Effects of crude oil contaminated sediment on the early life stages of lumpsucker (*Cyclopterus lumpus* L.).

Master thesis in Biology
Field of study: Marine Ecology (60 ECTS)

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Abstract:

Lumpsucker (*Cyclopterus lumpus*) is a demersal and shallow water spawning fish species with a relatively long egg incubation period. In case of acute oil pollution the embryos will be exposed to PAHs in the water for a relative long period. In this experiment we made a flow through rock column incubation system with oil contaminated gravel and exposed fertilized lumpsucker eggs to the water soluble fractions (WSF) of North Sea crude oil. During the incubation period we looked for differences in embryo development, mortality, hatching success, larvae length and size, embryo/larvae histology and possible abnormalities within oil exposed (High dose tPAH average 3110 µg L^{-1}, low dose tPAH 210 µg L^{-1}) and control group (Average tPAH 18 µg L^{-1}). We found no significant differences in either of the checked for parameters, except for the hatching success being much higher in the control treatment vs. the two oil exposed treatments. Despite the fact that we found no significant treatment effect in the oil exposed groups, the observed hatching success in the control units makes us believe that oil has an effect on the embryos ability to hatch and may thus affect the recruitment of lumpsucker in case of exposure towards oil polluted sediments or water during egg incubation.

Keywords: Lumpsucker; early development; crude oil; PAH; histology.

Introduction:

Oil pollution in the Arctic and Norwegian waters

The petroleum activity on the Norwegian continental shelf is already high, with four new oil and gas fields initiating production in 2007 and another three fields expected to start production in 2008. One of these fields is the Snøhvit gas field located near shore at the edge of the Barents Sea. Also the Goliat field in the same area has been discovered to contain large amounts of oil, but it is not clear when and if this oil is going to be exploited. In all a total of 32 search wells was drilled in 2007 and the search for oil will continue in 2008 (Norwegian petroleum directorate http://www.npd.no). Within the sub-Arctic and Arctic region there has been petroleum production for some years and the interest in these areas are believed to increase due to the rising energy demand of the world (Engelhardt 1985, AMAP 2007). The increase in oil exploitation in northern areas intensifies the risk of accidents causing oil spill. The traffic of Russian oil tankers along the Norwegian coast constitutes an additional risk of oil accidents as the shipping is believed to increase in the future (Hjermann et al. 2007).

As petroleum operations intensify the importance of understanding the impact this may have on northern marine ecosystems is apparent. According to the Norwegian petroleum directorate (http://www.npd.no) the pollution from the petroleum activities is mainly reflected by the production, where the risk of pollution is increasing as a result of longer distances to the market and prolonged exploit of the fields. At sea the major environmental threat is considered to be large oil spills (AMAP 2007). Most of the studies carried out on oil spills and oil degradation are from temperate and subtropical environments, whereas the lower temperatures in sea and air and the possible presence of ice in the Arctic makes the fate of an oil spill in the Arctic environment poorly understood (AMAP 2007). The lower temperatures in the north probably make the physical and biological oil degradation slower compared to the rates experienced in temperate regions (Garrett et al. 2003, Hjermann et al. 2007) meaning that hydrocarbons will persist longer and have more time to be taken up by plants and animals in the environment (AMAP 2007). The strong inter annual fluctuation in production means that the pelagic ecosystem may be largely affected in the summer, while winter accidents may cause only smaller impacts. While for the stationary inhabitants such as marine benthic animals that will be present all year round, the Arctic species are found to be more vulnerable

towards oil pollution than similar organisms from temperate regions (Olsen et al. 2007). Studies show that benthic organisms may experience reduced reproduction, abundance and growth (Johnson 1977) as well as increased heart rate (Camus et al. 2002) and respiration rate (Olsen et al. 2007) when exposed to Polycyclic Aromatic Hydrocarbons (PAH).

PAH in the marine environment

Polycyclic Aromatic Hydrocarbons (PAH) are considered to compose the more toxic part of the chemicals found in fossil fuels such as oil, natural gas and coal (Hylland 2006). The two major sources of direct PAH pollution into the marine environment is from the inshore smelter industry and offshore oil and gas production activities (Hylland 2006). But also spills of crude oil may contribute to the total PAH input in marine ecosystems. Crude oil is a mixture of different hydrocarbons with variable structures and properties and the composition changes with oil field and time. PAHs are ranging from two ring naphthalenes to complex ring structures containing up to 10 rings, (Hylland 2006, Hjermann et al. 2007). PAHs have a high affinity for particles and organic material and even the light oil can sink when adsorbed onto sediment particles (Hylland 2006, Hjermann et al. 2007). PAHs are degraded both through chemical and biological processes in the water. The lighter PAHs are more water soluble than the heavier ones and are therefore more bio available (Hylland 2006) while the larger ones are considered the most toxic (Black et al. 1983).

PAHs are taken up by the marine organisms either directly from water or through diet, but unlike most Persistent Organic Pollutants (POPs) such as PCB, DDT etc. the PAHs are not biomagnified through food webs because of the ability of both vertebrates like fish and invertebrates to metabolize PAHs (Hylland 2006). As mentioned, a major oil spill may contribute to the PAH levels in affected areas but the actual effects of a possible oil spill on the environment are dependent on several factors, including; (1) The chemical components and the physical properties of the released oil, (2) quantity of oil and duration of the spill, (3) seasonal oceanographic and meteorological conditions, (4) nature of the exposed biota, (5) habitat type and substrate, (6) geographic location and (7) type of spill cleanup employed (Clark and Finley 1977, Boehm and Page 2007, Hjermann et al. 2007).

Oil and fish

There are a number of studies done on effects of oil contamination on early life stages of various fish species (Lønning 1977, Cameron and Smith 1980, Hawkes and Stehr 1982, Carls et al. 1999, Heintz et al. 2000, Incardona et al. 2004). The studies have involved different oil components and have revealed various effects of oil contamination on the development and survival of fish embryos and larvae. Experimental studies done by Lønning (1977) on the eggs and larvae of cod (*Gadus morhua*), plaice (*Pleuronectes platessa*), and flounder (*Platichthys flexus*), showed that crude oil exposure over short periods led to deformed notochords, abnormalities in head region, and various levels of hatching inhibition and breakdown of yolk in these species. Cameron and Smith (1980) found inter- and intra- cellular spaces in brain and muscle tissues of oil exposed Pacific herring (*Clupea harengus pallasi*) larvae but not in control larvae. While Hawkes and Stehr (1982) found morphological abnormalities in the forebrain and neuronal layers of the retina and also lowered hatching success when exposing Surf smelt (*Hypomesus pretiosus*) embryos to seawater-accommodated fractions of crude oil. A study by Carls et al. (1999) revealed malformations, genetic damage and mortality at larval stages of Pacific herring (*Clupea pallasi*) when exposing embryos to weathered crude oil. Heintz et al. (2000) found that exposure to crude oil during early development of pink salmon (*Oncorhynchus gorbuscha*) can reduce growth and survival rates at early stages as well as lower the fecundity as adults for this species. Whereas Incardona et al. (2004) registered that zebrafish (*Danio rerio*) embryos show various abnormalities like cardiac dysfunction, edema, spinal curvature and reduction in size of some craniofacial structures when exposed to PAHs from petrogenic sources.

Adult and juvenile fishes appear to be able to avoid waters with high hydrocarbon concentrations whereas the eggs (both pelagic and demersal) and larvae that are not able to escape the pollution will be exposed to any toxic compounds the water may contain (Stene and Lønning 1984, Hjermann et al. 2007). Demersal eggs will be most affected when PAH contaminated particles sinks to the bottom sediments where such eggs are attached and developed, and one could expect those eggs to experience the same contamination level as the rest of the benthic community. Brown et al. (1996) and Hose and Brown (1997) found that the PAH levels in mussels could be correlated to genetic damages found in Pacific Herring (*Clupea pallasi*) embryos and larvae hatched at the same site. The eggs and larvae of fish are more vulnerable towards PAH exposure than the adults as they show slow elimination rates of

aromatic hydrocarbons, meaning that accumulated lipophilic contaminants will be stored in their bodies for a long time (Davenport et al. 1979, Solbakken et al. 1984). Although a study done by Solangi and Overstreet (1982) on adult fish has revealed pathological changes in liver, olfactory organ and gills after exposure to different concentrations of whole crude oil and its water-soluble fraction.

*Lumpsucker (*Cyclopterus lumpus *L.)*

The lumpsucker (*Cyclopterus lumpus*, Linnaeus 1758) is a semi-pelagic fish species (Cox and Anderson 1922). Adult females can be up to 63 cm long and weigh 5.5 kg while male lumpsucker can grow to 55 cm length. Colours are variable but males are often red while females are more blue-green (Moen and Svensen 2003). They are distributed along the eastern coast line of the North Atlantic from Portugal to the White Sea (Moen and Svensen 2003) as well as in the Atlantic littoral of North America from New Jersey to Greenland and even extending its range into the Canadian inland sea Hudson Bay (Cox and Anderson 1922). Spawning takes place in January to May in Europe and the female fecundity is up to 200,000 eggs (Sunnanå 2006). Lumpsucker spawn in shallow water masses and lays demersal eggs in large batches containing several thousand eggs, which adhere strongly to each other and to the rocky sea bed substrate (Zhitenev 1970, Davenport 1983, Kjørsvik et al. 1984). According to Zhitenev (1970) one female may lay up to 2-3 egg batches and the eggs in one nest can be either a single batch from one female or consist of several batches of different females. After spawning the female leaves the spawning ground and the eggs are guarded by the male throughout the whole embryo period (Zhitenev 1970, Davenport 1983). The male is protecting the eggs from predators and is also known to ‘fan’ the eggs with his pectoral fins during the developmental period to increase the oxygen uptake within the eggs of the batch (Davenport 1983).

Despite the fact that there are observed funnel- shaped depressions in the egg batches enabling the water to penetrate the deep and middle layers of eggs, large variations in development of the embryo within the batch is observed (Zhitenev 1970). Depending on the size of the batch and tight attachment between the eggs, some delayed development and decomposition of the inner layers of eggs is seen while the outer layer eggs often develop

more rapidly (Zhitenev 1970). The fertilized eggs range between 2.2-2.5 mm in diameter and contain several oil droplets which fuse into one single oil globule during embryo development (Zhitenev 1970). The incubation period can last up to 60 days (Sunnanå 2006). The newly hatched larvae average 5.6 mm SL and 2.4 mg, with the adult complement on 20-21 pectoral fin rays and a functional ventral suction disc according to rearing studies done by Benfrey and Methven (1986).

The lumpsucker is of commercial importance to the northern Norwegian coastal fisheries and the product utilized is the roe. Fishing for lumpsucker has been going on since the 1950s and the season is restricted to the period in the spring when the lumpsucker comes into shore to spawn (Sunnanå and Albert 2004). The fish is captured in very shallow areas (5-40 m depth) and the best fisheries take place on the outermost parts of the coast that is exposed to the open sea (Sunnanå and Albert 2004). The level of catches depends largely on the international market situation and the participating vessels, but the latest calculations indicate that the stock size of lumpsucker is relatively low and some regulations on the fisheries have to be made (Sunnanå and Albert 2004). Since 1996 there have been between 827 and 226 participating vessels in the lumpsucker fisheries, and for the fishermen the first hand value of roe has been between 7.1 and 38 mill. NOK (Sunnanå 2006). This is indicating that for many fishermen the lumpsucker fisheries are an important part of their yearly income. The recruitment of lumpsucker is for the most part unknown. It seems like the population in the later years are lower than earlier and this might be explained by natural variations in recruitment (Sunnanå 2006). The recruitment of lumpsucker and other species which spawns on relatively shallow locations might be lowered as a result of habitat disturbances such as oil pollution.

Project

The current project was carried out with Akvaplan niva AS in Tromsø and was financially supported by NOFO **(**Norwegian Clean Seas Association For Operating Companies). The main objective was to examine possible effects of PAH contamination on the early life history of fish with demersal eggs, such as lumpsucker, herring (*Clupea harengus*) and capelin (*Mallotus villosus*). More particularly, we wanted to study the effects of oil contamination on the embryo development and survival through hatching by exposing lumpsucker eggs to the water soluble fraction (WSF) of crude oil in a flow trough rock column system. Due to the

increased oil and gas activities in the Lofoten-Barents sea area (Hjermann et al. 2007) where lumpsucker are distributed and fished, gaining knowledge on possible harmful effects of oil on their development is important. The rationale for choosing lumpsucker ahead of the other representatives of fish with demersal eggs is the long incubation period (42 days (at 5 °C) for lumpsucker vs. 15 days (at 8 °C) for herring and 21 days (at 7.2 °C) for Capelin (Fridgeirsson 1976, Kjørsvik et al 1984, Stene and Lønning 1984, Hill and Johnston 1997, Sunnanå 2006), making them especially vulnerable to exposure if an oil flake should be drifting ashore during the egg incubation period.

Hypothesis

Our hypothesis is:
H_0: Exposure to crude oil hydrocarbons during the egg incubation period, does not affect lumpsucker (*Cyclopterus lumpus*) embryo development and survival.

H_1: Exposure to crude oil hydrocarbons during the egg incubation period affects lumpsucker (*Cyclopterus lumpus*) embryo development and reduces survival.

Materials and methods:

Experimental design and location

The exposure experiment with lumpsucker eggs was carried out during the spring of 2006, at the seawater laboratory at the Norwegian College of Fishery Science. The start of the experiment was set when the spawning of lumpsucker was on its peak. The experiment was prepared according to the methods developed by Carls et al. (1999) and the oil concentrations used were based on concentrations used by Carls et al. (2005). This set up mimics a natural oil spill by washing the different oil components gradually out of the sediments as the water inlet is placed under the gravel in the experimental containers.

The experiment was carried out in nine 50 l polypropylene containers each containing 35 kg washed 4-8 mm gravel (Figure 1). The control, low dose and high dose oil treatment were made by adding 0, 2 or 4 grams of North Sea crude oil per kg^{-1} gravel in the respective containers (Carls et al. 2005). The oil covered gravel was allowed to dry for 48 hours before starting the water flow. Each treatment was replicated three times. To make sure that the oil in the gravel would be washed out into the water the water intake was placed in the bottom of each container (Figure 1). This water flow was set to 2 l min^{-1} during the experimental run and was started 13 days prior to the start of the experiment to wash out the lighter naphtalenes (Carls et al. 2005).

Figure 1. Experimental design. Photo a, Each experimental unit with lids on marked with treatment code (C= Control, L= low dose, H= High dose oil), main water inlet (Mwi), container water inlet (Wi) and water outlet with three tubes (Wo). Photo b, the experimental units viewed from above: Plankton netting dish (P), Water inlet (Wi), Water outlet (Wo), Eggs (E) on the plankton net, Air supply (A) and Gravel (G). Photo c, Smaller Cups with plankton nets (C) to collect larvae escaping the main container.

Male and female lumpsuckers were collected 22nd May 2006, outside Hekkingen (69º36'N, 17º45'E) in Troms, Northern Norway by the help of a local fisherman using fishnets. Onboard the boat 4 female and 4 male lumpsuckers were stripped, and the eggs and sperm were collected. Both the eggs and sperm from the four individuals of each sex were pooled together. The eggs were fertilized in a polyethylene bag, by adding the sperm and some seawater and mixed carefully. The fertilized eggs were transported back to the laboratory in a styrofoam box with cold seawater to reduce heating during transport. By arrival at the seawater lab the eggs had adhered strongly to each other and had formed one large clump of eggs. In the laboratory the eggs were stored in running seawater in one of the control

containers overnight, before starting the experiment on the next day, the 23rd of May. The fertilization percentage was found to be ~100 % 24 hours after fertilization. In order to distribute the eggs into each experimental container and to increase the water flow to the inner layers of eggs, we broke the egg mass in to smaller egg clumps containing approximately 280 eggs each. In the 9 containers 28-30 randomly selected clumps of eggs were placed onto a plankton net that was lowered onto the gravel in the containers (Figure 1, photo b.) making approximately 8000 eggs in each container. To be able to collect hatched larvae small plastic cups with plankton net outlets was placed under the main water outlet (Figure 1, photo c.).

Experimental procedures

The experiment lasted for 50 days from the eggs were collected on May 22nd until no more larvae hatched by July 10th. The water flow and temperature was measured daily, and the water flow was kept as stable as possible around 2 l min^{-1} in each container. The temperature regime was natural i.e. the same as in the sea outside Tromsø, starting at 6 ºC, and increasing to 7 ºC on day 30 and to 8 ºC on day 41. In order to ensure enough aeration of the eggs in case of accidental problems with the water flow each container was equipped with an air supply (Figure 1, photo b). The air supply and the general conditions of the eggs were controlled daily. Approximately every 6th day during the incubation period one random clump of eggs was collected from each of the buckets. These clumps were then divided into two; one piece was stored on 4% buffered formaldehyde for later analysis while the other was studied under the stereomicroscope to estimate the amount of dead eggs among 25 randomly selected eggs. When the embryos started to hatch on day 38, the larvae were collected every day until no more hatching was observed (Day 49, July 9th). The larvae were collected from the water outlet of the experimental containers into small plastic cups with a plankton net (see Figure 1, photo c) or they were collected along the walls or on the net in the experimental containers by use of pipettes. The hatched larvae were also stored on 4% buffered formaldehyde for later measurements and histological studies.

Water sampling and hydrocarbon analysis

In this study we have analysed the PAH (Polycyclic aromatic hydrocarbons) concentrations of the water as an indicator of oil exposure level. The toxicity of fresh oil is correlated to the PAH content, which is considered to be the primary toxins of oil (Hjermann et al. 2007). Water samples from all the containers were taken four times during the experimental period, at days 5, 19, 33 and 47. The samples were filled in 2.5 l sterilised amber bottles and were frozen until analysis. The water analysis for PAH content over time was done by UniLab AS in Tromsø by Gas Chromatography and Mass Selective Detector (GC-MSD) methods, to analyse for Polycyclic aromatic hydrocarbons (PAH) all of the 16 PAHs found in the Environmental Protection Agency protocol 8310 (16 EPA) and also Naphtalene, Phenanthrene, Dibenziotiophene (NPD) are included in this analysis.

The GC-MSD procedure includes the following steps: One litre of water sample measured in a measuring cylinder was transferred to a separatory funnel, and the pH was measured (of remaining water in the measuring cylinder). First 1 ml of a PAH internal standard was added to the sample and then 60 ml of dichloromethane (DCM) was added. This chemical will extract the PAHs in the water sample. The water sample was shaken and the pressure was released a couple of times before it was placed on a shaking machine for 10 minutes. The volume of the DCM phase containing the PAH was measured in a new measuring cylinder before the DCM-phase was transferred to a 250 ml cylindrical bottle. Then another 60 ml DCM was added to the separatory funnel and the above procedure was carried out three times totally. Finally, 3-4 teaspoons $NaSO_4$ were added to the DCM phase to remove the last remains of water in the sample and the sample was stored in the refrigerator overnight. The next step was to filter the samples through filter paper in a glass funnel, and transfer it to turbovap tubes. The samples were evaporated down to about 1 ml (could take up to one hour). Then the samples were transferred to marked vials with conical bottoms, and evaporated carefully to dryness by nitrogen. To preserve PAH, 100 micro litres of Isooctane was added to the sample and the samples then placed in a freezer, before analyses in the GC/MSD instrument (Hawlett-Packard MS 5971 with Hawlett-Packard 5890 gas chromatograph and Hawlett-Packard G 1034 B software for Chem station).

Egg analysis

For the first two samplings at day 5 and day 11, the egg clumps were collected from each container, and divided in two. One was preserved on formaldehyde (4%) in the refrigerator and one live sample was placed in seawater in a small ziplock bag and kept cool during the transport to the laboratory at Akvaplan niva for further study and photographing. In the laboratory we stage determined the eggs, measured the diameters and took pictures of 25 eggs per container to estimate mortality and possible abnormalities. For the rest of the sampling dates (day 17, 23, 30, 36, 42 and 46) live samples were not studied in the same way as previously because it turned out to be quite time demanding; the only analysis carried out on live eggs was the mortality registrations on 25 selected eggs done in the seawater laboratory at the sampling time. The rest of the egg clump was preserved on formaldehyde for later analysis. From the first two sampling dates (day 5 and 11) only the results from the live sample analysis were used because the preservatives had resulted in collapse of the eggs, making it very hard to stage determine the eggs at this early stage in development. The remaining analyses were done solely on preserved eggs.

During the egg analysis the following egg characteristics were estimated in each sample: stage of development, mortality, unfertilized eggs, eye pigmentation (also used as a parameter in the stage determination), and abnormalities. Because of only few incomplete descriptions of lumpsucker egg development (Ehrenbaum 1904, Zhitenev 1970) the analysis of early developmental stages was based on our own material (from control groups) and developmental studies done on other fish species (Kimmel et al. 1995). The development was divided into 16 different stages from fertilization to hatching and defined by changes in early and late embryo characteristics such as pigmentation of eyes, otolith formation, skin pigmentation, fin development and other characteristic morphological changes observed during the incubation period (see Appendix B).

Larvae analysis

All the hatched larvae fixed on 4% buffered formaldehyde when collected were later studied more closely under the stereomicroscope. Their body length, body height over the anal opening and yolk size were measured and possible deformations were noted.

Histology

Histological studies were carried out to reveal possible differences in internal organ structure and differentiation between embryos and larvae from the control groups and the different oil exposures. Three apparently normal fixed eggs were selected from all the nine experimental units (n= 54) from days 17 and 30. Also 23 fixed larvae (9 from Control, 8 from Low dose and 6 from High dose treatment) from the peak hatching on days 44 and 45 were embedded in paraffin (see methods in Appendix A), sectioned with the rotation microtome (Leica RM 2255) and placed on micro slides and stained with Haematoxylin-Eosin (H&E-stain) (Amin, Mortensen and Poppe 1991) (see Appendix A). Samples were then studied and photographed (Olympus Camedia C-5060 Wide zoom, digital compact camera) in the microscope (Leitz dialux 22EB). The histology of the eyes, liver, intestine, kidneys and yolk were examined more thoroughly.

Statistical analysis

Statistical analyses were performed using Statistica® (Ver. 6.1). Normality and homogeneity of variance inspection revealed no serious deviations from the assumptions for using parametric tests, so parametric tests were used on all data.

For the embryo data a factorial 2-way ANOVA was used to test for differences in mortality among treatments. When a significant treatment effect was found, a post-hoc test (Unequal N) was applied to distinguish differences, with $\alpha = 0.05$. The generalized linear model nested ANOVA (nested in treatment) was used to check for differences in developmental stage at each sampling date and to check for container effects ($\alpha = 0.05$).

For the larvae data the generalized linear model nested MANOVA (nested in treatment) was used to check for differences in developmental stage and to check for container effects ($\alpha = 0.05$).

Figures and tables are made using Sigma plot, version 10.0, Systat software,inc. and Microsoft office Excel 2007.

Results:

Hydrocarbon analysis

PAH concentration of the seawater in the experimental units varied, both between the different containers which were added the same concentration of crude oil and also, as expected, PAH levels changed during the time of the incubation.

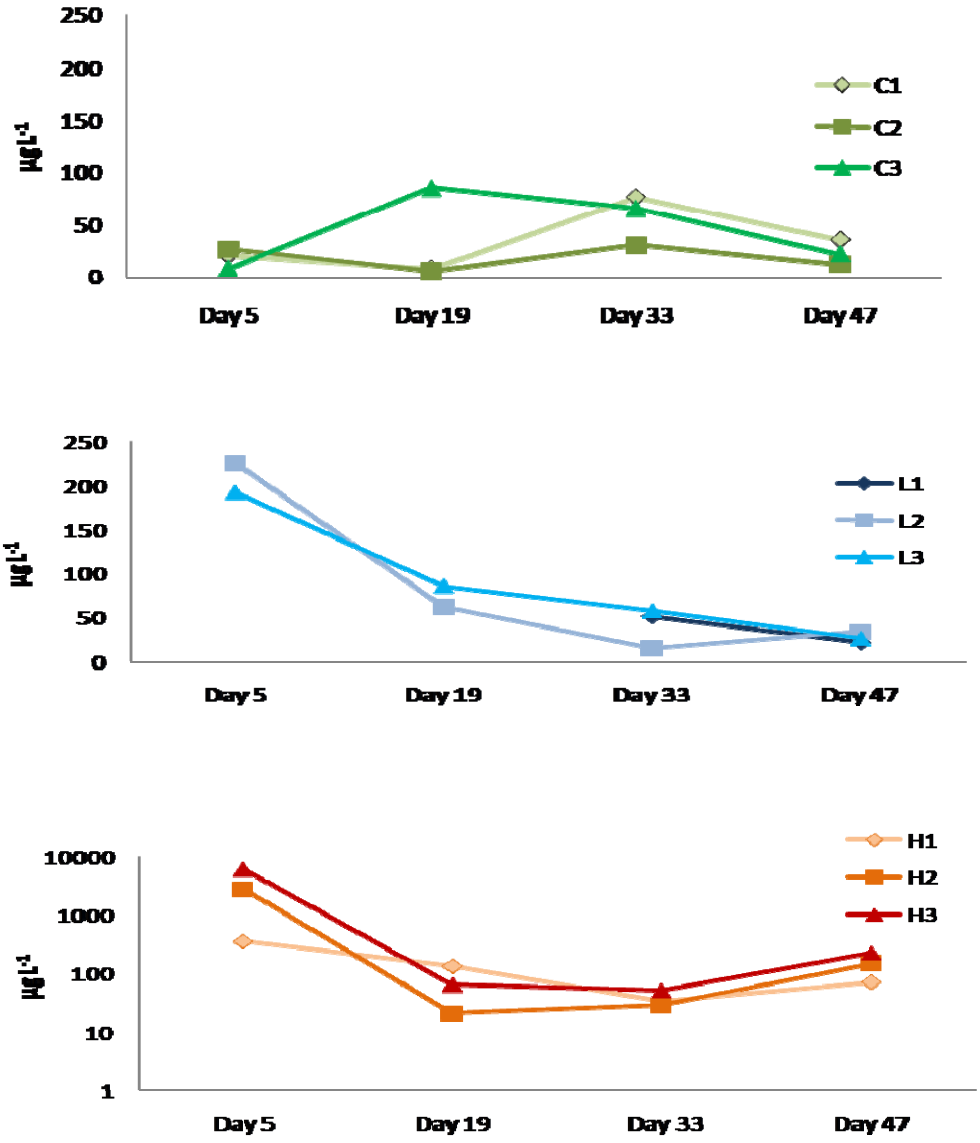

Figure 2. Total PAH concentrations (µg L^{-1}) in water samples taken from the water outlet of each container during the incubation period. Notice the logaritmic scale on the high concentration. The reason for the incomplete result from L1 is accidentally breaking of the sampling bottle.

Total PAH levels in the experimental units showed an average of 18 µg L^{-1} in the control units, 210 µg L^{-1} in the low concentration units and 3110 µg L^{-1} in the high concentration units at the first week (day 5) of the experiment (Figure 2).

The different components of the oil was distributed as shown in Figures 3,4,5 and 6, with the oil components having an increasing weight from the lighter naphthalene to the heavier Dibenzo[a,h]anthracene along the x-axis. The components followed the expected trend with the lighter naphtalenes present at the beginning of experiment (Figure 3), and the heavier ones more prominent later in the experimental run (Figure 4 and 5). The lighter PAHs had another peak towards the very end (Figure 6).

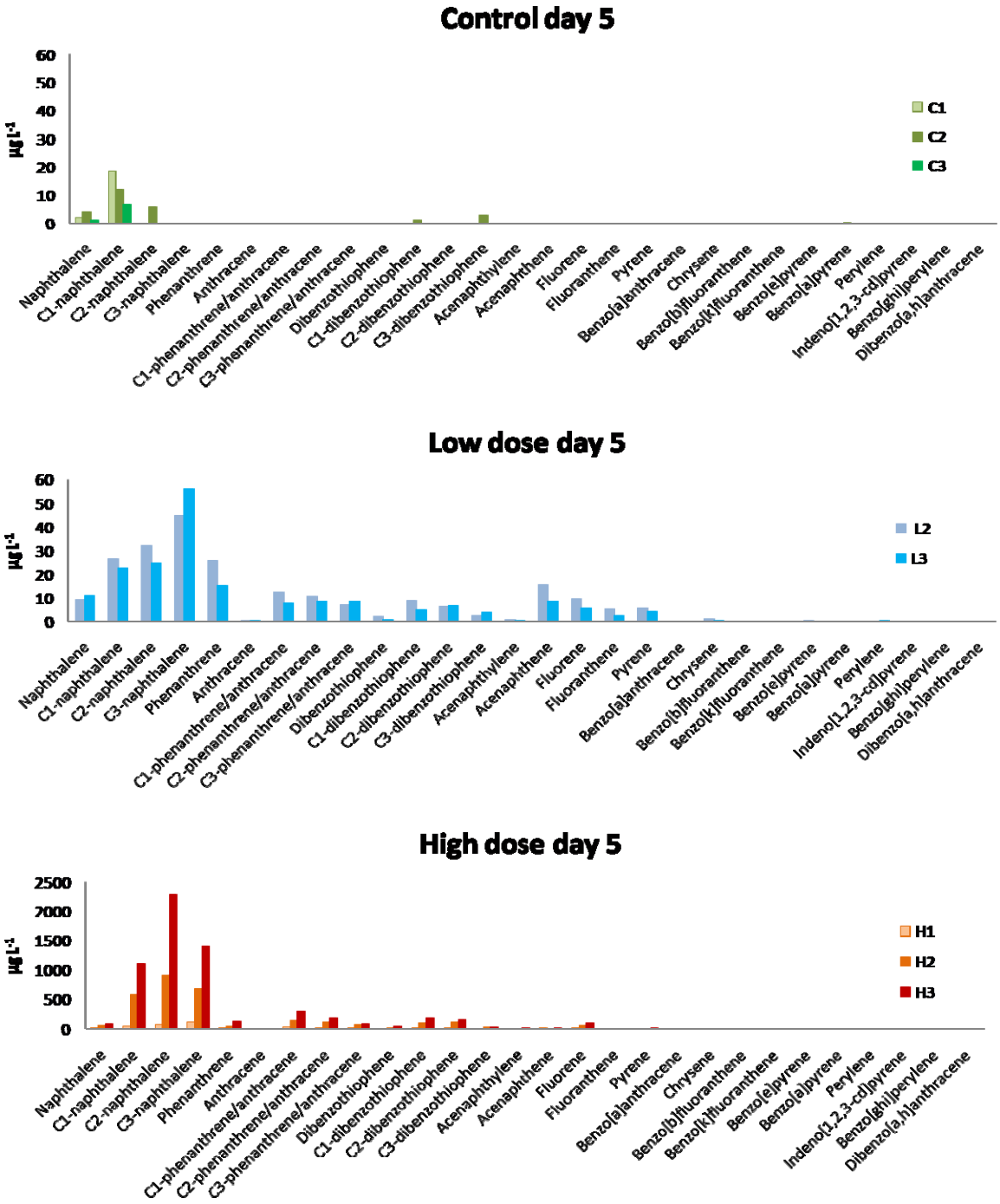

Figure 3. The composition of PAH in the the different containers in first week of the experiment (day 5). Notice the different scale on high dose panel. One sample (L1) was accidently broken and could not be analysed.

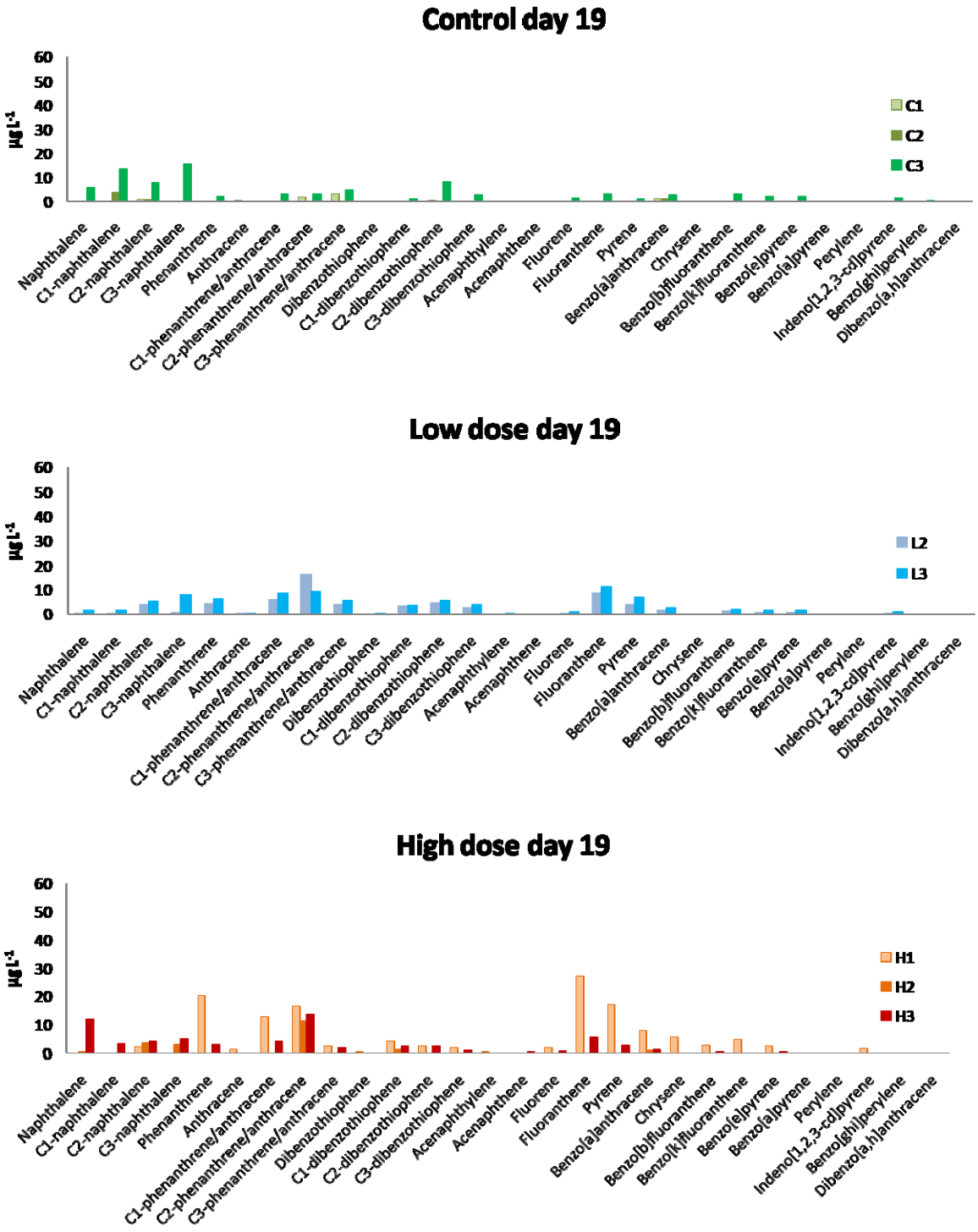

Figure 4. The composition of PAHs in the the different containers on day 19. One sample (L1) was accidently broken and could not be analysed.

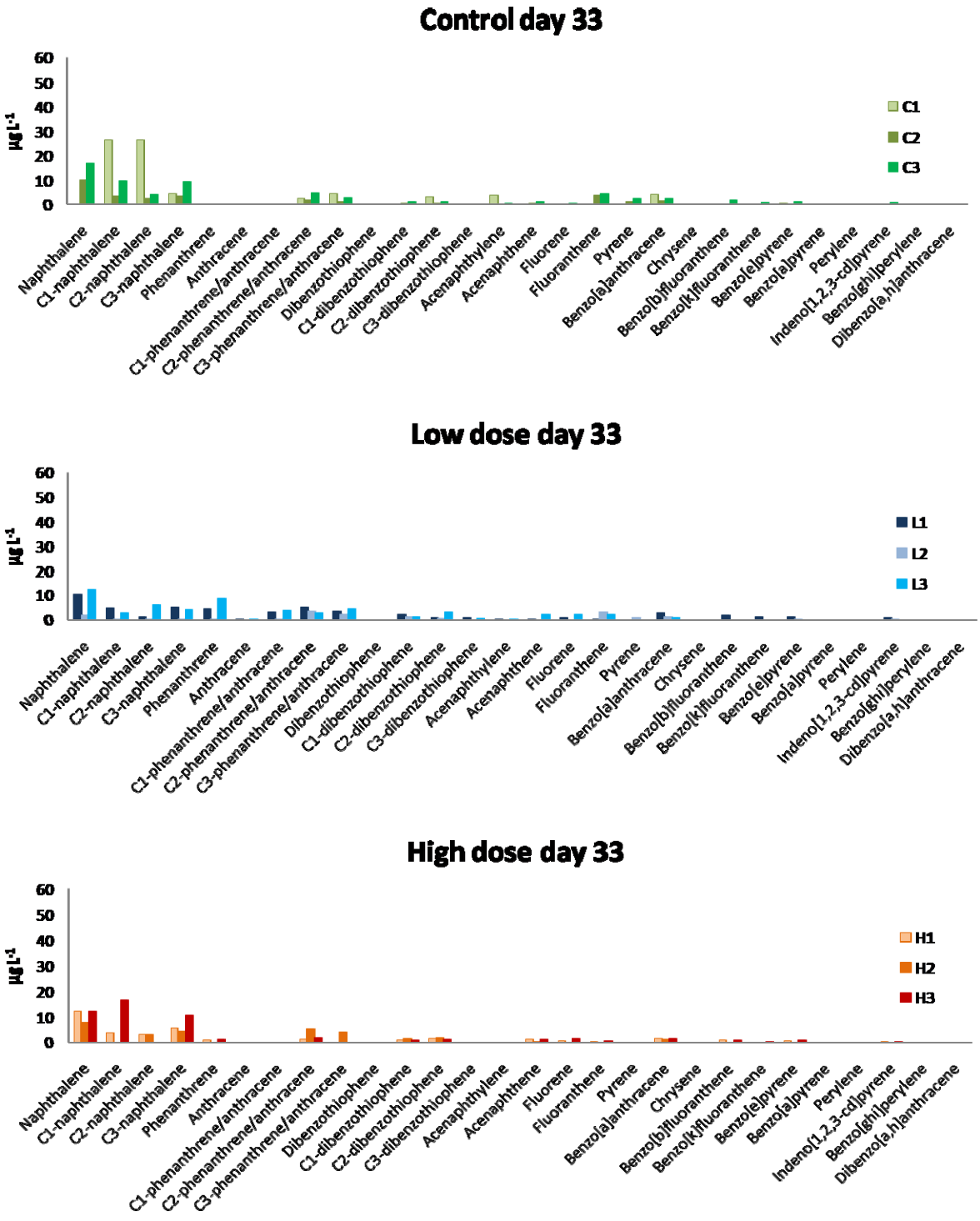

Figure 5. The composition of PAHs in the the different containers on day 33.

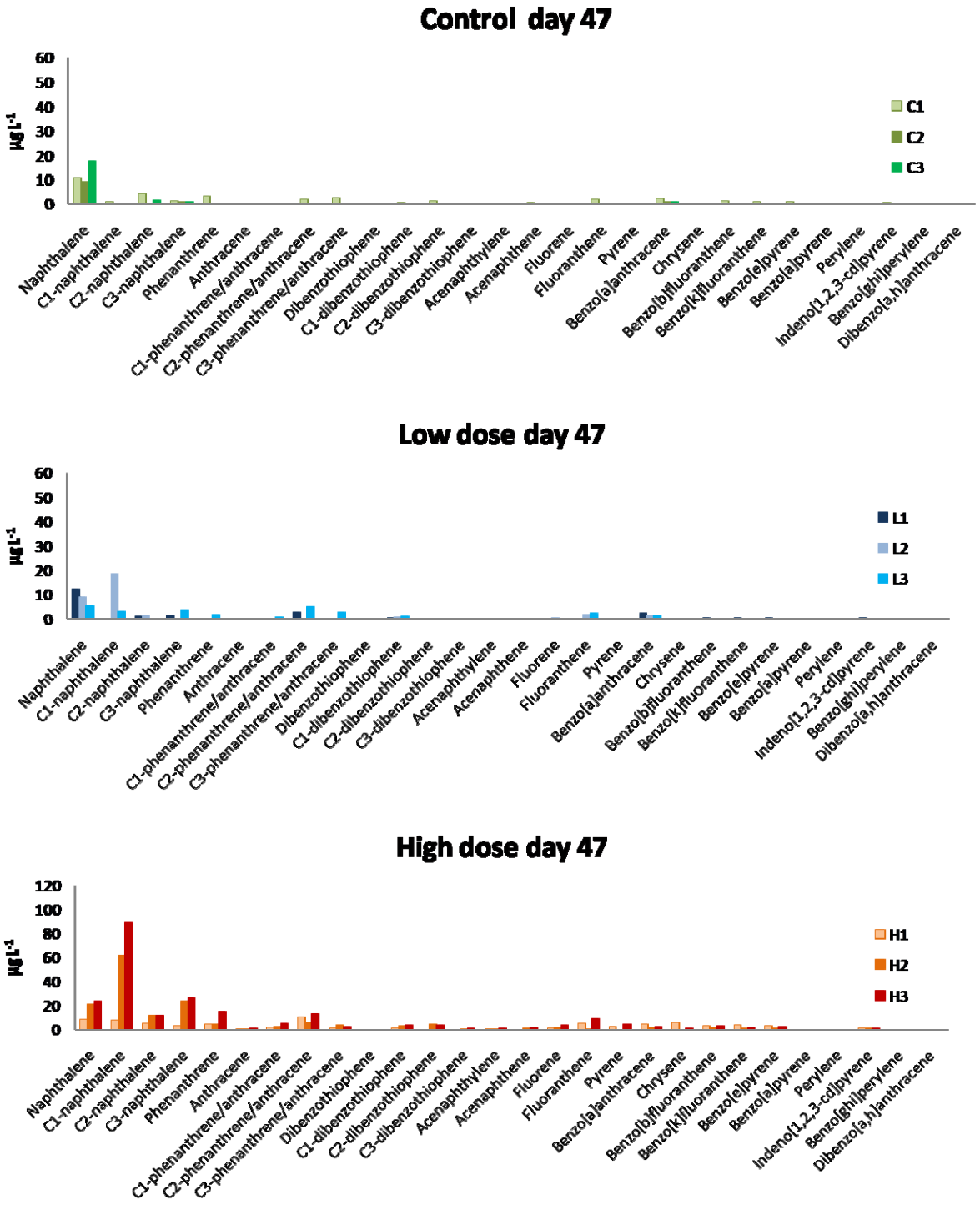

Figure 6. The composition of PAHs in the the different containers day 47, 3 days before the experiment was ended. Notice the different scale on high dose panel.

Differences between the treatment replicates were found on some of the dates and for some of the PAHs. At the start of the experiment (Figure 3) the replicates showed a similar level of PAHs within the same treatment in control and low dose but for the high dose treatment the

variance between the three replicates was fairly large. Still the lowest concentrations found in high dose treatment were higher than concentrations in low dose and control group.
For the control groups on day 19, a higher level of especially naphtalenes in one of the containers (Control 3) is observed, while the low dose and high dose replicates are showing small peaks of the C1-C3 phenanthrene/anthracene and the heavier fluoranthene and pyrene. On day 33 both low dose and high dose treatment show low values for almost all of the PAHs with only a slighly higher level of the lighter PAH compounds, while in the control group at that date we observed two rather high peaks of C1 and C2- naphtalenes. When looking at the last date (day 47) that was sampled for PAHs, the lighter naphtalenes once again showed a small peak in all of the treatment groups whereas for the high dose we also find some heavier PAHs.

Egg development

Developmental stages of lumpsucker eggs, embryos and newly hatched larvae are illustrated in Appendix B and the photos in Figure 8. Approximately every 7th day throughout the incubation period developmental stages of living eggs/embryos were determined in each experimental replicate. Variations vere noted, but for the embryos we found no significant differences in development stage among treatments and dates (GLM Nested ANOVA $p \geq 0.25$, $F = 5,14$). Not surprisingly there were significant differences in stage between all dates, with higher stage for each date (unequal N post-hoc test $p \leq 0.00026$) as the embryos are developing over time (Figure 7).

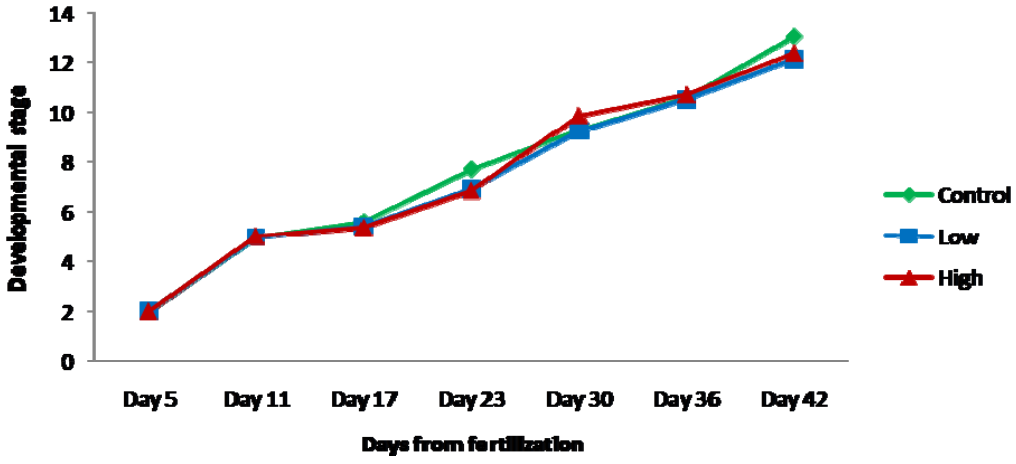

Figure 7. Mean development stage reached throughout incubation time for each of the three treatments. For explanation of the developmental stage numbers please see Appendix B.

As we expected the development is progressing with time. But there is no evidence that oil exposure slows down the development process as there were no differences in development between the treatments.

The photos of eggs/embryos presented in Figure 8 are from live samples of unexposed embryos while the hatched control larvae have been fixed before photo was taken. These photos are representing some of the stages described in Appendix B and show some of the features used for stage determining of the embryos, such as eye pigmentation, otocysts, body pigmentation as well as the fusing of the yolk oil globules.

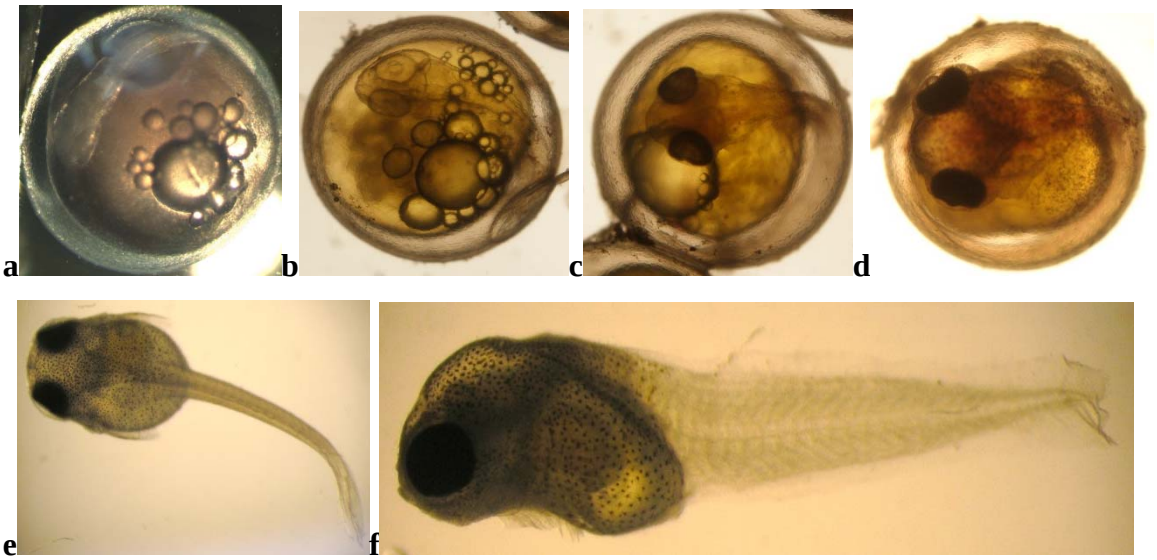

Figure 8. Photos of selected stages of lumpsucker embryo development, a) stage 4, b) stage 5, c) stage 7, d) stage 9 and e) newly hatched lumpsucker larvae, viewed from above and f) from the side. For more extensive information see Appendix B and text below.

Stage 4 (Figure 8 a) is reached approximately 7-8 days after fertilization and the embryo is starting to take shape, there is still no eyes present but the optic primordia is formed. At this stage as well as at stage 5 (Figure 8 b) there are still many small oil droplets in the yolk. Approximately 12 days after fertilization the embryo has reached stage 5, heartbeats are observed and the eyes are visible but lack pigmentation. Stage 7 is reached approximately 18 days after fertilization. The eyes are pigmented, olfactory pits and paired pectorals are developed. Blood circulation over the yolk can be observed with a pale pink color. The most obvious feature at this stage is the relatively large otocyts with particularly visible otoliths inside. Stage (9) is reached approximately 28 days after fertilization (Figure 8 d) and the embryo is now more heavily pigmented particularly on the head and dorsal part. The small oil droplets have fused into one larger globule, and the blood vessels are more evident in the embryos and surrounding the yolk. The last two photos (Figure 8 e and f) are of the newly

hatched larvae. Lumpsucker larvae are well developed at hatch, with large eyes, round body and long slender tail they resemble a tadpole. The ventral sucker is developed, and is fully functional at this point (Cox and Anderson 1922).

Mortality

During incubation period there was registered differences in embryo mortality with time and treatment (2 way ANOVA, $p \leq 0.026$), with a significantly higher mortality in High dose ($p \leq 0.019$) compared to low dose, but no other significant differences in mortality between the treatments ($p \geq 0.2$). Interaction term not significant ($p=0.757$).

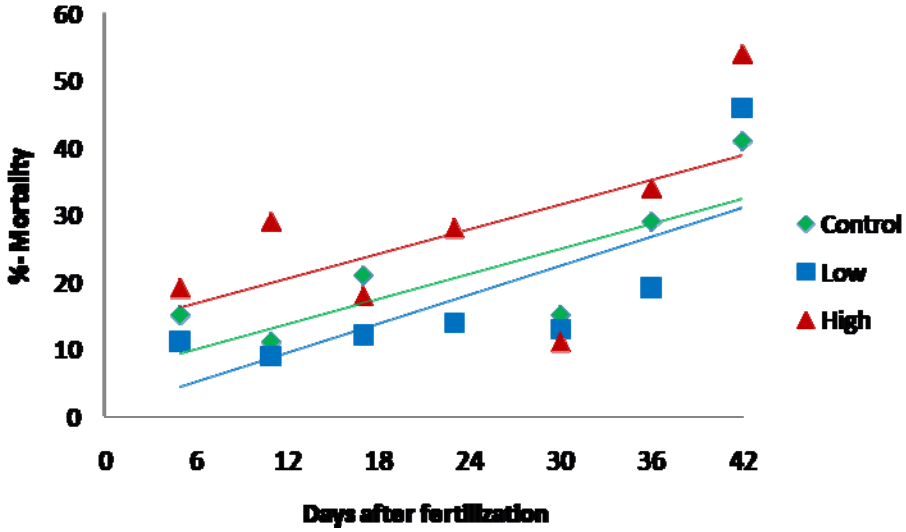

Figure 9. Embryo mortality of control and exposed groups at different days during the incubation period, shown as means of triplicate.

Microorganism growth was found in the containers during incubation period; it first started to appear on day 36 in a few of the containers, but soon spread to all of the containers. For all treatments the mortality at day 42 was significantly higher ($p \leq 0.019$) compared to all previous sampling days.

Hatching

Hatching started on day 38 in the control and high dose oil exposure containers and continued in all containers until day 49. In total 135 larvae hatched in all the experimental containers; 99 hatched in the control units, 13 in the low concentration units and 23 in the

high concentration units. Despite the large differences in hatching success the hatching trend was the same in all treatments with a maximum hatching on days 44 and 45 (Figure 10).

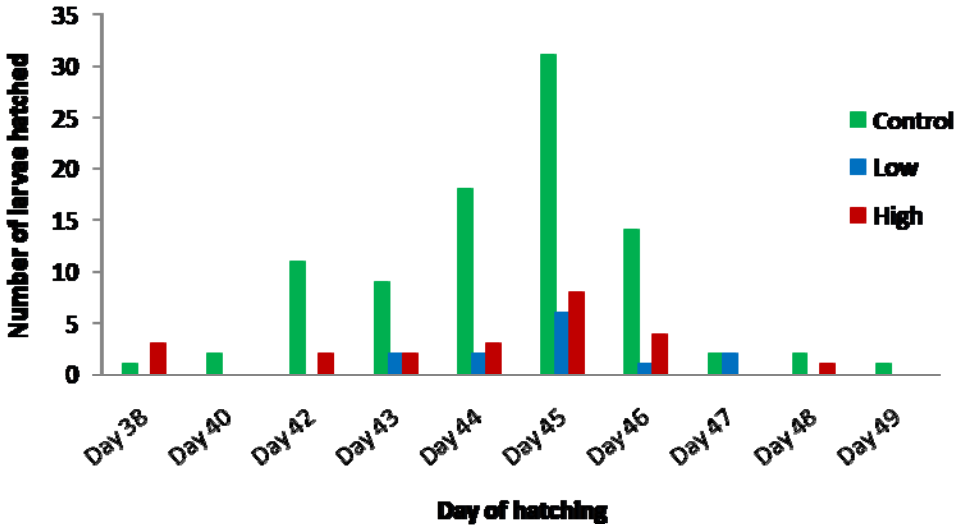

Figure 10. Number of larvae hatched in control and exposed groups during the hatching period from day 38 to 49.

The length of hatched larvae were measured together with the yolk height and body height over the anal opening. Observations on possible deformations were also made. We found no significant differences in larvae length, yolk height and height over anal opening between the treatments (GLM nested MANOVA p= 0.380, F= 1.071) (Figures 11, 12 and 13). No container effects were found (p= 0.180, F= 1.307).

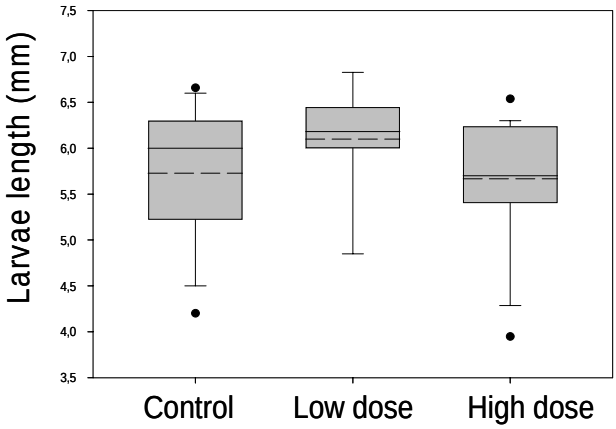

Figure 11. Box plot showing larvae length (mm) in control, low dose and high dose for all larvae hatched. Median (-), Mean (---), outliers (●), 5^{th}/95^{th} percentiles are shown.

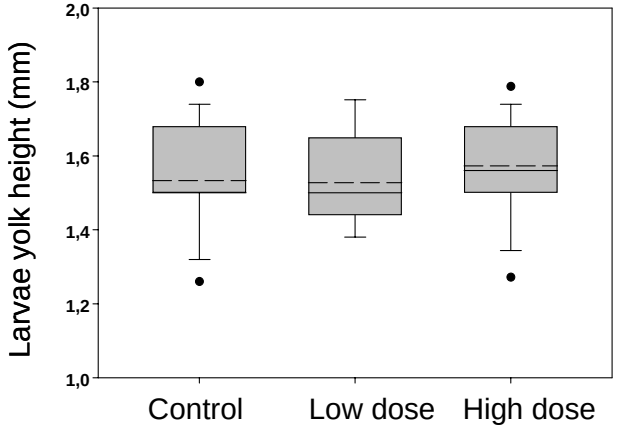

Figure 12. Box plot showing larvae yolk height (mm) in control, low dose and high dose for all larvae hatched. Median (-), Mean (---), outliers (●), 5th/95th percentiles are shown.

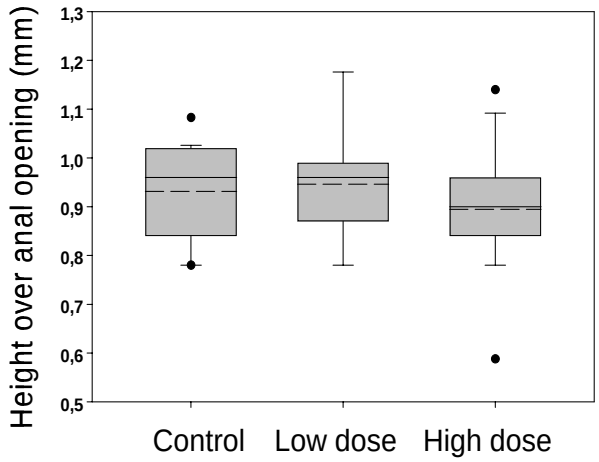

Figure 13. Box plot showing body height over anal opening (mm) in control, low dose and high dose for all larvae hatched. Median (-), Mean (---), outliers (●), 5th/95th percentiles are shown.

The larvae hatching at the peak did not differ from the larvae found before or after as no significant differences in larvae length, yolk height and body height over anal opening was found (GLM nested MANOVA, p=0.343, F=1.103) suggesting similar length, yolk and anal opening body height independent of hatching time. There were no container effects either (p= 0.243, F=1.194).

Abnormalities

The abnormal embryos observed during the incubation period (50 of 1575 eggs examined) were in most cases at a less advanced developmental stage or were in the same stage in development as the others on that date but clearly smaller in size. Only 17 of the 50 deformed embryos were alive when registered (Table 1). It was generally very difficult to observe minor

deformations during the incubation period on the fixed eggs but some were clearly abnormal and have been registered.

Table 1: Observations on mortality and deformations of eggs/embryos during the incubation period.

	Normal (1525 eggs)		Abnormal (50 eggs)	
	Alive	Dead	Alive	Dead
Control treatment	374	134	5	12
Low dose treatment	392	113	9	11
High dose treatment	329	183	3	10
Total	*1095*	*430*	*17*	*33*

Of the embryos surviving through incubation 135 larvae hatched and 40 of these showed some kind of deformations. How these larvae were distributed between treatments and average measurements on length and yolk is presented in table 2.

Table 2: Length, yolk height and number of deformed larvae among the hatched larvae in control, low dose and high dose treatments.

	Number of larvae	Deformed larvae	Average length (mm)	Average yolk height (mm)
Control treatment	99	32	5,73	1,53
Low dose treatment	13	4	6,1	1,52
High dose treatment	23	4	5,66	1,57

In most cases deformations of the larvae were noted in the tail region in the shape of a short malformed tail (in total 25 larvae, 22 from control, 2 from low dose and 1 from high dose treatment). More rarely a deformation of the yolk sack (in total 9 larvae, 5 from control, 1 from low dose and 3 from high dose) was found and in some cases also operculum deformations, small size, small head, deformed snout, crocked body or combinations of these malformations were observed (Figure 14).

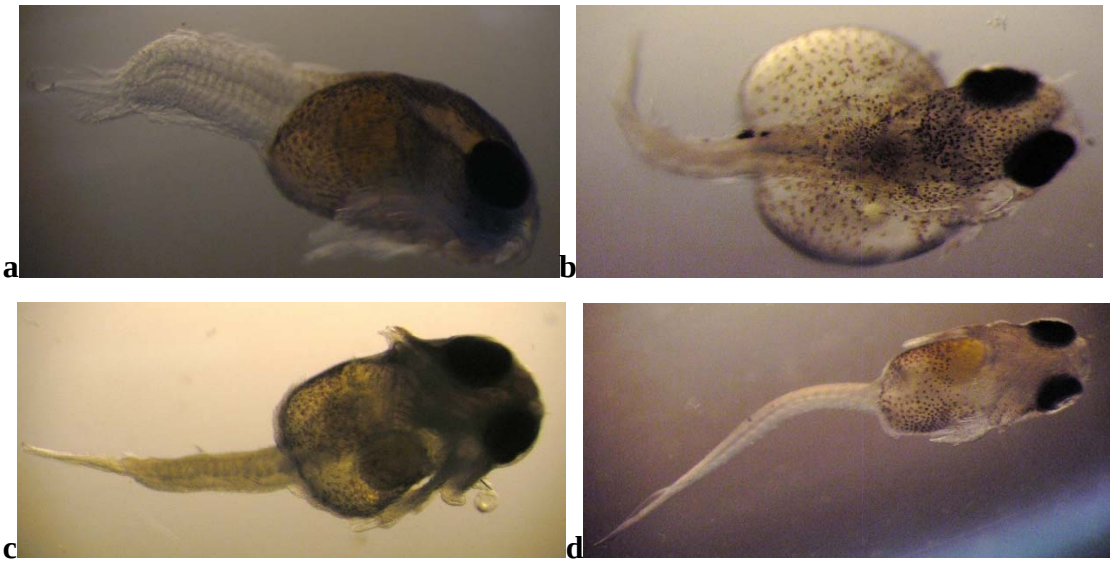

Figure 14: Deformed larvae. a) shows a rather short and deformed tail viewed from the side, b) deformation of the yolk viewed from above.c) deformed operculum viewed from below, d) crocked bodyshape viewed from below. These larvae are all from control group (the first larvae (a) hatched on day 43, and the other three hatched on day 45).

We were unable to carry out statistical analyses on the data of abnormal larvae because of the low number of hatched larvae in the oil exposed groups. Due to the fact that approximately one third of the hatched larvae from the control group were abnormal or deformed, we really can not conclude that such abnormalities were an effect of oil exposure.

Histology

Intentionally we wanted to study histology of embryos from both day 17 and 30. Two eggs were embedded and sectioned from each container on both dates, but very few sections turned out to be good enough for comparison between treatments. For day 17 we did not obtain enough material in control group to be able to show a normal histology, whereas from day 30 we got a fairly good normal histology from control group but unfortunately not any good sections from oil exposed embryos to be able to compare control vs. oil exposed. The embryos at these stages are very fragile and almost impossible to separate from the chorion without the embryo breaking or dissolving. So the sectioning was made with the embryo still within the egg shell. Because of the large yolk reserves of lumpsucker embryos there were some additional difficulties sectioning the eggs.

The most successful histological sections of lumpsucker embryos from day 30 (8 days before first hatching was observed) are shown in Figure 15. All the photographed embryos are from

the control group at this date and the embryo was in stage ~9 (heavily pigmented eyes, head and back as well as blood).

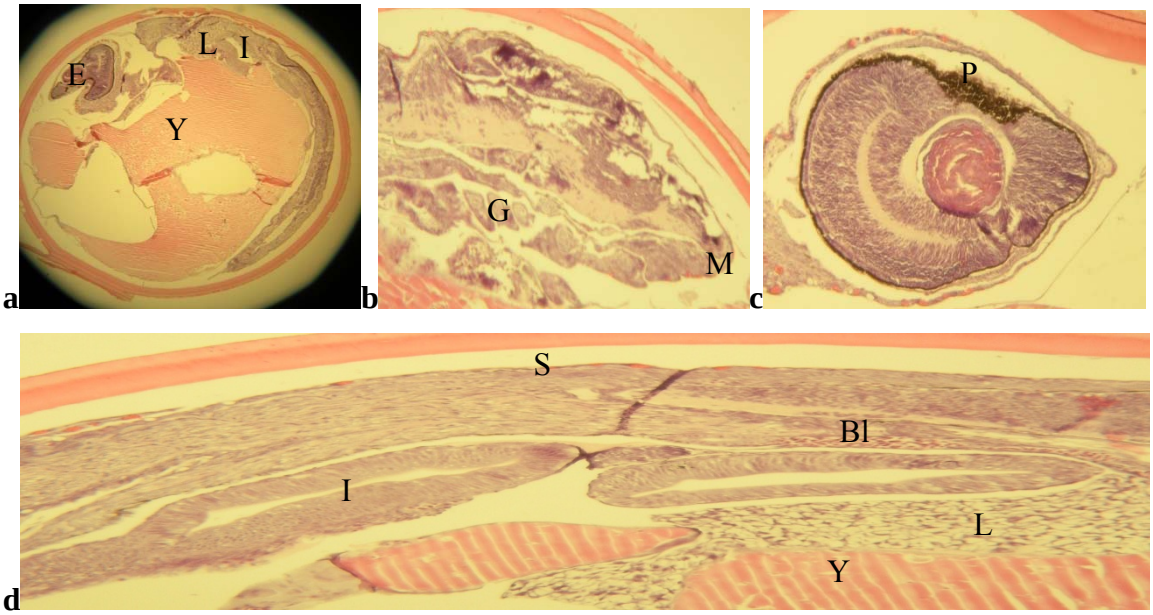

Figure 15. Longitudinal section of lumpsucker embryo inside the egg, 30 days after fertilization when the embryo has reached stage 9. a) Overview of Eyes (E), intestine (I), Liver (L) and Yolk (Y). b) Head with gill arches (G) and mouth (M). c) Eye with pigmented cells (P). d) Mid-body with Blood cells (Bl), Intestine (I), Liver (L), Skin cells (S) and Yolk (Y).

As shown in figure 15a a large yolk reserve dominates the volume of the lumpsucker egg The 30 day old embryo is well developed with liver (L), intestine (I) and eyes (E) at this stage. The close up photo of the head region of the embryo (Figure 15 b), shows the gill arches (G), and the mouth (M), while photo c, shows the eye of the embryo at this point in development with pigmentation degree 4. Internal organ developed at this stage is illustrated closer in picture d) showing the intestine (I) with a smooth surface, liver (L) is well developed, blood cells (Bl) are present, and the skin (epidermis) (S) with mucous cells are noted at this stage. The few sections studied of embryos from exposed groups did not reveal any abnormalities in those organs that were observed, but as mentioned before, there was not sufficient data to compare and to draw conclusions from this material.

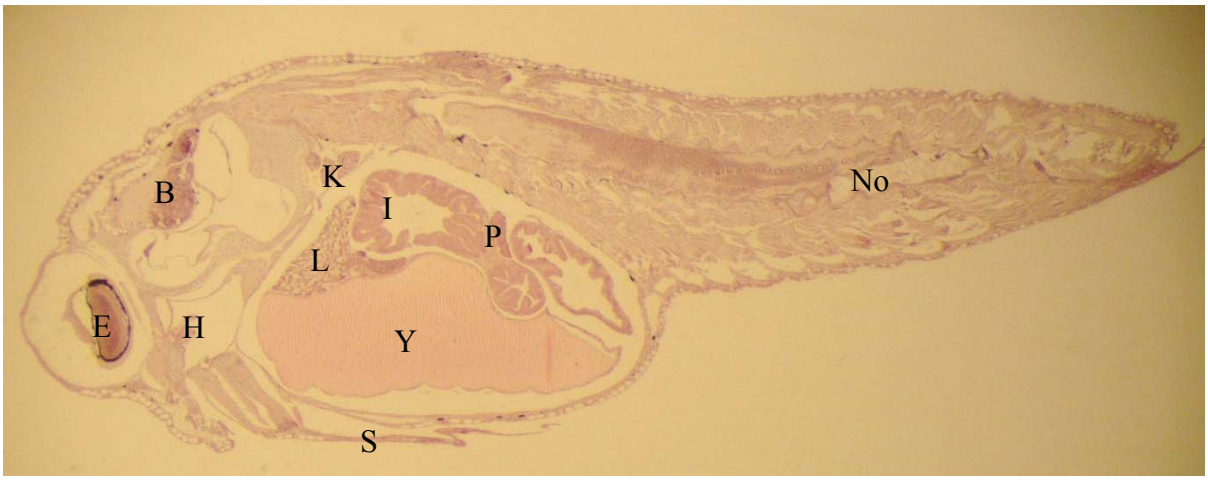

Figure 16. Longitudinal section of a newly hatched lumpsucker larva. Brain (B), eye (E), heart (H), intestine (I), kidney (K), liver (L), notochord (No), pancreas (P), ventral sucker (S) and yolk sac (Y).

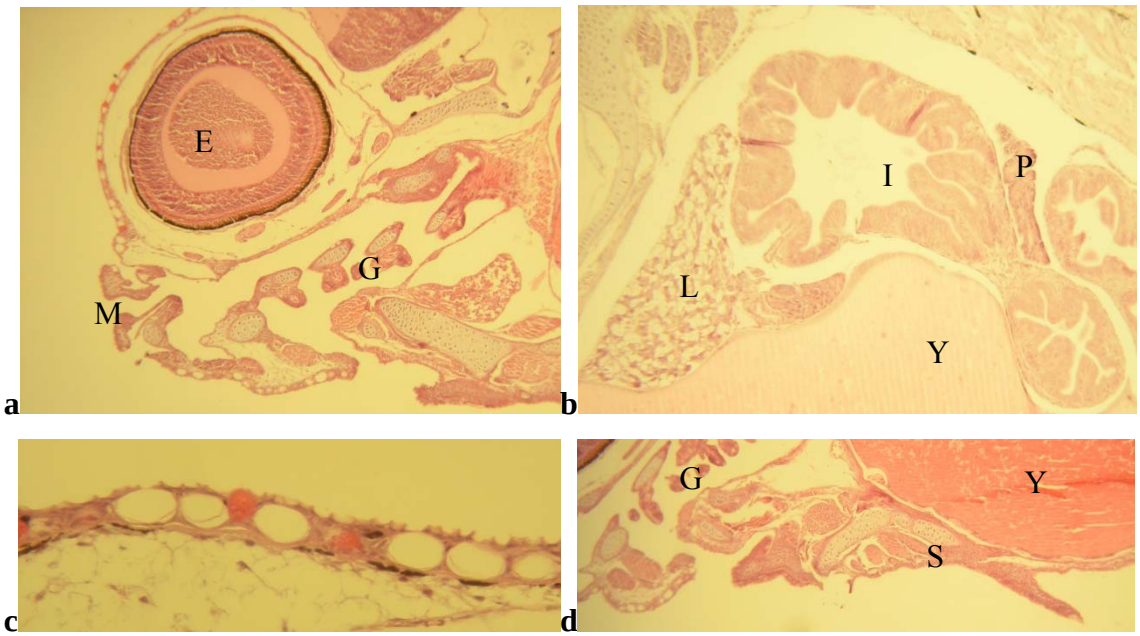

Figure 17. Enlarged sections of control group lumpsucker larva at hatching. a) Eye (E), 4 gill arches (G) and mouth (M). b) intestine (I), liver (L), and pancreas (P). c) Three different skin cells on the back of lumpsucker larvae. d) gill arches (G), ventral sucker (S), and yolk (Y).

Histological sections of hatched lumpsucker larvae showed that this species is well developed at the time of hatching.They still have a fair amount of yolk reserves (figure 16). They have a well developed intestine, with folded and differentiated mucosa, the liver is relatively large and has vacuolated and hepatic cells, several kidney tubules are observed and also pancreatic tissue is prominent close to the intestine. Sensory organs appear well developed. The eyes have differentiated retina with a pronounced pigment cell layer, and there are two olfactory pits between the eyes. The epidermal skin of the lumpsucker larvae consisted of several cells

of different size, including numerous mucous cells, and also pigment cells (see Figure 17 c). The sucker disk (Figure 17 d), which represents modified pelvic fins, is very characteristic of this species (Cox and Anderson 1922). The main phases of sucker development takes place around the last week before hatching in the stages named 13, 14 and 15 (se Appendix B). At these later stages of embryo development the pelvic fins are closing up and create the round sucker disk recognized at hatching.

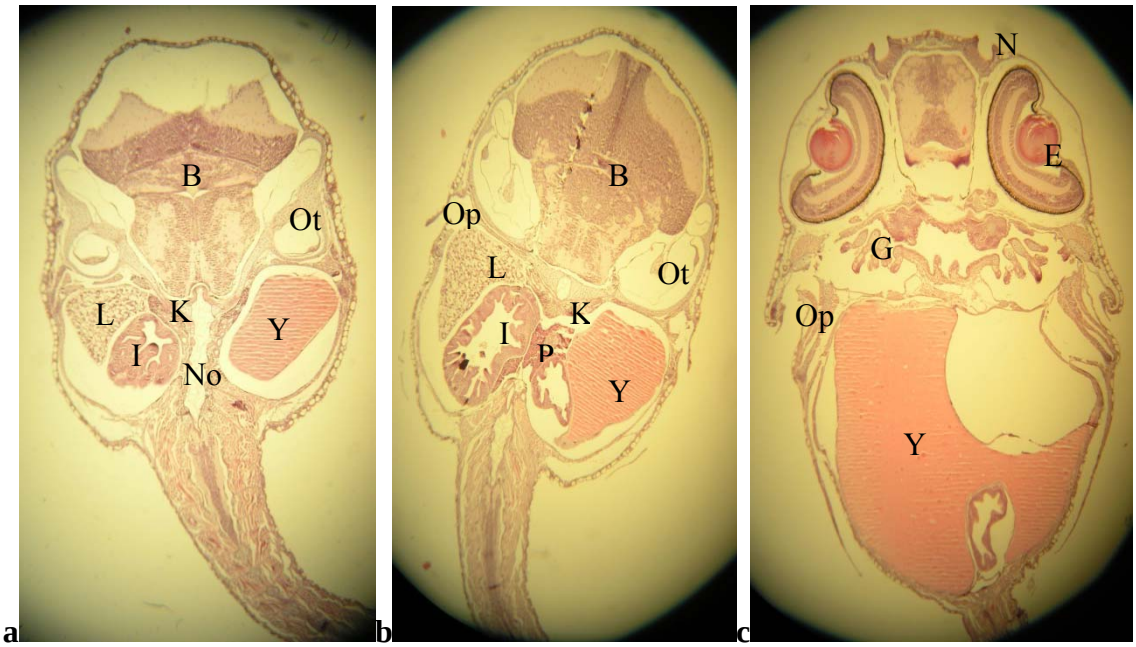

Figure 18. Photo a,b and c) Dorsoventral sections from lumpsucker larva at hatching. Brain (B), eyes (E), gill arches (G), intestine (I), kidney (K), liver (L), olfactory pits/nose (N), notochord (No), operculum (Op), otocysts (Ot), pancreas (P), and yolk (Y).

The sections of the larvae shown in Figure 18 are from the Low dose treatment group. We can see a clear left/right division in abdomen, with liver (L) and intestine (I) on left side and yolk reserves (Y) on the right side. Notochord (No) and kidney tubules (K) are also very distinct along the middle of the larva. The brain (B) is fairly dominant in the first sections taken (Figure 18, a and b), and the large otocysts (Ot) can be seen on each side of the brain. The deeper section (b) shows the pancreas tissue (P) as well and the operculum (Op). Photo c) shows an even deeper section of the lumpsucker larvae, with very noticeable olfactory pits (N), large eyes (E), four gill arches (G) and the rather large yolk (Y) reserves that are present at hatching.

When comparing the sections from the different treatments, we especially checked for histological differences in the eye, skin, liver, kidney, intestine and yolk.

Sections were made from nine control larvae six high dose and eight low dose oil exposed larvae in total. The sections from exposed larvae did not show any obvious differences from the control larvae (Figure 17 for control and Figure 19 for oil exposed larvae). Figure 19 shows sections from eye, liver, skin and intestines from the high exposure group larvae.

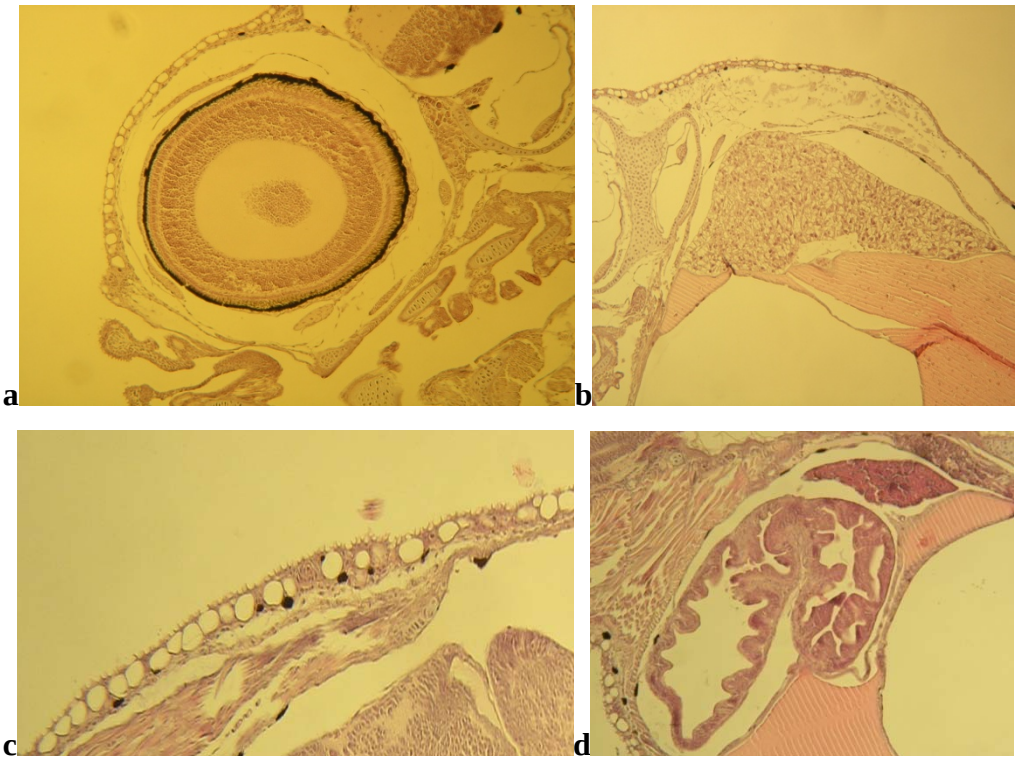

Figure 19. Longitudinal sections of a) eye, b) liver, c) skin and d) intestine from high exposure group larvae. No abnormalities compared to sections of larvae from the control group were noted.

Discussion:

Hydrocarbon analyses

Total PAH levels in the experimental units showed an average concentration of 18 µg L^{-1} in the control units, 210 µg L^{-1} in low concentration unit, and 3110 µg L^{-1}in the high concentration units at the beginning of the experiment. As expected, these levels gradually decreased towards the end of the experiment, this was especially observed in the low dose treatment groups. In high dose groups we saw a slight increase in total PAH levels on days 33 and 47 (Figure 2). This may be explained by the co-occurring temperature increase after day 26 as higher temperature increases the solubility of PAHs (Viamajala et al. 2007). The reason for the elevated PAH concentration in one control replicate on day 19 (Control 3), and on day 33 (Control 1) (Figure 2) is not clear, but for the latter date the elevated PAH might be explained by water sample analyses. The water sample from control 1 container had to be reanalysed as a result of extremly diverging results within control group replicates when looking at the heavier PAHs. The peak of tPAH on day 19 might also be explained by water sample analyses as control 3 replicate showed much higher values than replicate 1 and 2. For the other sampling dates the replicates was following the same tPAH level within the treatments. Due to the water system available in the laboratory and the fact that we did not choose to distribute the water by controlled pumping, the water flow in the containers varied between days. Although the flow was roughly 2 litres pr minute in each container, varying flow regimes may alter the outwash of PAHs and thus concentrations found in our samples. Another possible explanation is that there could have been sudden wash of the gravel in a few containers at some point during the experiment, or the waterflow is shifting within the rock column and this could have lead to higher levels of some components on some of the days.

In all of the water samples from control group replicates we found a relatively low level of PAHs throughout the incubation period, which probably represents contamination either from water or sediments. We used lids on the containers so the contamination is most likely not from air. Presumably the observed contamination is from the gravel as the pattern in PAH composition follows similar trends found in Carls et al. (2005) with smaller naphtalenes most abundant at the beginning and more substituted PAHs detected towards the end of the experiment. Carls et al. (1999) found a PAH start concentration of 0.04-0.05 ppb, which indicates that our experiment probably has been contaminated with PAHs from some

unidentified sources. This background level of PAHs do not change the baseline conditions of the experiment as we still observe the expected differences in PAH levels between treatments. In order to reveal possible sources of contamination, the water samples should probably have been taken in triplicates, at least to eliminate errors in the PAH analysis, but this was not economically feasible with our project budget.

The PAH levels of the water had an obvious difference in start concentrations as we wanted to achieve by using different oil doses. There were some small variations between the replicates of each treatment, but over all the total PAH within replicates followed the same trend (see Figure 2). Because the results from our PAH analyses showed some confusing variations and unwanted contaminations in control units, the results from the PAH analysis should really be considered rough guidelines of what is present in the oil that was added to the gravel when preparing the experiment.

Development of lumpsucker eggs and larvae

By making a developmental series based on the development in the control we felt confident to identify possible differences between control and oil exposed eggs and larvae. The observations on developmental stages were done by the same person making the same judgment and analyzing the samples in a randomly manner. The egg development between the groups followed the same curve (Figure 7) which indicated that the embryos were developing normally even in contaminated sea water. This was also supported by the fact that we found no significant differences in development between the embryos within the different containers. According to Stene and Lønning (1984) the lumpsucker egg has a particularly thick and complex chorion making the eggs less permeable towards 2-metylnaphtalene. Our results indicated that a thick chorion may also exclude other PAHs, and that oil exposure had limited effects on the development of lumpsucker embryo. According to recent studies done by Carls et al. 2008 on the toxicity of oil and the effects on developing zebrafish embryos, the toxicity of oil is related to the PAH dissolved in the water and not the PAH attached to particles. The chorion and its pore size is the only protection the eggs have against pollution in the water. So a study of the chorion structure of lumpsucker eggs, and measurements of potential levels of PAHs inside the egg and/or in the perivitelline fluid might reveal if PAHs are entering the eggs and at which amount.

Mortality

Significant differences in mortality between the different exposure regimes were observed with the mortality in high dose treatment being significantly higher than low dose. Also there were significant differences in mortality over time as we observed higher mortality on the final sampling date compared to the earlier sampling dates. The latter of these observations can be explained by the microorganism growth that occurred during the experiment and became extensive at the end of the incubation period. There were no difference between the replicates within each exposure group with regard to mortality, development, or larvae size which indicates that the larvae within the same exposure have been experiencing the same environment and thus are true replicates of the treatment.
Early life stages are found to be more sensitive to hydrocarbon pollution than adult fish because they are less mobile, do not have chemoreceptors to detect pollution, have a larger surface to volume ratio and easily accumulate toxins in the yolk (Glamuzina et al. 1990, Stephens et al. 1997). According to Stene and Lønning (1984) it seems like eggs are more resistant towards hydrocarbon pollution than larvae, this is also supported by other studies indicating that yolk sac larvae and larvae represent the most sensitive early life stage to hydrocarbon exposure and the WSF's of crude oil (Carls and Rice 1988, Stephens et al. 1997).

One common risk we face when having live biological material in the laboratory is that large amounts of decaying material facilitate microorganism growth. Unfortunately microorganism growth started to appear in our experiment around day 28. At that point it was only observed in three of the containers (Low 1, Low 2 and High 3) but soon it developed also in the rest of the containers. The issue of microorganism growth in the containers and the effect this might have had on the observed mortality in our experiment is debatable. The discussion on whether treating the microorganism growth or not was conferred within the group responsible for this experiment. We did not know what effect disinfectants would have on the eggs of this fish species and however, because we wanted to do this experiment as natural as possible (to be able to reproduce the experiment and get comparable results) the decision was made by the responsible person at Akvaplan niva that treatment with disinfectants should not be carried out. At that point we did not know how extensive this growth problem could be and we were hoping that it would keep to a minimum until hatching. Unfortunately within a week there was microorganism growth in all experimental containers and it progressively covered all of

the egg clumps, when at this point it was too late to start treatment. This microorganism growth could have an effect on the mortality of the embryos observed at the end of the experiment, as there was significantly higher mortality on day 42 than previous sampling dates. Microorganism growth was also observed in other parts of the seawater laboratory where excess eggs were incubated. This microorganism growth weakens some of the conclusions drawn from the experiment especially after day 28. But as microorganism growths were found equally in all containers we are still confident that the data produced are satisfactory.

Hatching

The first few lumpsucker larvae started hatching 38 days after fertilization (June 28th). The hatching of the first few larvae might be introduced by accidental disturbances when tending the containers causing some of the fragile eggs to open as the main hatching did not start until 42 days after fertilization (July 2nd). The peak hatching was observed at day 44 and 45 after fertilization (July 4th and 5th). All the hatched larvae were alive when collected. Statistical analyses revealed no significant differences in length or yolk size between the larvae hatched during the hatching peak and the larvae hatched on the other days during the hatching period. This indicates that the larvae that hatched early are fully developed and not premature hatched. Further, we found no significant differences in larval and yolk size between the different treatments indicating that it probably was the most normal and healthy larvae that hatched within each treatment and the others did not survive through hatching. We only observed 135 hatched larvae in total, and compared to the amount of eggs fertilized (approximately 72000) the overall hatching success was low. The hatching in control containers (n=99) was higher than in oil exposed containers (low dose n=13, high dose n=23) and it seems like oil had an effect on lumpsucker hatching success. Reduced hatching and also delayed or premature hatching have been found in other studies with oil exposed fish eggs (Kühnhold 1974, Hawkes and Stehr 1982, Brown et al.1996). The effects of crude oil on the hatching ability of lumpsucker is a great concern if an oil spill happens during lumpsucker egg incubation or if the lumpsucker eggs were deposited on contaminated bottom sediments. The stock size of lumpsucker is dependent on the recruitment, and for the later years the population of lumpsucker has decreased, most likely because of natural variations (Sunnanå 2006). If there were to be a long lasting oil contamination of lumpsucker spawning grounds,

the consequences for the recruitment could be fatal as the hatching success would lower considerably compared to hatching in uncontaminated areas.

Abnormalities

The morphological abnormalities found on the embryos during egg incubation were in most cases size or developmental differences, while any minor deviations from the normal morphology was difficult to document because of the state of the fixed eggs. The morphological abnormalities in the hatched larvae were more obvious and in most cases too severe to expect the larvae to survive long after hatching. The severely deformed tail that was observed on several individuals would reduce the larvae's swimming ability to the extent that the larvae would soon die as a result of predation or lack of food. The deformed yolk sac would probably cause problems with friction during swimming as it was rather large and bulky, whereas abnormalities like defect operculum would cause problems with the respiratory function. Similar abnormalities on the yolk and spinal deformation have also been observed on Pacific herring larvae in the study by Carls et al. (1999). Other abnormalities like the crocked or less pigmented body is considered to be less severe as the larvae would probably survive for much longer time after hatching. Some of the differences observed such as pigmentation variations might as well be caused by different genetic combinations making some of the larvae less pigmented than others. The deformation of the larvae cannot be related to oil exposure in this experiment, due to the high number of deformed larvae in the control groups. The few larvae hatched in the oil exposed groups made it difficult to compare the data.

Histology

The histological sections did not reveal any differences in organ structure or tissue differentiation between oil exposed and control group embryos and larvae. The histology of the embryos within the eggs was rather difficult to analyze due to the difficulties mentioned earlier but the results from the few sections studied showed no obvious abnormalities in the eyes, liver, intestine, skin pigments or yolk. Comparative studies of sections of hatched larvae showed no obvious differences between control larva and oil exposed larvae when looking at

the eyes, skin, intestine, liver, kidneys and yolk either. The sections made of hatched larvae are furthermore important documentation on organ structure for later studies on lumpsucker histology and development.
The histological sections showed that the lumpsucker larvae have well differentiated eyes, liver, intestine, pancreas and kidneys at the time of hatching, and that they are ready to capture and digest external food, compared to other less developed species such as halibut (Lønning et al. 1982, Pittman et al. 1990) and cod (Hall et al. 2004), which depend on their yolk reserves for weeks or days before being able to digest exogenous food.
From other histological studies done on fish embryos, larvae and adult fish exposed to oil hydrocarbons one could expect to find pathological changes in the olfactory organ, liver, gills, pancreas, eyes and brain (Hawkes and Stehr 1982, Solangi and Overstreet 1982) but no such abnormalities were found in our material.

Methods

A way of limiting the variations in water flow between the containers and maybe lower the differences in PAH levels between the replicates is to use water pumps pre set to 2 litres of water per minute throughout the incubation period (and by this exclude the uncertainties of constant water flow).
We used formaldehyde to preserve the eggs until further analysis in the laboratory but unfortunately fixed eggs from the early embryo stages were less transparent and also very difficult to dissect for further histological embedding. The basic observations on development also became somewhat difficult on fixed eggs. The best would have been to study developmental stages on live samples but as this procedure turned out to be fairly time consuming after the two first sampling dates we had to rely on preserved samples.
Microorganism contamination of the eggs was also an issue we had to face as the incubation period proceeded. Because we did not know the effects of a possible disinfectant on the eggs we chose not to treat them. We considered that if we were to treat the eggs the results we found would not be suitable to compare to later studies where contamination might not be an issue, so we ran the experiment as ”natural“ as possible. To avoid microorganism growth in experiments on embryo development the water should be UV treated before injection into the system.

The histological studies of the eggs on two selected dates during incubation gave unsatisfactory results due to difficulties in sectioning the eggs. The best procedure would have been to dissect the embryos out of the egg chorion and remove the yolk reserves before embedding and sectioning. But as this procedure turned out to fail, due to the extreme fragility of the embryos, and the egg chorion was tough to remove we decided to section the eggs whole. The reason for the reduced quality of the sections when having whole eggs is that the yolk reserve at early stages in lumpsucker development is very large. After egg preparation and paraffin embedding the yolk reserves have become very hard and this makes the yolk fall out or scratch the microtome knife when trying to section the embryos. For the eggs sectioned on day 17 we found no good slides that showed the internal embryonic structure at this stage in development. Fortunately, the eggs from day 30 turned out to be more co-operative and we got a fairly good normal histology from embryos in the control group, but only a few good sections from low 1, 2 and 3, as well as high 2 and 3 for the oil exposed groups for comparison. The hatched larvae were much more robust and contained smaller amounts of yolk so the sectioning was much easier. For the hatched larvae comparative studies of selected organs was carried out, but no differences in tissue or organ structure was observed between control and oil exposed larvae.

Conclusion:

Basically the lumpsucker egg development seems to be little affected by oil exposure during early life stages, but still it is not safe to conclude that there are no effects of oil contamination as we observed reduced hatching success in eggs from oil exposed containers. Such results are important to keep in mind as it is the hatching success and survival through early larval stage that may determine the recruitment of a species (Fuiman 2002)

Further studies should focus on possible effect mechanisms and influence on later life stages of oil exposed lumpsucker eggs. How do the larvae develop further; through first feeding and early growth? Does an oil exposure during early life have an effect on the survival and reproductive success of an individual; do they have a reduced immune system or other weaknesses as an effect of the exposure during the embryo stage? Many questions are still to be asked and many answers to be found.
It is important to study the lethal and sub lethal effects of hydrocarbon exposure on the eggs and larvae of lumpsucker as well as on other northern marine fish species, so that in the case of an oil spill on lumpsucker spawning grounds we would be able to foresee the consequences this would have on lumpsucker recruitment and population existence.

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Appendix A

1. Paraffin embedding and slides colouring procedure:

Before starting the paraffin embedding of the samples, the larvae/eggs were put in a marked plastic cassette and placed in 70% alcohol for 10 minutes x 2, (changed the alcohol after 10 minutes), and then for 30 minutes, to dry out the formaldehyde from the samples. After this was done the samples were placed in the tissue processing machine (Citadel 1000, Shandon). Checked that there were sufficient fluids in all the "baths" that the samples were going to rotate for the next 16 hours. For this machine we used a pre- set program A, including 10 separate baths throughout the whole process. First the samples were placed for 2 hours in 96% ethanol, before rotating to

2 hours in 96% ethanol
2 hours in 100% ethanol
2 hours in 100 % ethanol
1 hour in 100% ethanol and xylene (1:1)
1 hour in xylene
1 hour in xylene
1 hour in xylene
1 hour in xylene and paraffin wax (1:1) and then at least 3 hours in paraffin wax.

After this procedure the samples are ready to be embedded in paraffin. The larvae and egg samples were placed in small metal boxes, and melted paraffin was added, the samples were cooled before being sectioned on the microtome (Leica RM 2255).
The samples were sectioned at 5 μm, put in a water bath at 43 Cº, and then placed onto micro slides. These slides were dried in a cabinet holding 63 ºC overnight, before starting the colouring and placing of cover glass the next day.

For colouring we used the Haematoxylin Eosin procedure where the slides were added to a series of fluids as described below:

2 minutes in Xylol
2 minutes in Xylol
1 minute in 100% ethanol
1 minute in 100% ethanol
2 minutes in 96% ethanol
10 minutes in running Water
15 minutes in Haematoxylin colouring bath
15 minutes in running water
2 minutes in Eosin colouring bath (consisting of 40 ml Eosin stem solution (1 gram Eosine in 20 ml distilled water, added 80 ml 96% ethanol) 100 ml 96% ethanol, 20 ml distilled water and 0,8 ml glacial acetic acid).

2 minutes in 96% ethanol
2 minutes in 96% ethanol
2 minutes in 96% ethanol
2 minute in 100% ethanol
2 minute in 100% ethanol
2 minutes in Xylol
2 minutes in Xylol
2 minutes in Xylol

Time of colouring was 1 hour 8 minutes. Then eukitt glue was used to fasten the cover glass before leaving the slides to evaporate on a well ventilated place for 1-2 days before studying in the microscope.

Appendix B

Descriptions of the different stages of lumpsucker early development:

1. Morula with small cells, approximately 2 days after fertilization.

2. Blastula, early gastrula, approximately 4 days after fertilization.

3. Gastrula.

4. Segmentation, 3-somite stage (also see photo in Figure 8).

5. Embryo with heartbeat, between 6 and 10 somite stage. Eyes with no pigments (0), approximately 15 days after fertilization (also see photo in Figure 8).

6. Eyes with weak pigmentation (1). Small otocysts are visible. Embryo is moving, and the yolk oil globules are almost fused into one. Approximately 17 days after fertilization.

7. Eyes are more pigmented (2), the otocysts are clearly visible and small otholits can also be seen. Olfactory pits, pectoral fins are being developed. Pink colored blood circulation over the yolk can be seen at this stage. Approximately 19 days after fertilization (also see photo in Figure 8)

8. Some pigments are now visible on the head region, but the rest of the body is transparent. The blood has a darker pink/red color, and eye pigmentation is darker (3). Stage reached approximately 24 days after fertilization.

9. More pigments on the head and also on the back and eye pigments (4). Large capillaries and blood veins over the yolk, with only one oil drop. Stage reached approximately 31 days after fertilization (also see photo in Figure 8).

10. Pigments on the head, back and also down over the yolk. Eye pigments (5) and tail fins covering half of the tail. Mouth and operculum are visible but cannot be opened.

11. Eye pigment (6), pigments over the whole body is developing. The mouth and operculum can be opened. Embryo is beginning to take a larval shape, it is growing wider in the head and body region, and the tail fin is reaching two thirds of the tail length.

12. Head is bigger, and the tail appears slimmer and longer with fins all the way to the abdomen. Chin is rising from the yolk.

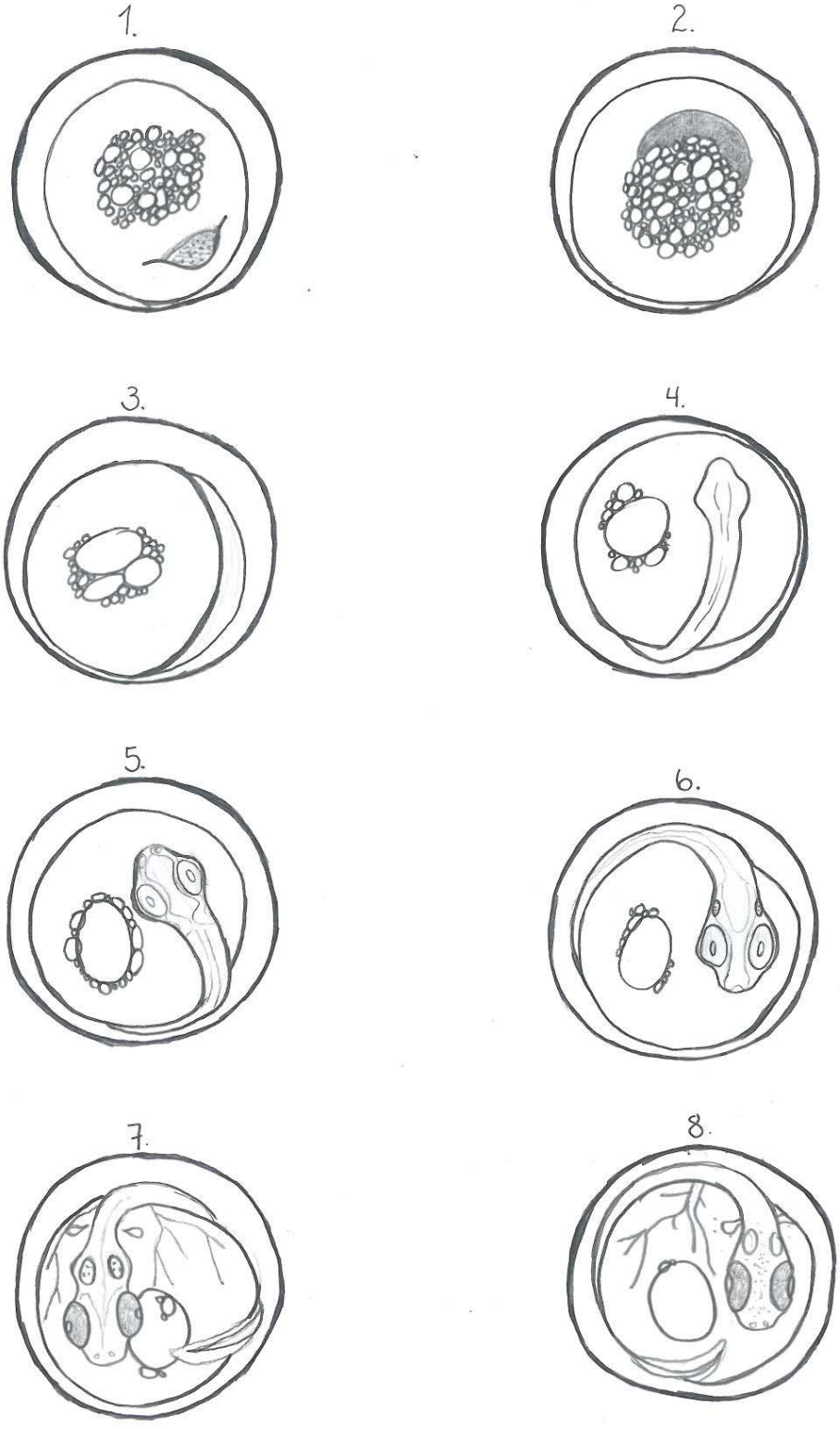
13. pelvic fin modifications, see illustration

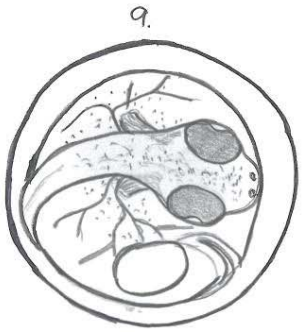
14. pelvic fin modifications, see illustration

15. pelvic fin modifications, see illustration

16. Fully developed larvae at hatching (also see photo in Figure 8).

Illustrations of the different stages in lumpsucker early development.

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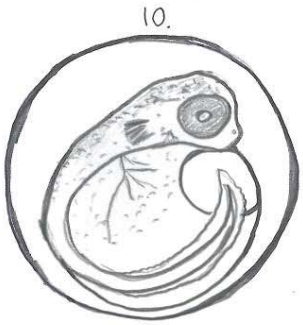
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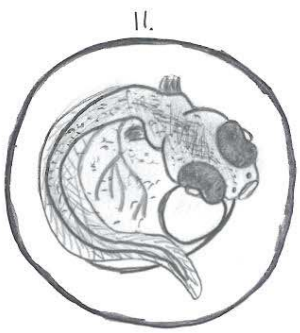
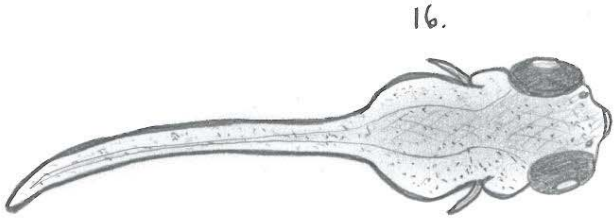

11.


12.


13.


14.


15.


16.

Appendix C

Temperature in the water during the experimental run