salar	Oil+Dispersant	29	195.50	0.80	M	wounds	5.24
20110120 - 235	Salmo salar	Oil+Dispersant	29	196.60	0.81	M		3.78
20110120 - 236	Salmo salar	Oil+Dispersant	29	213.20	0.87	F		4.24
20110120 - 237	Salmo salar	Oil+Dispersant	28	191.40	0.87	F		2.43

Sampling sheet for Blue mussels:

yyyy.mm.dd - ind.-no	Species	Treatment	Size (cm)	NRRT (min)	Comments
20110120 - 001	Mytilus edulis	1. Reference	6		no cells
20110120 - 002	Mytilus edulis	1. Reference	5		no cells
20110120 - 003	Mytilus edulis	1. Reference	5		no cells
20110120 - 004	Mytilus edulis	1. Reference	6	120	
20110120 - 005	Mytilus edulis	1. Reference	5	150	
20110120 - 006	Mytilus edulis	1. Reference	7	120	
20110120 - 007	Mytilus edulis	1. Reference	6	120	
20110120 - 043	Mytilus edulis	1. Reference	5	60	
20110120 - 044	Mytilus edulis	1. Reference	7	60	
20110120 - 045	Mytilus edulis	1. Reference	5	60	
20110120 - 046	Mytilus edulis	1. Reference	5	90	
20110120 - 047	Mytilus edulis	1. Reference	5	90	
20110120 - 048	Mytilus edulis	1. Reference	6	120	
20110120 - 049	Mytilus edulis	1. Reference	5	60	
20110120 - 050	Mytilus edulis	1. Reference	6	90	
20110120 - 008	Mytilus edulis	2. Oil	5	120	
20110120 - 009	Mytilus edulis	2. Oil	6	150	
20110120 - 010	Mytilus edulis	2. Oil	5	150	
20110120 - 011	Mytilus edulis	2. Oil	5	150	
20110120 - 012	Mytilus edulis	2. Oil	5	120	
20110120 - 013	Mytilus edulis	2. Oil	6	120	
20110120 - 014	Mytilus edulis	2. Oil	5	150	
20110120 - 059	Mytilus edulis	2. Oil	5	30	
20110120 - 060	Mytilus edulis	2. Oil	5	90	
20110120 - 061	Mytilus edulis	2. Oil	6	15	
20110120 - 062	Mytilus edulis	2. Oil	5	30	
20110120 - 063	Mytilus edulis	2. Oil	6	30	
20110120 - 064	Mytilus edulis	2. Oil	6	30	
20110120 - 065	Mytilus edulis	2. Oil	5	60	
20110120 - 066	Mytilus edulis	2. Oil	6	60	
20110120 - 015	Mytilus edulis	3. Oil+Dispersant	6	150	little colour
20110120 - 016	Mytilus edulis	3. Oil+Dispersant	6	150	little colour
20110120 - 017	Mytilus edulis	3. Oil+Dispersant	6	150	little colour
20110120 - 018	Mytilus edulis	3. Oil+Dispersant	6	150	
20110120 - 019	Mytilus edulis	3. Oil+Dispersant	6	150	
20110120 - 020	Mytilus edulis	3. Oil+Dispersant	5	150	
20110120 - 021	Mytilus edulis	3. Oil+Dispersant	6		no cells
20110120 - 067	Mytilus edulis	3. Oil+Dispersant	6	60	
20110120 - 068	Mytilus edulis	3. Oil+Dispersant	5	90	
20110120 - 069	Mytilus edulis	3. Oil+Dispersant	5	60	
20110120 - 070	Mytilus edulis	3. Oil+Dispersant	5	90	
20110120 - 071	Mytilus edulis	3. Oil+Dispersant	5	90	
20110120 - 072	Mytilus edulis	3. Oil+Dispersant	5	60	
20110120 - 073	Mytilus edulis	3. Oil+Dispersant	5	60	
20110120 - 074	Mytilus edulis	3. Oil+Dispersant	5	60	
20110120 - 022	Mytilus edulis	4. Dispersant	6	150	
20110120 - 023	Mytilus edulis	4. Dispersant	7	150	
20110120 - 024	Mytilus edulis	4. Dispersant	6	150	
20110120 - 025	Mytilus edulis	4. Dispersant	5	150	
20110120 - 026	Mytilus edulis	4. Dispersant	6	150	
20110120 - 027	Mytilus edulis	4. Dispersant	6	180	
20110120 - 028	Mytilus edulis	4. Dispersant	5	150	
20110120 - 051	Mytilus edulis	4. Dispersant	6	60	
20110120 - 052	Mytilus edulis	4. Dispersant	6	90	
20110120 - 053	Mytilus edulis	4. Dispersant	6	90	
20110120 - 054	Mytilus edulis	4. Dispersant	5		no cells
20110120 - 055	Mytilus edulis	4. Dispersant	6	60	
20110120 - 056	Mytilus edulis	4. Dispersant	5	90	
20110120 - 057	Mytilus edulis	4. Dispersant	5	90	
20110120 - 058	Mytilus edulis	4. Dispersant	5	90	

Statistical raw data for Atlantic salmon.

yyyy.mm.dd---ind.-no	Species	Treatment	Size-(cm)	Weight-(g)	condition-factor	Sex	Liver-weight-(g)	LSI	Biliverdin-abs	290/335	log-290/335	341/383	log-341/383	380/430	log-380/430	micromol/l	protein,-mg/ml	EROD,-pmol/min	Log-EROD
20110120---210	Salmo-salar	Reference	31	281.9	0.946258937	F	5.53	1.96	0.07	23.38	1.37	3.29	0.52	0.98	-0.0074	0.0027	30.60	0.0021	-2.6855
20110120---211	Salmo-salar	Reference	27	161.4	0.819996952	M	4.33	2.68	0.11	17.54	1.24	2.94	0.47	0.98	-0.0079	-0.0017	32.60	-0.0012	#NUM!
20110120---212	Salmo-salar	Reference	31	257.7	0.86502635	F	5.83	2.26	0.05	18.47	1.27	2.89	0.46	0.88	-0.0560	0.0100	25.66	0.0090	-2.0475
20110120---213	Salmo-salar	Reference	31	242.2	0.812997214	M	5.78	2.39	0.12	20.84	1.32	3.14	0.50	0.99	-0.0035	0.0010	29.30	0.0008	-3.0946
20110120---214	Salmo-salar	Reference	29	220.8	0.905326172	F	4.44	2.01	0.07	31.84	1.50	4.18	0.62	1.65	0.2167	0.0069	33.10	0.0048	-2.3202
20110120---215	Salmo-salar	Reference	29	241.4	0.989790479	M	5.69	2.36	0.03	19.48	1.29	3.05	0.48	1.00	0.0013	0.0164	27.36	0.0138	-1.8606
20110120---216	Salmo-salar	Reference	29	228.2	0.935667719	F	3.83	1.68	0.03	18.96	1.28	2.86	0.46	0.92	-0.0381	0.0401	31.60	0.0292	-1.5352
20110120---217	Salmo-salar	Dispersant	30	275.6	1.020740741	F	5.04	1.83	0.02	15.25	1.18	2.80	0.45	0.91	-0.0434	0.0161	34.59	0.0107	-1.9713
20110120---218	Salmo-salar	Dispersant	28	207.3	0.94433309	F	4.25	2.05	0.06	16.42	1.22	2.93	0.47	0.87	-0.0615	0.0178	32.43	0.0126	-1.8986
20110120---219	Salmo-salar	Dispersant	29	220.2	0.902866046	F	4.12	1.87	0.13	21.94	1.34	3.20	0.50	0.91	-0.0419	0.0060	27.97	0.0050	-2.3044
20110120---220	Salmo-salar	Dispersant	29	224	0.918446841	M	5.35	2.39	0.12	12.82	1.11	2.50	0.40	1.09	0.0354	0.0046	27.00	0.0039	-2.4111
20110120---221	Salmo-salar	Dispersant	27	188.5	0.957679216	M	4.25	2.25	0.13	18.69	1.27	2.96	0.47	0.76	-0.1175	0.0125	23.58	0.0122	-1.9149
20110120---222	Salmo-salar	Dispersant	26	143.9	0.818730086	F	2.26	1.57	0.14	18.10	1.26	3.27	0.51	0.90	-0.0482	0.0067	26.86	0.0058	-2.2403
20110120---223	Salmo-salar	Dispersant	27	187.2	0.951074531	M	2.79	1.49	0.06	14.36	1.16	2.87	0.46	0.81	-0.0899	0.0366	25.69	0.0328	-1.4844
20110120---224	Salmo-salar	Oil	31	277.8	0.932496392	M	5.13	1.85	0.06	68.42	1.84	7.20	0.86	1.25	0.0959	0.0164	22.44	0.0169	-1.7732
20110120---225	Salmo-salar	Oil	30	265.3	0.982592593	F	3.31	1.25	0.02	36.74	1.57	7.66	0.88	0.87	-0.0620	0.1133	25.71	0.1013	-0.9943
20110120---226	Salmo-salar	Oil	32	272.1	0.830383301	F	7.06	2.59	0.15	67.99	1.83	7.35	0.87	1.08	0.0330	0.0105	31.31	0.0077	-2.1137
20110120---227	Salmo-salar	Oil	29	226.6	0.929107384	F	3.97	1.75	0.07	73.25	1.86	7.78	0.89	1.25	0.0976	0.0432	26.67	0.0372	-1.4289
20110120---228	Salmo-salar	Oil	29	234.9	0.96313912	F	5.2	2.21	0.19	52.60	1.72	5.63	0.75	0.88	-0.0540	0.0156	19.80	0.0181	-1.7415
20110120---229	Salmo-salar	Oil	30	263.1	0.974444444	M	6.37	2.42	0.06	44.58	1.65	5.32	0.73	0.99	-0.0031	0.0285	10.76	0.0609	-1.2153
20110120---230	Salmo-salar	Oil	27	187.2	0.951074531	F	3.04	1.62	0.10	60.57	1.78	6.63	0.82	1.02	0.0082	0.0308	14.35	0.0493	-1.3069
20110120---231	Salmo-salar	Oil+Dispersant	27	201.5	1.023726058	F	3.45	1.71	0.05	48.37	1.68	6.10	0.79	1.30	0.1146	0.0266	25.90	0.0236	-1.6264
20110120---232	Salmo-salar	Oil+Dispersant	26	164	0.933090578	M	3.51	2.14	0.10	56.69	1.75	6.44	0.81	1.44	0.1590	0.0260	23.07	0.0260	-1.5856
20110120---233	Salmo-salar	Oil+Dispersant	29	201.4	0.825782115	F	3.79	1.88	0.14	52.83	1.72	5.92	0.77	1.07	0.0282	0.0191	20.94	0.0209	-1.6791
20110120---234	Salmo-salar	Oil+Dispersant	29	195.5	0.801590881	M	5.24	2.68	0.12	52.71	1.72	6.22	0.79	1.09	0.0390	0.0090	19.76	0.0105	-1.9780
20110120---235	Salmo-salar	Oil+Dispersant	29	196.6	0.806101111	M	3.78	1.92	0.08	60.33	1.78	7.63	0.88	1.46	0.1632	0.0088	23.34	0.0087	-2.0625
20110120---236	Salmo-salar	Oil+Dispersant	29	213.2	0.874164582	F	4.24	1.99	0.03	35.17	1.55	4.73	0.67	1.08	0.0350	0.0359	23.63	0.0350	-1.4565
20110120---237	Salmo-salar	Oil+Dispersant	28	191.4	0.871902332	F	2.43	1.27	0.05	21.86	1.34	3.60	0.56	1.29	0.1109	0.0407	22.56	0.0414	-1.3825

Appendix B

SOP-FF

SOP - FF & SFS PAH metabolite screening in unhydrolysed fish bile

Table of content

Theme: Standard Operating Procedure (SOP) for an analysis used at IRIS-Akvamiljø.		Document: Identity xxx
Based on: Previous SOPs on fish bile fixed fluorescence (FF) and synchronous fluorescence spectroscopy (SFS) (Endre Aas 2000 & 2001)		Revision nr.: xxx
Scope: This document describes semiquantitative fluorometric methods for determination of PAH metabolites in fish bile.		Replacing: Identity / Rev. yyy Valid from: xx.xx.200x
Revised by (date/sign): 29.08.2007 Jonny Beyer	Controlled by (date/sign): xx.xx.200x enter your name here	Approved by QM (date/sign):

1 Introduction

PAH metabolites in bile can be measured semi quantitatively by means of fluorescence detection at certain fixed excitation and emission wavelength pairs (fixed fluorescence detection, or FF) or by fluorescence scanning (emission scans, excitation scans or synchronous scanning, or SFS) (Aas et al. 2000; Ariese et al. 2005). FF detection is suited for providing semiquantitative concentration levels of PAH metabolites. SFS is suited for investigating the source of contamination (i.e. discriminating between pyrogenic or petrogenic PAH mixtures). Pyrene is a strong fluorophore and can be used as a surrogate standard for expressing FF signals as pyrene equivalents.

Data on the general concentration of the bile fluid is required when bile fluorescence detections are used for assessment of PAH exposure, this type of information is analyses of biliary biliverdin or general proteins content (see separate SOPs).

PAHs in native bile are present as conjugated structures which are not available as standards. Hydrolysis is sometimes used for providing deconjugated structures which can be identified and quantified (see separate SOPs).

2 Other relevant documents and files

Doc / file	*Where to find it:*
SOP- necropsy of fish	GLP SOP folder
SOP – Biliverdin analysis in fish bile	GLP SOP folder
SOP - protein (Bradford)	GLP SOP folder
SOP - FF & SFS PAH metabolite measurement in hydrolysed (deconjugated) bile	In prep
SOP – HPLC-F detection of PAH metabolites in fish bile	In prep
SOP – GC-MS detection of PAH metabolites in fish bile	In prep

3 HSE risk associated with this procedure

Fish bile spoiled on the operator's eye may cause severe damage to the sight, therefore always use protection glasses when working with gall bladder and bile.

4 Equipment

Equipment	*Where to find it:*	*Comments:*
Fluorometer (Perkin Elmer LS50B)	Chemistry lab	Warm up instrument before use
Quartz cuvette (4 or 1 ml)	Chemistry lab	Respective sample volume: 3 and 0.8 ml
Pipettes (10-40, 40-200, 100-1000 and 1000-5000 µl)	Chemistry lab or ecotox lab	Ensure calibration
Table centrifuge	Ecotox lab	Cool the centrifuge

		before use

5 Chemicals

Chemical	*Where to find it:*	*Source:*
Methanol	Volatile chemicals cabinet	
Distilled water	Chemistry lab	
Pyrene (surrogate standard)	Chemicals cabinet	Sigma, St.Louis, USA

6 Buffers and solutions

Buffers and solutions	*Preparation and storage:*
Diluting solvent	Methanol mixed 1:1 with distilled water (= 50% MeOH)

7 Procedure

1. Obtain bile samples from freezer (see note A) and thaw them on ice. Ensure that you include house control bile samples for quality control.
2. Turn on and warm up instruments. Set the fluorometer to correct fluorometrical settings (see note B).
3. Dilute the bile in 50% MeOH in order to prepare for biliverdin analysis (see note C for dilution range in biliverdin analysis).
4. Centrifuge the pre-diluted bile sample (5000G for 5 minutes) (see note D).
5. Carry out the Biliverdin analysis and register the result (use the SOP for analysis of biliverdin).
6. Dilute the bile sample further in diluting solvent to the appropriate measurement dilution (see note E).
7. Transfer the diluted bile to quartz cuvette, and put it into the pre-warmed fluorometer.
8. Measure the fluorescence (FF or SFS, or both) of the analysis mixture and register the result.

8 Notes

A. Bile samples are best stored in freezer (-20°C or –80°C), as pure bile without the gall bladder. In cases of small bile volumes (< ca 30 µl), an initial dilution of the bile in distilled water is sometimes done during the necropsy procedure, this to simplify the handling of the sample during the later analysis (should be noted in the sampling description).

B. Fluorometer settings:

   a. Slit widths: Normal settings are 2.5 nm or 5 nm for both excitation and emission light. Consider the outcome to confirm that your signal is within the measuring range of the fluorometer, and adjust to other slit width if necessary. NB, always register which slit width you choose to use.

   b. Fixed Fluorescence settings of excitation and emission wavelengths

      i. 290/335 nm for detection of naphthalene type of metabolites

      ii. 341/383 nm for detection of pyrene type of metabolites

      iii. 380/430 nm for detection of benzo[a]pyrene type of metabolites

   c. For synchronous fluorescence spectroscopy: Simultaneous scanning of both excitation and emission wavelengths with constant difference between these ($\Delta\lambda$). A $\Delta\lambda$of 42 nm is suitable for the detection of naphthalene, pyrene and benzo[a]pyrene type of metabolites. Peaks in the scan can be attributed naphthalene, pyrene and benzo[a]pyrene metabolites. In particular conjugated 1-OH pyrene has a particular well defined peak at 341/383 nm (excitation/ emission).

C. Biliverdin measurements generally need more concentrated bile than FF and SFS detections do. Hence, a two step dilution procedure is practical with biliverdin analysis first and FF/SFS analyses afterwards in the same but further diluted bile specimens. Depending on how much bile is available for analysis the typical dilution for biliverdin analysis range in the 40x - 400x dilution (the less dilution the better).

D. Samples should be centrifuged prior to analysis to get rid of eventual proteins or precipitation in the bile sample. (Apply 5000G for 5 minutes). Performed before biliverdin analysis.

E. Normally, concentrated bile will need a dilution of approximately 1:1000 – 1:2000 in order to avoid inner filter effects in fluorescence measurements (Aas et al. 2000). A dilution - response curve should be made from bile of an exposed individual before a sample series is measured, to decide for the appropriate sample dilution. A too high dilution may reduce the sensitivity of the method.

9 Quality control & method validation elements

The use of house control bile samples FF signals in reference bile samples

Both a positive and a negative control reference bile sample should be analysed for each measurement series carried out. Reference standards should be kept in –80°C freezer.

Present reference samples:
Negative control: HAK Control
Positive control: HAK Exposed
Biliverdin should be measured prior to FF analysis according to the SOP for biliverdin analysis.

Measure the fluorescence in 1:1600 times diluted samples in quartz cuvette, slit widths 2.5/ 2.5 nm (ex/em) at the wavelength pairs 290/335 nm, 341/383 nm and 380/430 nm. Slit widths might be set differently on different fluorometers to get within measuring range of the instrument.

Old Signals in HAK96 control samples

		Biliverdin	Pyrene	Naphthalene	BaP
		Abs 660 nm	341/383 nm	290/335 nm	380/430 nm
		100x Dil.	1600x Dil.	1600x Dil.	1600x Dil.
HAK96 control dilution 1600x					
22.12.97	JB	0,178	9,4	31	3,4
22.12.97	JB	0,176	8,9	30	3,3
02.01.98	JB	0,156	9,3	29	3,7
Mean signal		**0,17**	**9,2**	**30**	**3,5**
HAK96 exposed dilution 1600x					
22.12.97	JB	0,166	425	56	23
22.12.97	JB	0.16	414	54	23
02.01.98	JB	0,152	440	55	25
06.01.98	JB		465		
Mean signal		**0,159**	**436**	**55**	**24**

10 References

Ariese, F., J. Beyer, G. Jonsson, C. P. Visa and M. M. Krahn, 2005. Review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile. ICES-TIMES Techniques in Marine Environmental Sciences, pp 41.

Aas, E., J. Beyer and A. Goksøyr, 2000. Fixed wavelength fluorescence (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish: an evaluation of compound specificity, inner filter effect and signal interpretation. Biomarkers 5, 9-23.

SOP-NRRT

SOP Neutral Red Retention Time assay In *Mytilus Edulis*

Table of content

Theme: Standard Operating Procedure (SOP) for Neutral Red Retention Time in *Mytilus Edulis*, Blue mussel		Document: IRIS 2.2-433
Based on: Method decribed in (Lowe, Fossato et al. 1995)		Revision nr.: 001
Scope: This document describes the use of Neutral Red assay as an indicator of contaminant exposure in invertebrate cells		Replacing: Valid from: 24.01.2011
Prepared/revised by (date/sign): 16.01.2009 Solveig Apeland	Controlled by (date/sign): 14.07.2010 Anne Helene S. Tandberg	Approved by QM (date/sign): 24.01.2011 Kjell Birger Øysæd

1 Introduction

The Neutral Red Lysosomal Retention Assay

The ability of cells to take up and retain the cationic vital dye, neutral red, has been used in several studies as a measure of cytotoxicity (Dierickx and Vandevyver 1991). Work by Lowe *et al.* (1992) demonstrated the use of the neutral red assay as an indicator of contaminant exposure in fish(Lowe, Moore et al. 1992). Further work has developed the technique for use on invertebrate digestive cells (Lowe and Pipe 1994) and on invertebrate haemolymph cells (Lowe, Fossato et al. 1995) as a reliable indicator of invertebrate health in laboratory and field studies.

2 Other relevant documents and files

Doc / file	*Where to find it:*
Picture collection (with descriptions) from NRRT assay run on other relevant species: Amphipod, Scallops, Sea urchin etc?	P\KHMS\4-2 - Control Charts & Reference materials\NRRT

3 HSE risk associated with this procedure

HSE: Special precautions to be taken when carrying out this work						
Procedure	Volume	R/S phrases and MSDS link	Handling, storage and registration	Protective clothing	Waste treatment and disposal	Internal Risk Assessment Health (1-7)
Neutral red	20 mg	Not classified as dangerous according to Direktiv 67/548/EØF. There may be potential harmful effects to respiratory system, skin, eyes and by swallowing. MSDS Neutral red	Chemistry lab, ECE, Bridge.	Nitril gloves, eye protection and respiratory protective equipment is recommended.	Special waste treatment	not yet assessed
DMSO	1 ml	Not classified as dangerous according to Direktiv 67/548/EØF. There may be potential harmful effects to respiratory system, skin, eyes and by swallowing. MSDS DMSO	Chemistry lab	Nitril gloves, eye protection and respiratory protective equipment is recommended.	No special requirements	not yet assessed

4 Equipment

Equipment	*Where to find it:*	*Comments:*
1 ml Syringes	Ecotox lab	
Needles (0,6 x 25 mm)	Ecotox lab	
Eppendorf tubes, preferably siliconised	Ecotox lab / Chemistry lab	
Microscope slides	Ecotox lab	
Cover slip 22 x 22 mm	Ecotox lab	
Scissors/tweezers, dissection tray	Ecotox lab	
Stopwatch	Ecotoxlab	
Light proof humidity chamber (black box)	Microscopy room	
Pipette and pipette tips for 30 µl	Ecotox lab / Chemistry lab	
Light microscope, magnification 10/20 and 40	Microscopy room	
NRRT-sheet for correct organism	NRRT-folder	

5 Chemicals

Chemical	*Where to find it:*	*Source:*
DMSO dried (Dimethylsulfoxid) (FW= 78,13)	Chemistry lab	VWR 1.02931.1000
HEPES (FW = 238,30)	Chemistry lab	VWR 1.10110.0250
$CaCl_2$ dihydrate (FW=147,01)	Chemistry lab	
MgSO4 heptahydrate (FW = 246,47)	Chemistry lab	VWR 1.06067.1000
NaCl (FW = 58,44)	Chemistry lab	VWR 1.06404.1000
KCl (FW = 74,56)	Chemistry lab	Sigma P9541.500G
Neutral Red (FW= 288,78)	Grovkjøkken 313 (4°C)	Sigma N7005-5G

6 Buffers and solutions

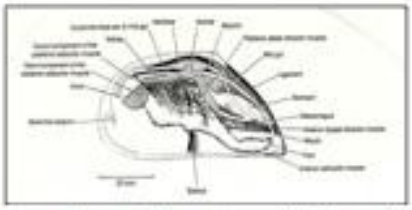
Buffers and solutions	*Preparation and storage:*
Physiological saline (Peek and Gabbott 1989)	4.77 g Hepes (20mM) 1.47 g $CaCl_2$ x $2H_2O$ (10mM) 13.06 g $MgSO_4$ x $7H_2O$ (53mM) 25.48 g NaCl (436mM) 0.75g KCl (10mM) into distilled water to give a 1 L solution. The solution is adjusted to pH 7.36 with 1 M NaOH, and stored in fridge. If you want to store the solution for more than 5-7 days, check the pH before reuse.
or	or
Filtered sea water	Freshly sampled and filtered seawater from where the animals have been collected to make sure you have the same salinity as in the animals. The sea water must be filtered through a Syringe Filter 0,2µm.
Neutral Red Stock Solution	A stock solution of the dye Neutral Red is prepared by dissolving 20 mg of dye in 1 ml of Dimethyl sulfoxide (DMSO). This stock can be stored in the fridge, in a light proof bottle, for up to 3 weeks, and for a longer period of time if stored in freezer. NB! It thaws slowly, take it out of the freezer 1 hour before use. **See note 2.**
Neutral Red Working Solution	5 µl is added to 995 µl filtered sea water or physiological saline. The working solution is also stored in a light proof bottle/vial, and is made up on the day of use. Fresh solutions are used for each experiment. **See note 2.**

7 Procedure

1. Haemolymph Extracting Procedure

   a. For *Mytilus edulis*, haemolymph is drawn from the posterior adductor muscle (PAM). This is the compact, slightly translucent or white, collection of filaments, attached to the inside of the upper and lower valves. **See note 1 and 3.**

PAM



   b. Gently prising open the shell (using a small scalpel or scissors) will reveal the muscle located at one end of the shell, lying adjacent to the hinge.

c. For *M. edulis*, a syringe should be filled with 0.1 ml of the physiological saline or filtered sea water, then 0.1 ml of haemolymph is withdrawn from the muscle (PAM) using a 1 ml syringe with a 0.6 x 25 mm needle. If 0.1 ml cannot be obtained, or more haemolymph is required, then the target should be to achieve a **50:50** mixture of haemolymph and saline/sea water. If repeated bleeding is required from an individual then extra care should be taken during haemolymph extraction. Smaller specimens, particularly in the case of *M. edulis*, may prove difficult to rebleed and it is suggested that adequately sized animals are chosen to suit the experimental requirements (> 4 cm in length).

d. After removal of the needle (to reduce shear stress) haemolymph samples should be rapidly dispensed into a (siliconised) eppendorf tube after each bleeding. The haemolymph extracting procedure should be carried out in a rapid, efficient manner, the aim being to remove the haemolymph without unduly stressing the specimen and compromising the sample. It should not take an experienced operator more than 1-2 minutes to extract haemolymph from one individual.

2. Slide preparations

a. Mix the cell suspention by inverting the eppendorf tube a few times, firmly, but gently, to avoid damaging the cells. **30µl** of cell suspension (haemolymph/saline mixture) is then transferred from the eppendorf and onto the centre of a clean microscope slide. The cell suspension must be left in a light proof humidity chamber at 20°C (room temperature) for **15 minutes** to allow adherence of the cells. A humid atmosphere can be obtained by placing wet paper towels in the base of the chamber. The chamber is supposed to prevent the haemolymph samples from drying out and photo-activation of the neutral red probe.

b. After 15 minutes the slides are removed individually from the chamber and the excess suspension is carefully tapped off. Holding one end of the slide, it is tapped lightly on a paper towel on the bench top. This will leave a monolayer of cells on the slide. The slides are quickly returned to the humidity chamber.

3. Probe treatment

a. A working solution of the probe, Neutral Red, should be prepared as described above.

b. **30µl** of the working solution (same volume, or slightly smaller volume, as cell suspension added) is then added to the cell layer, and finally a 22 x 22 mm cover slip placed on the slide. Slightly smaller volume is to be used if the air humidity is high – this is done with adding a larger volume than **30µl** of the cell suspension.

4. Visualisation of Neutral red retention time

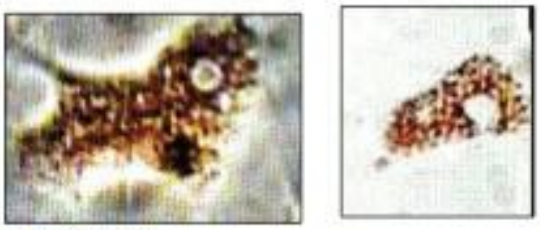
   a. After 15 minutes incubation in the lightproof humidity chamber, slides are examined systematically using light microscopy. The coloured cells are located using lower power (x10/20) and then examined using x40/100 magnification. The light level is kept to a minimal tolerable level, recorded and maintained throughout the subsequent analysis. Examination time for each slide should be kept to less than a minute.

   b. Following a further 15 min incubation, the preparation are examined again and thereafter systematically at 30 minutes intervals to determine at what point in the time there is evidence of dye loss from the lysosomes to the cytosol.

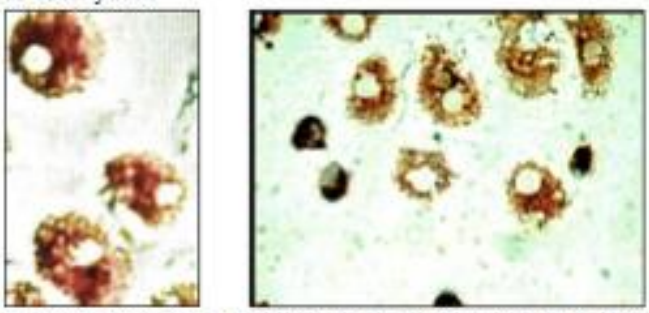
   c. The test for each slide terminated when dye loss is evident in 50% (numerically assessed within each field view) of the granular haemocytes, and the time is recorded when this occurs.

   d. The mean retention time (NRRT) is then calculated for each sample set. A healthy sample set of *M. edulis* normally has a retention time from 150-180 minutes.

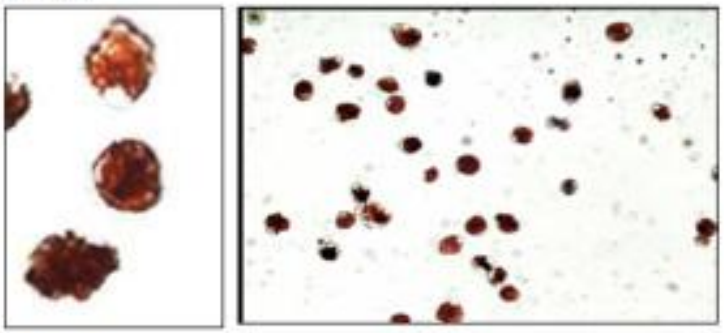
Examples



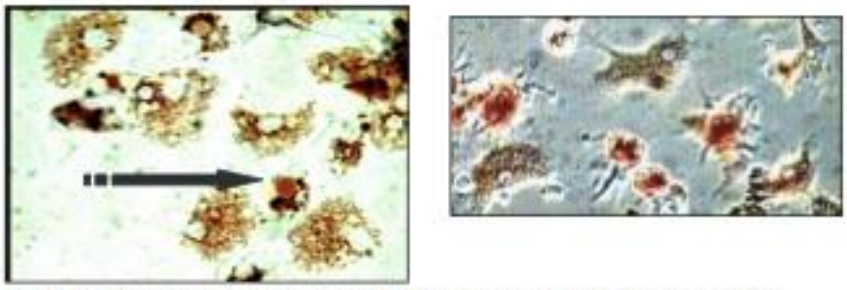
A: Healthy cells



B: Dye loss is evident in some of the cells and the cell morphology is changing from irregular to round.



C: Dead cells



D: Enlarged lysosomes from a spawning mussel. E: Endpoint; >50% dead cells.

8 Notes

1. Spawning animals should not be included in any tests, because NRRT will normally decrease dramatically when the animals are spawning. The lysosomes are enlarged and will start leaking were quickly.
2. After discussions with D. Lowe it has been worked out that we can use this concentration of the Neutral Red working solution, although it is a different one than in (Lowe, Fossato et al. 1995). The difference in concentration has no influence on the results.
3. 15 individuals must be used in each group, due to the high inter-individual variation for this assay.

9 References

Dierickx, P. J. and I. E. Vandevyver (1991). "Correlation of the Neutral Red Uptake Inhibition Assay of Cultured Fathead Minnow Fish Cells with Fish Lethality Tests." Bulletin of Environmental Contamination and Toxicology **46**(5): 649-653.

Lowe, D. M., V. U. Fossato, et al. (1995). "Contaminant-induced lysosomal membrane damage in blood cells of mussels *Mytilus galloprovincialis* from the Venice Lagoon: An in vitro study." Marine Ecology-Progress Series **129**(1-3): 189-196.

Lowe, D. M., M. N. Moore, et al. (1992). "Contaminant Impact on Interactions of Molecular Probes with Lysosomes in Living Hepatocytes from Dab *Limanda-Limanda*." Marine Ecology-Progress Series **91**(1-3): 135-140.

Lowe, D. M. and R. K. Pipe (1994). "Contaminant Induced Lysosomal Membrane Damage in Marine Mussel Digestive Cells - an in-Vitro Study." Aquatic Toxicology **30**(4): 357-365.

Peek, K. and P. A. Gabbott (1989). "Adipogranular Cells from the Mantle Tissue of *Mytilus-Edulis*-L. .1. Isolation, Purification and Biochemical Characteristics of Dispersed Cells." Journal of Experimental Marine Biology and Ecology **126**(3): 203-216.

Neutral red retention time (mussels)

Date: _____ Project:_____
Operator:_____
Comments:_____

Time									
Slide	15 min	30 min	60 min	90 min	120 min	150 min	180 min	NRRT (min)	Comment
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
14									
15									

Average NRRT
Median NRRT

NB: If the data are normally distributed, average NRRT should be calculated, otherwise median NRRT is more correct.

Information to hand to Emily Lyng:

Experiment + Responsible person + Analyst	
Species	
Exposure type and concentrations (Oil, drilling mud, produced water. At least nominal concentrations)	
Mean and median control (Time to 50% dead control cells)	
Control/Exposed (Relative difference: Did the exposed cells die twice as fast as the control cells – or just a few percent faster?)	
Statistics (Did we conclude that it was a significant response? Yes/no) **Type of statistical test used.**	

SOP-Histology

SOP - Collection and preparation of histology samples

Table of content

Theme: Standard Operating Procedure (SOP) for collection and preparation of histology samples at IRIS Biomiljø.		Document: IRIS 2.2-422
Based on:		Revision nr.: 2
Scope: This document describe the preparation of histology samples for both frozen sections and wax sections.		Replacing: 1 Valid from: 01.10.2009
Prepared/revised by (date/sign): 30.09.09 Nadia Aarab Brit Fjone Godal	Controlled by (date/sign): 01.10.09 Brit Fjone Godal	Approved by QM (date/sign): 01.10.2009 Kjell Birger Øysæd

1 Introduction

In order to prevent the appearance of post mortem artefacts, it is essential that the specimen is handled with extreme care. All tissues need to be placed into histological fixative for wax sections or frozen in liquid nitrogen immediately after dissecting mussels or fish.

If visible abnormalities are present, a section should also be taken through the entire depth of the affected area(s), including, where possible, adjacent to normal tissue. At this juncture, it is important that accurate notes are taken to describe the gross features of the lesion so that it may be confidently related to its microscopic appearance after the sample has been processed in the laboratory.

2 HSE risk associated with this procedure

Formaldehyde 37 %:
May cause cancer. Vapor harmful. Harmful if inhaled or absorbed through skin. Causes irritation to skin, eyes and respiratory tract. May be fatal or cause blindness if swallowed. Use local exhaust ventilation and chemical safety goggles. Flammable liquid and vapor. Gas vaporizes readily from solution and is flammable in air. Containers may explode when involved in a fire. Harmful to aquatic life in very low concentrations. Hazardous waste.

Calcium chloride:
Causes irritation to skin, eyes and respiratory tract. Harmful if swallowed or inhaled. Use local exhaust ventilation and chemical safety goggles. Hazardous waste.

3 Equipment

Equipment	*Where to find it:*	*Comments:*
Balance	Ecotoxicology lab	
Ruler	Ecotoxicology lab	
Al-foil	Ecotoxicology lab	
Parafilm	Histology lab	
Cutting board	Ecotoxicology lab	
Forceps	Dissection kit	
Scalpel	Dissection kit	
Scissors	Dissection kit	
Cryostat chucks	Histology lab	
Histocassette	Histology lab	Different colour for different tissues.
Freezer -40°C	Old histology lab	Contact histology group before putting samples in the freezer.

4 Chemicals

Chemical	*Where to find it:*	*Source:*
Frozen Section Medium	Histology lab	Richard-Allan Scientific
Formaldehyde	Chemistry lab	Merck
Calcium chloride	Chemistry lab	vwr
Sodium chloride	Chemistry lab	Merck
Anaesthesia (fish)		Metakain, Benzokain, Etomidat, Metomidat or Klorbutanol. (Brattelid, T.)

5 Buffers and solutions

Buffers and solutions	*Preparation and storage:*
Baker's solution (fish).	100 ml formaldehyde (37%) + 10 g calcium chloride ($CaCl_2$). Up to 1L with distilled water. Stored at 4 °C.
Baker's calcium-solution (mussel)	100 ml formaldehyde (37%) + 25 g sodium chloride (NaCl) + 10g calcium chloride ($CaCl_2$). Up to 1L with distilled water. Stored at 4 °C.

6 Sampling procedures

6.1 Fish

After collection of the fish, the individuals are immediately transferred to plastic containers with seawater collected from the sampling site. The fish are kept in containers until dissection.
For gill histopathology, it is important to anaesthetize the fish before sampling. Lamellar clubbing and lifting of the epithelium can be induced if the fish are killed by a blow to the head or simply if they are handled roughly prior to dissection.

Regulation on Animal Experimentation (Forskrift om forsøk med dyr): *§14 Painful experimentation: Should an experiment be assumed to be painful, anaesthesia must be used, except if the aims of the experiment prevent this and the National Animal Research Authority has approved the absence of anaesthesia in the particular experiment. The experiment must not be commenced before the anaesthesia has taken effect and must be concluded before its effect has worn off.*

The tissues should be dissected within 24 h after sampling. Length, liver weight, gonad weight, gross external disease or parasites in individual fish should be recorded.

6.2 Bivalves

The bivalves should be kept in seawater or on ice after sampling, and the dissection should be made within 24 h after sampling. Measure the shell dimensions and wet and dry weights. Insert scalpel into ventral byssal cavity and move knife down so it cuts the posterior adductor muscle. Open shell and remove byssal thread (Figure 1). Analyse tissue for the presence of parasites, pearls or other abnormalities.

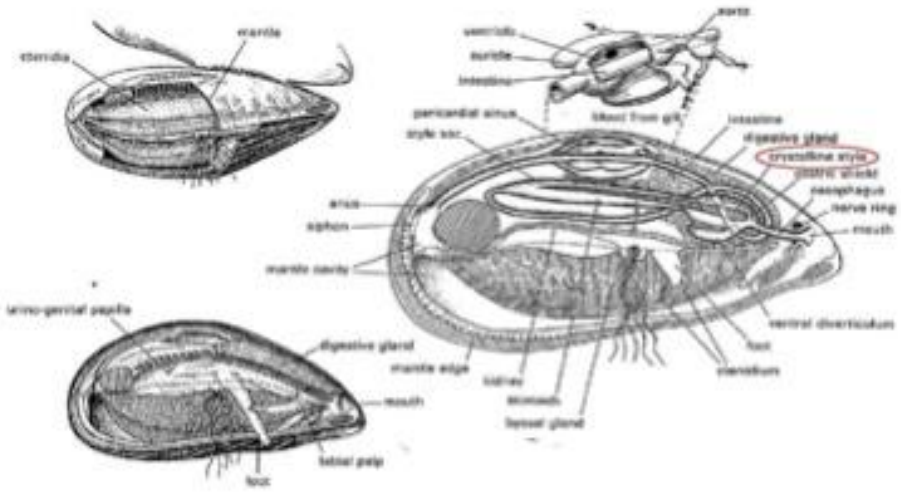


Figure 1 Anatomy of Mussel

6.3 Labelling

All samples should be labelled in the same way.

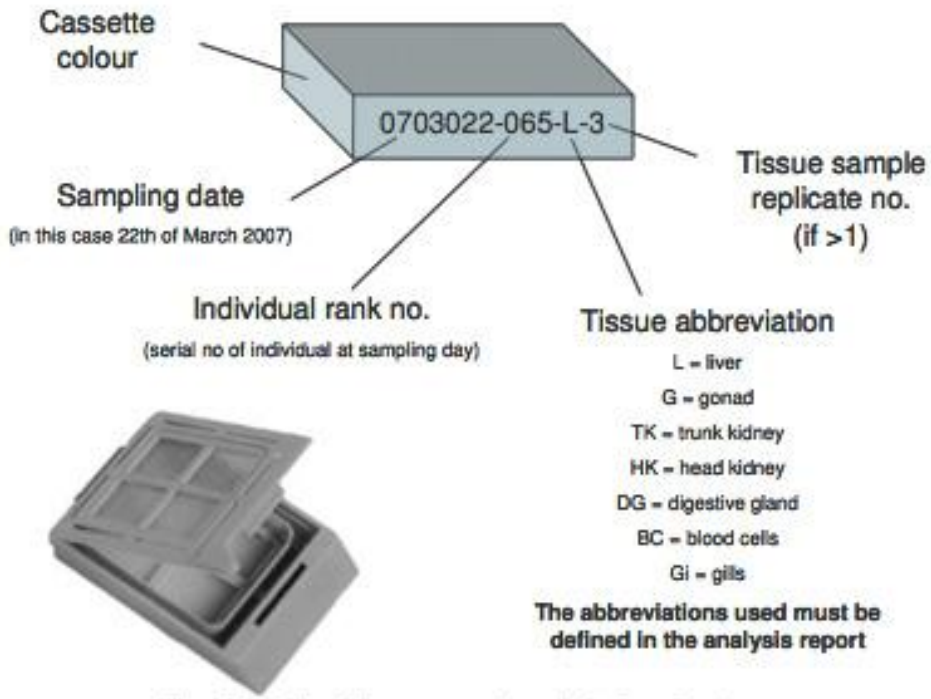


Figure 2: Labelling of histo cassettes and cryo-chucks (Jonny Beyer)

7 Procedure

7.1 Frozen sections

For fish, a piece (5 mm x 5 mm) of the central part of the liver (Figure 3) is put in a cryo vial and immediately frozen in liquid nitrogen. For bivalves, a piece of the digestive gland or gonads is put in a cryo vial and immediately frozen in liquid nitrogen. The cryo vials are stored at -40°C or -20°C until cutting. Three frozen tissue samples are fixed on a cryostat chuck (Figure 4). During this procedure, keep the cryo vials and chucks inside the cryostat at -20°C. Cryostat sections (10 µm) are then cut in a cryostat with the cabinet temperature below -25°C with object cooling -18°C and knife cooling -16°C. The sections are transferred to "warm" slides (20°C). The slides can be stored in the freezer at -40 °C or -20 °C before use. After cutting, the chucks are sealed by wrapping in parafilm and then in Al-foil and stored at – 40°C -20°C.

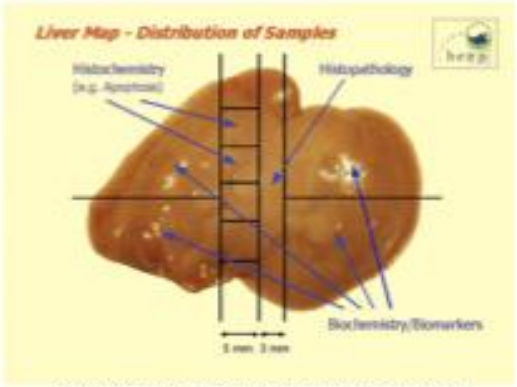


Figure 3: Liver map according to Feist et al. 2004.

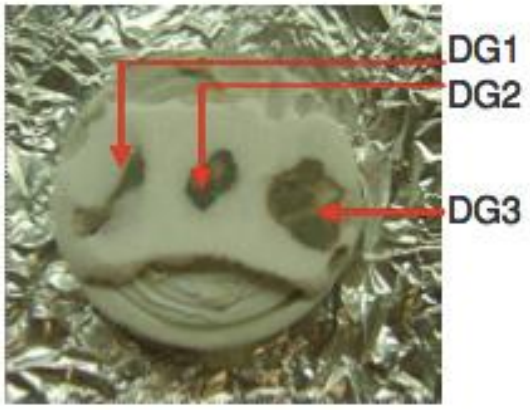


Figure 4: Chuck preparation.

7.2 Wax section

The target organs are dissected and put in a pre-labelled histocassette. Use a pencil to label histocassettes. All histocassettes (Figure 5) are placed in a plastic container of Baker's solution (fish or mussel). Fix the tissue samples with 10 times their volume with Baker's solution. The tissue samples should be no thicker than 1 cm so they will fix properly, but long and wide enough to represent the different areas of a tissue. If the sample contains any abnormalities, dissect the abnormal tissue and let it be surrounded with some normal tissue.

Samples should be handled carefully, do not scrape surfaces of tissues or compress the tissue with the forceps. The samples should be stored in Baker's solution at 4°C until embedding.

Figure 5: Histocassette for wax section

8 Note

Caution: prolonged storage at -40 °C or -20°C may cause the sections to become fragile with a resulting loss of tissue.

9 References

Brattelid, T. (1999). Forsøksdyrlære for fiskeforskere. Norges veterinærhøgskole. 84-91.

Feist, St., Lang, Th., Stentiford, G.D., Koehler, A. (2004). Biological effects of contaminants: The use of liver pathology of flatfish for monitoring biological effects of contaminants. ICES techniques in marine environmental sciences, 38, 1-42.

Forskrift om forsøk med dyr (Regulation on Animal Experimentation): Fastsatt av Landbruksdepartementet 15.1.1996.