Faculty of Biosciences, Fisheries and Economics
Department of Arctic and Marine Biology

Cardiotoxic Effects from the Water-Soluble Fraction of Crude Oil on an Arctic Keystone Species – Polar Cod (*Boreogadus saida*)

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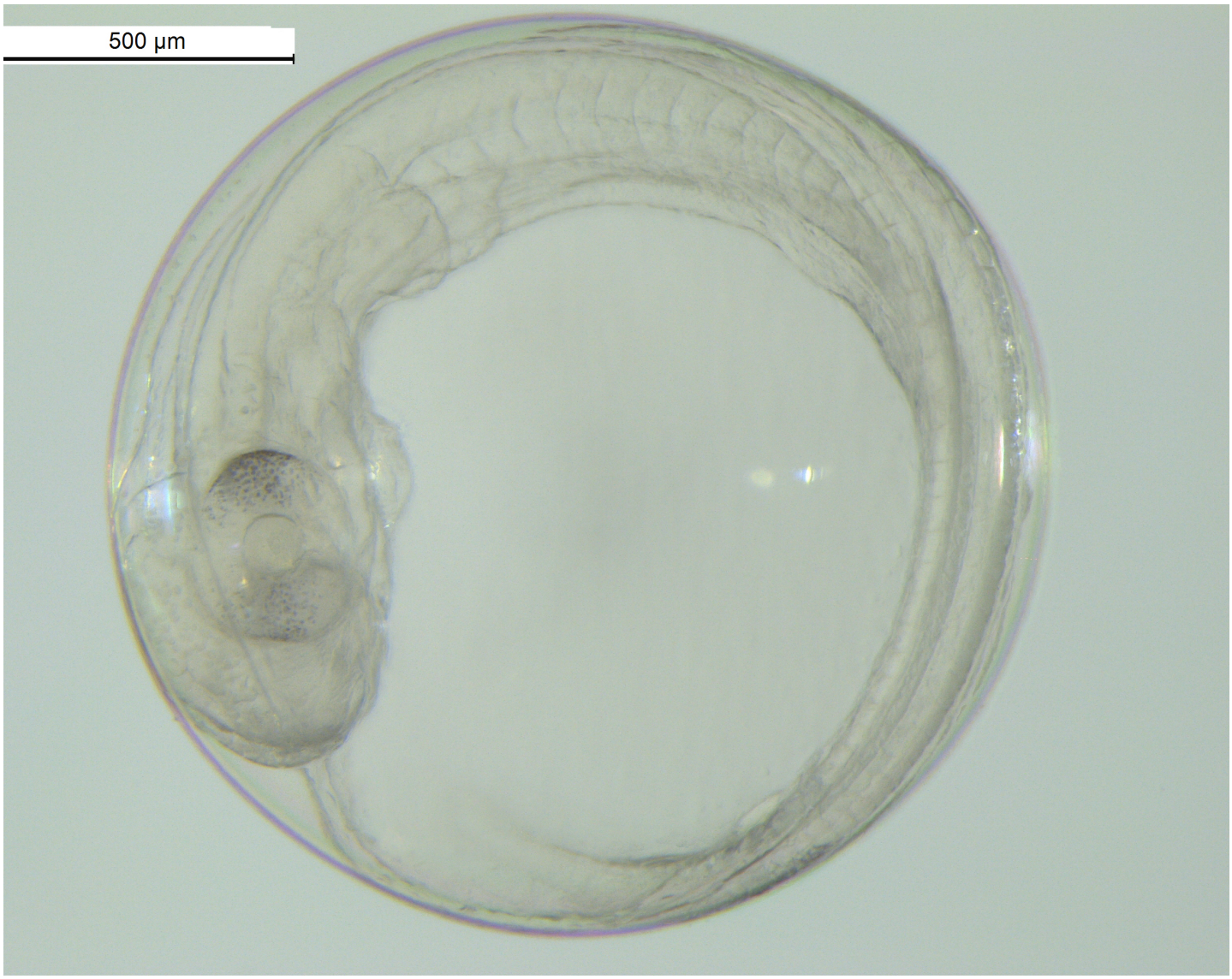

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Abstract

The Arctic ecosystems and its species are under increased anthropogenic pressure from both climate change and industrial activities. Of special interest is pollution from petrogenic substances, and in particular accidental oil spills, that pose a risk to arctic marine organisms. An oil spill in the Arctic may see crude oil encapsulated into the sea ice and over time leaching to surface waters in the vicinity.

For all sympagic species this is a threat, and for polar cod (*Boreogadus saida*) with buoyant eggs aggregating in surface waters it is of imminent importance to understand how they are affected. A wide range of early life stages of marine fish have shown sensitivity to exposure from the WSF of crude oil, including polar cod. The effects from exposure involves symptoms like pericardial edema, yolk-sac alterations, malformations, bradycardia and arrhythmia.

Polar cod is a keystone species in the Arctic pelagic ecosystem. The species feeds upon smaller zooplankton and is very effective at assimilating this energy into reproduction and growth, and thus funnels energy from lower trophic levels all the way up to marine mammals and seabirds.

We exposed buoyant polar cod eggs to environmentally realistic concentrations of the water-soluble fraction (WSF) of crude oil through an oiled rock column experiment over 79 days. The experiment mimicked a potential spill in the Arctic exposing the buoyant polar cod eggs to a continuous release of water-soluble oil components from the sea ice. The total amount of the polycyclic aromatic hydrocarbons (sum of 44 PAHs) in the water were in the ng/L range and the highest concentration measured in eggs were roughly at 1100 ng/g wet weight. In addition to crude oil exposure. The embryos were maintained under two different temperature regimes, 0 °C and 3 °C respectively to determine if additional pressure from elevated temperatures could affect the severity of toxicity.

This is the first study to document dose dependent effects in cardiotoxicity for an arctic keystone species, but also underlines previously observed effects regarding toxicity from the WSF of crude oil in early life stages of marine fish (e.g. yolk-sac alterations, pericardial edema, deformities, increased mortality etc.). The study couldn't observe a clear increased toxicity between temperature regimes, but it's is not ruled out as a potential stressor for polar cod and the Arctic system in general. Thus, further research is needed to understand how temperature effects polar cod embryos in general and specifically when exposed to additional stressors.

Effects from very low environmentally realistic concentrations of the WSF of crude oil seen in this study may have implications for the arctic keystone species – polar cod. This short-lived species whom invests large amounts of its energy into reproduction may have impaired recruitment due to oil spills and pollution and thus, it most likely will affect food web dynamics and the Arctic system as a whole.

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1 Introduction

1.1 Anthropogenic Interests in a Changing Arctic

The warming of our planet due to increased levels of greenhouse gases in the atmosphere and feedback mechanisms that accelerate this process, is of imminent importance for humans (Masson-Delmotte et al., 2018). Changing weather patterns, ocean acidification, melting of glaciers and increasing sea levels, absence of sea ice are some of the challenges facing ecological systems all over the globe (Masson-Delmotte et al., 2018). The globally averaged combined land and ocean surface temperature data as calculated by a linear trend, shows a warming of 0.85 °C from 1880 to 2012 (Masson-Delmotte et al., 2018). However, this global mean is not reflecting the pace of changes happening in the Arctic. Symon et al. (2005) reports that the temperature changes in the Arctic are happening twice as fast compared to the rest of the globe; and the Arctic will be deprived of sea ice during summer within 2050 – making it a seasonal ice sheet (Notz & Stroeve, 2016).

An ice-free Arctic has large ecological implications for an array of endemic and specialized species that rely on multiyear ice for survival (Gradinger, 1995; Kohlbach et al., 2017; Rass, 1968). For instance, polar cod (*Boreogadus saida*) use the ice as a "top down spawning substrate"; i.e. early life stages of polar cod use it as a nursery ground, but also for protection (David et al., 2016; Kohlbach et al., 2017). Polar cod also have temperature preferences in relation to how effectively they utilize nutrients, and where growth performance is highest (Kunz et al., 2016; Rass, 1968). In addition, invasive species may put additional pressure on population dynamics through interspecific competition (Renaud et al., 2012).

The changes in the Arctic are associated with human activity around the globe. The changes themselves are ironically making the area accessible for increased shipping, oil explorations and prospecting for minerals (Peimani, 2012). These activities are all potential sources of pollution from petrogenic substances, both chronic and acute. Of explicit interest is of course the vast gas and oil reserves in the Arctic, which amounts up to 30 % of the remaining gas, and 13 % of the remaining oil in the world (Gautier et al., 2009).

In this context the Arctic is a potential subject for spill events similar to accidents of recent history — e.g. the Exxon Valdez oil spill (1989), or the Deepwater Horizon oil spill (2010). Both spills released large volumes of crude oil into the marine environment and they had implications for surrounding ecosystems and biota and especially early life stages of marine fish (Beyer, Trannum, Bakke, Hodson, & Collier, 2016; Peterson, Rice, Short, & Esler, 2003). Increased pressure from human activity, alongside predicted long-term changes in the Arctic region is therefore an important subject of study and knowledge gaps in this regard needs to be addressed.

1.2 Crude oil, the weathering process and oil in ice interactions

Crude oil is an organic compound composed of thousands of different chemicals (Fingas, 2011). It's a mixture of a wide range of hydrocarbons, polycyclic aromatic hydrocarbons and

other non-hydrocarbon compounds such as heterocycles. These petroleum related compounds vary in molecular size and number of isomers. In addition, some of them also contains oxygen, nitrogen, sulphur and different metals and chemical groups (e.g. copper, uranium and alkylation.) (Symon, Skjoldal, Arctic, & Assessment, 2010). Furthermore, the composition of crude oil changes with locality and also depends on when it was initially formed (Fingas, 2011).

In acute oil spill events like the Exxon Valdez oil spill the weathering of oil plays a key role for the bioavailability of chemicals to marine organisms. In particular, the dissolution of toxic compounds into surrounding waters that has been a concern for many researchers (Carls & Meador, 2009).

Weathering of oil involves macro-scale processes (e.g. natural dispersion, advection) to specific chemical processes (e.g. dissolution, evaporation, emulsification). The pace and character of these processes are bound by the physical forcing and environment of where the crude oil is released – e.g. stormy weather would enhance natural dispersion by wind forcing, or high temperature would increase the pace of evaporation (Symon et al., 2010). The weathering process is thoroughly described by Symon et al. (2010) and illustrated in **Figure 1**.

Dissolved chemicals from crude oil are of special biological interest because they are more bioactive compared to their undissolved counterparts. The dissolution of nonpolar compounds are dependent on their hydrophobicity (K_{ow}) of the given substance. Crude oil with its large number of different constituents naturally show a degree of solubility for each chamical. (Fingas, 2011). For instance, the solubility of PAHs decreases with increasing number of aromatic rings, i.e. the two-ringed naphthalene are more soluble compared to phenanthrene, and the four ringed pyrene is again less soluble compared to phenanthrene (J. P. Incardona, Collier, & Scholz, 2004). Differences in solubility and properties of compounds (i.e. alkylated compounds) affect how they interreact with biota and their toxicity – e.g. the more soluble naphthalene, although it can bioaccumulate, shows very low toxicity, while phenanthrene is believed to disrupt cardiac function by affecting specific ion-channels in cardiomyocytes (J. Incardona, 2017).

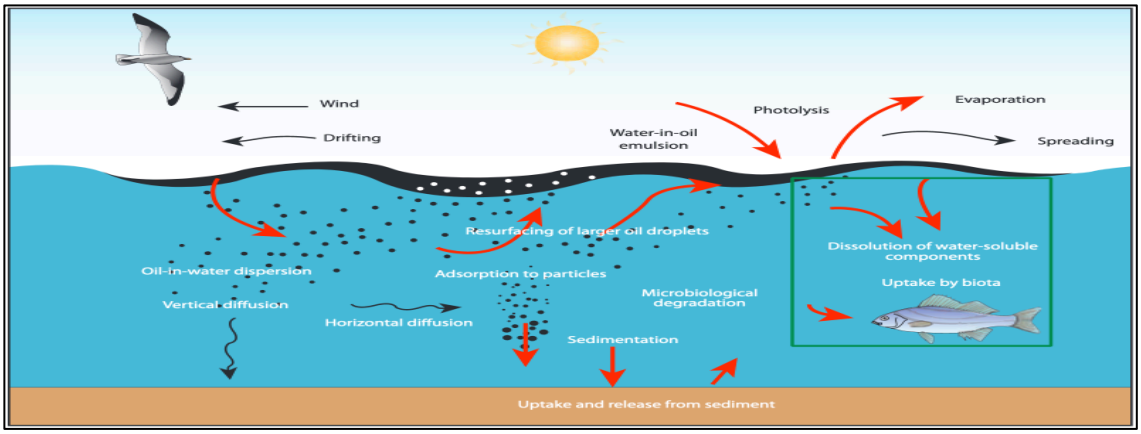

Figure 1. *Schematic representation of the weathering processes of surface-spilled crude oil in open waters. In this thesis, the dissolution of water-soluble components is the most relevant process in regard to WSF-experiments (see Materials and Methods section). The process of dissolution is important because it enhances the bioavailability of potential toxic components (Symon et al., 2010).*

Furthermore, natural dispersion effects the rate of solubility because of the area of interaction between the water and crude oil. This may be of significance for oil trapped in refuges where natural dispersion is hindered, like in ice covered waters (Symon et al., 2010).

In the Arctic the weathering of oil expresses a different character (**Figure 2**.). Incorporation of crude oil into the ice potentially prolongates the exposure of biota to the water-soluble fractions (WSF) of crude oil. The weathering processes in presence of ice are slowed, dispersion is not uniform and trapped oil may be released as melting commences during spring (Brandvik & Faksness, 2009). This inclines that the timing of spills in the Arctic may be irrelevant in terms of biological consequences – since spills potentially will impact the most productive period of the year regardless of when it occurred.

A large-scale experiments in ice covered waters have been conducted to observe weathering of crude oil in presence of sea ice; and PAHs related to toxicity in the µg/L scale has been detected in spills with 7000 L of crude oil (L. G. Faksness, P. J. Brandvik, R. L. Daae, F. Leirvik, & J. F. Børseth, 2011). *L.-G. Faksness, P. J. Brandvik, R. L. Daae, F. Leirvik, and J. F. Børseth (2011)* concluded that the acute toxicity in their experiment was low, but the volume applied was not comparable to a spill in the scale of e.g. Deepwater Horizon, where 3.19 million barrels of crude oil was released – equivalent to 500.000.000 L. Figure 2. exemplifies how oil may act within ice and this is of concern to species living in close relation to sea ice.

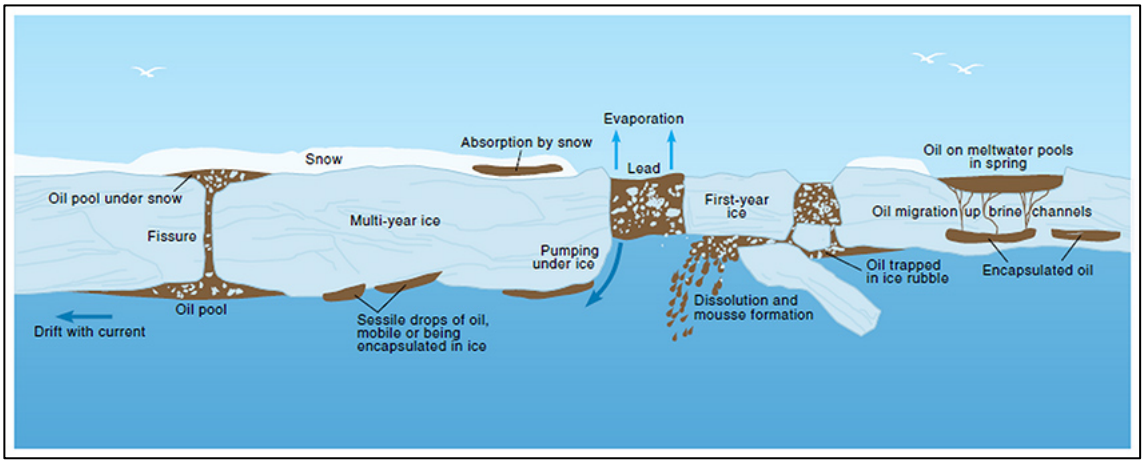

Figure 2. *Schematic illustration of probable paths for crude oil spilled; upon, under or in the vicinity of sea ice. The weathering processes are also shown (Symon et al., 2010).*

1.3 Early life stages of marine fish and their sensitivity to crude oil

Early life stages (ELS) of marine fish have shown a sensitivity to the WSF of crude oil and a suit of symptoms are documented, ranging from deformities (spinal, facial), dysfunctional hearts (bradycardia and arrhythmia) and different forms of vascular accumulations in the yolk and around the heart of fish larvae (i.e. pericardial edemas and yolk-sac edemas) (Carls & Meador, 2009; Hodson, 2017).

Symptoms seen have been shown in different species of fish, both from fresh water and in marine environments. The sensitivity to the dissolved fractions of crude oil seems to be a general toxicological effect for a wide range of ELS of fish, and has been documented in

japanese medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), pacific herring (*Clupea pallasii*), pink Salmon (*Oncorhynchus gorbuscha*) and polar cod (Carls, Rice, & Hose, 1999; Heintz, Short, & Rice, 1999; Nahrgang et al., 2016; Salaberria, Brakstad, Olsen, Nordtug, & Hansen, 2014)

The toxic effects seen in ELS of fish exposed to crude oil is in the literature credited to polycyclic aromatic hydrocarbons (PAH). PAHs are composed of multiple aromatic rings arranged in a planar structure; and especially the 3-4 ringed PAHs are assumed to be the most toxic constituents in crude oil, at least from information derived from single compound studies (J. P. Incardona et al., 2005; J. P. Incardona et al., 2004; Zhang, Huang, Zuo, Chen, & Wang, 2013).

There are well-balanced reasons to believe that PAHs have toxic impact on ELS of fish. Single compound studies have disclosed this fact (Hodson, 2017; J. Incardona, 2017). But, these studies have been used as screening studies to reveal mechanistic relationships; and the doses used are often not environmentally realistic (J. Incardona, 2017). And thus, when evaluating toxicity for crude oil the potential interplay (additivity, synergism, antagonism) between PAHs and chemicals that aren't measured traditionally in crude oils should be considered (Hodson, 2017).

1.4 Cardiotoxicity

Cardiotoxicity has been shown in several studies in a wide range of species. The specific symptoms in the heart is the accumulation of fluids (i.e. pericardial edema) around the heart and morphological changes in the heart characterized by poor looping and reduced cardiomyocyte proliferation – symptoms that on the organismal level have been seen are bradycardia and arrhythmia and eventually cardiac arrest.

For ELS of marine fish the heart is most likely a specific target for PAHs (J. Incardona, 2017) and several authors also claims that cardiotoxicity is the initial vector for secondary effects seen in ELS of fish (J. P. Incardona et al., 2004; Zhang et al., 2013). It is claimed that since secondary effects (e.g. skeletal deformities) isn't necessary for cardiac disruption, the secondary symptoms are derived from circulatory failure (Corinne et al., 2011; J. P. Incardona et al., 2015; J. P. Incardona et al., 2013) – i.e. the absence of secondary effects (when cardiotoxic effects are present) is used as evidence that circulatory failure is the main toxicological response to crude oil exposure.

The mechanism behind cardiotoxicity in ELS of marine fish is complex and is believed to involve different toxicological pathways. The most studied routes of toxicity are the aryl hydrocarbon receptor (AHR) dependent and AHR-independent pathways (J. Incardona, 2017). "The AHR is the ligand activated transcription factor that controls the metabolic detoxification of planar aromatic compounds by inducing expression of a gene battery encoding phase I and phase II enzymes (CYP1A activation, battery of genes involved in regulating PAHs and related compounds) (J. Incardona, 2017)." AHR-dependent cardiotoxicity was initially shown as a response to dioxin and PCB (blue sac disease in salmonids), but higher molecular weight PAHs (e.g. benzo(a)pyrene and dibenzanthracene) also produce similar defects (J. P. Incardona, Day, Collier, & Scholz, 2006). Exposure of strong AHR ligands and the toxicity of them is thought to be due to prolonged activation of AHR2 isoforms in cardiomyocytes, and the down regulation of transcription factors essential

for normal cardiomyocyte development. However, the actual mechanism behind AHR-mediated toxicity and effectors involved are still unclear (J. Incardona, 2017).

The AHR-independent cardiotoxicity is mainly credited to the tricyclic PAHs (J. P. Incardona et al., 2004). These compounds don't activate the AHR-ligand, but show similar symptoms as previously described. This path of toxicity is mainly believed to be caused by disruption of ion balance in the heart through blocking channels of Na+/K+-ATPases and Ca2+ LTCC channels in cardiomyocytes, and thus affecting cardiac excitation contraction coupling, but also potentially affecting SERCA ion channels in the sarcoplasmic reticulum, which also plays an essential part in the contraction of heart muscles cells (Elin et al., 2016; Fabien et al., 2017; J. Incardona, 2017; Zhang et al., 2013). However, these molecular initiating events also lack extensive evidence since single compound studies haven't showed this at the low doses of PAHs (µg/L range) claimed within a crude oil WSF.

For ecological systems it's the WSF-studies that presumably are most relevant for ELS of marine fish; since complex mixtures is what ELS meets in the wild. Pericardial edema, bradycardia, arrhythmia, and changes in the morphology of the heart is a well-documented cardiotoxic response to the WSF of crude oil (Carls et al., 1999; J. P. Incardona et al., 2009; J. P. Incardona et al., 2015; J. P. Incardona et al., 2014; Jung et al., 2013; Nahrgang et al., 2016). There is therefore, little doubt that the WSF of crude oil is cardiotoxic, and three decades worth of literature supports this (Carls & Meador, 2009; Hodson, 2017; J. Incardona, 2017).

1.5 Polar Cod a Keystone species in the Arctic

Polar cod is a fish in the order gadiformes, and the gadids inhabits polar to temperate waters in the northern hemisphere. It holds the position as the northern most occurring fish, and more specifically it's distributed from the central arctic basin, the Canadian archipelago, shelf areas throughout the Arctic Ocean southwards around Greenland and northern Iceland, the Newfound land banks, the Bearing sea, around Svalbard and the Barents Sea (Catherine W. Mecklenburg, Møller, & Steinke, 2011).

The species is a relatively small and short-lived fish, generally with lengths up to 30 cm and age up to 7 years. It's a cryopelagic and nerito-oceanic fish that has been documented at depths down to 800 m, with salinities and temperatures ranging from 31.3 to 34.9 ppt, and -1.9 to 7.9 °C(C. W. Mecklenburg et al., 2018). Furthermore, polar cod is physiologically adapted to subzero temperatures with production of anti-freeze proteins (Osuga & Feeney, 1978) and, Kunz et al. (2016) also reveales that feed conversion efficiency for polar cod were highest at 0 °C and the best growth performance was attained at 6 °C.

The vertical distribution of the species is dependent on the life history of the species. Young immature specimens and embryonic stages are often found higher in the water column and in close relation to sea ice (sympagic) (David et al., 2016; Rass, 1968). They use the ice for protection and as a nursery ground during their early development (usually the first year) (Haakon Hop & Gjøsæter, 2013). Kohlbach et al. (2017) have identified a strong link between ice algae produced carbon and young polar cod, which underlines polar cod sympagic relationship.

Figure 3. *"Distribution of polar cod based on participation in research sampling, examination of museum voucher collections and literature. The map shows the maximum distribution observed from point data and includes both common and rare locations (Eastman, 2018)."*

As they mature, polar cod depart from the sea ice and assemble in shoals in productive shelf areas and fjords around the Arctic Ocean (Falk-Petersen, Frivoll, Gulliksen, & Haug, 1986; H. Hop, Welch, & Crawford, 1992). At the initiation of spawning from late November to February polar cod aggregates deeper in the water column; often in fjords and shelf areas close to sea ice (Benoit, Simard, & Fortier, 2008, 2014). Polar cod is a synchronous broadcast spawner and with the fertilization and advection of buoyant polar cod eggs with the currents (Craig, Griffiths, Haldorson, & McElderry, 1982; Nahrgang et al., 2014), the embryonic stages and immature polar cod once again lives in close relation the sea ice.

The life history, distribution and abundance are important aspect of why polar cod is crucial to the Arctic system – a keystone (Craig et al., 1982; Haakon Hop & Gjøsæter, 2013). Christiansen (2017) describe the fish as a focal species that makes ecosystems swing and functions as a wasp-waste species; i.e. it affects top predators through bottom-up interactions

and prey through top-down interactions (Bakun, 2006). Predators, such as harp seal, Atlantic cod, white whales and sea birds' profits from polar cods' effective assimilation of energy and growth in cold waters. The energy funnels through the arctic ecosystem all the way from algae to different species of zooplankton (e.g. copepods, amphipods and euphausiides; i.e. their diet) and all the way up to the polar bear (Bradstreet, 1982).

The omnipresence and function of polar cod makes it an interesting and important subject for studies. With a changing Arctic and already documented sensitivity to WSF of crude oil (Nahrgang et al., 2016) Furthermore, Nahrgang et al. (2016) revealed that polar cod; an Arctic key stone species is sensitive to very low concentrations of the WSF of crude oil. Polar cod exposed to concentrations barely detectable in effluent water display sub-lethal dose-dependent effects like e.g; pericardial edema, retarded growth and increased spine curvature. However, cardiotoxic effects were not documented on the organismal level in their study, and thus further research on this omnipresent fish of the Arctic is needed.

1.6 Objectives

The goal of this study was to understand how the water-soluble fraction of crude oil affects ELS of polar cod during embryogenesis. We predict that i: The WSF of crude oil will cause cardiotoxic effects in a dose-dependent manner, that is bradycardia and arrhythmia in polar cod; ii: The WSF of crude oil will cause malformations, skeletal deformities and pericardial edema in polar cod larvae iii: Malformations (if detected) are directly correlated to cardiotoxic effect at individual level; iv: Is temperature causing adverse effects in relation to the WSF toxicity.

To answer these hypotheses, polar cod embryos were exposed to several concentrations of a crude oil WSF and studied at the larval stage for both cardiac activity and malformations. The third hypothesis was tested by studying larvae of different phenotypes (normal developed versus malformed) within a same crude oil treatment and relating the observed malformations, (or lack of) to the cardiac activity.

2 Materials and Methods

2.1 Ethical Statement

All work regarding this thesis was done in compliance with regulations enforced by the Norwegian Animal welfare authorities. The vessel, R/V Helmer Hanssen has all the necessary authorization from the Norwegian Fisheries Directorate to use bottom trawls to collect fish for scientific purposes. The author has appropriate training (FELASA C) to perform the work, and no harm considered a violation of present rules were done.

2.2. Sampling of Wild Polar Cod

Mature polar cod were sampled in Svalbard waters in November 2017 during a research cruise on the R/V Helmer Hanssen. The specimens were caught at depths of about 150 m, at about 2 knots and for 5 minutes at a time with a Campelen Super 1800 bottom trawl. The trawl has a fish lift to avoid injuring collected specimens. During the voyage polar cods were kept in a 500 L tank with inflow of sea water. Deceased and injured individuals were removed daily.

Upon arrival in Tromsø, the fish were further transferred to the biological research station at UiT; The Arctic University of Norway in Kårvika and maintained in a 2000 L holding tank with continuous inflow of seawater. The sea water at the research station was pumped from 50 m depth from the sea close to the station. Before the water enters the tank, it goes through a drum filter to remove particles, it is UV-treated to remove microorganisms and cooled to 2 °C to have an optimal temperature. Polar cod lives in total darkness during the polar night; hence, the room was kept dark before mature polar cod were stripped. The fish were treated with Halamid® to avoid microbial infections. Polar cods were also fed with natural prey Calanus spp. to keep them in good condition (bought from Calanus AS).

2.3 Experimental Design

The experiment was set-up using a similar exposure design as Carls et al. (1999) and previously used on polar cod ELS by Nahrgang et al. (2016). It's a design and system that delivers environmentally realistic water-soluble fractions of crude oil through oiled rock columns in a flow-through system (**Figure 4**.). The setup mimics a potential oil spill in the Arctic where crude oil incorporated into sea ice over time leach toxic fractions into surrounding waters.

The system consisted of 32 incubators divided between a 0 °C and 3 °C temperature regime (16 incubators each). Moreover, for each temperature regime, the 16 incubators were further separated into four crude oil treatments (control [C], low [L], medium [M] and high [H]), in four replicates. Each of the four crude oil treatments per temperature regime was generated using oiled-gravel columns filled with 11 kg of gravel. Briefly, the gravel was sieved (size 7-11 mm), washed (which involved, rinsing with water and 96 % ethanol, and soaking in HCl), dried and mixed with different amounts of crude oil. The gravel was then left to dry for 72 hours at ambient temperature. The amount of crude oil/kg gravel was 0, 0.19, 0.75 and 3 g oil per kg gravel for the C, L, M and H treatment groups respectively.

Three days before initiation of the experiment gravels were inserted in each columns, connected to the seawater and flushed for 72 hours in order to remove the highly volatile, and acutely toxic BTEX compounds.

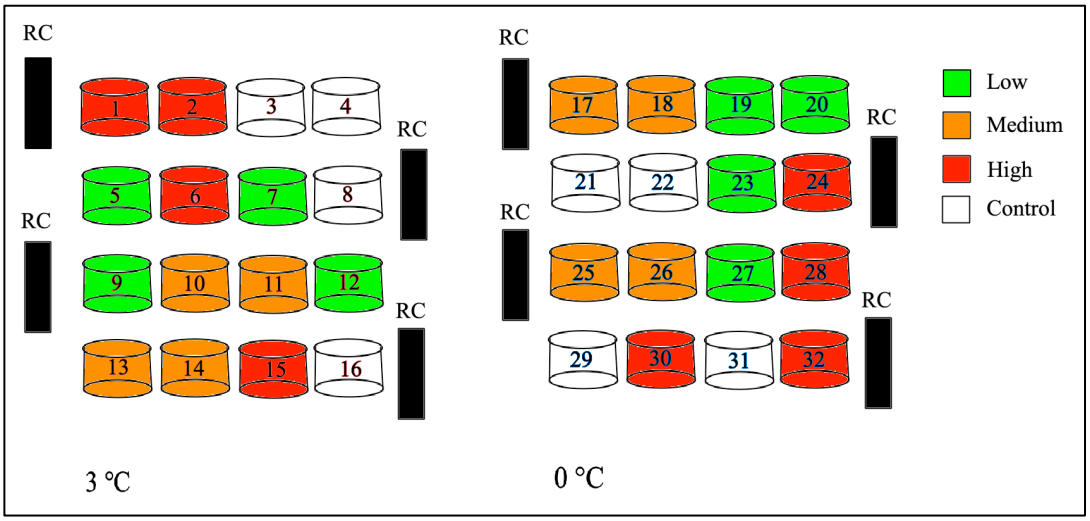

Figure 4. *A simple representation of the experimental design. The number from 1-32 shows every single incubator in the experimental set-up. RC represents the oiled-gravel columns (C: 0g, L: 0.19, M: 0.75 and H: 3g crude oil/kg gravel), i.e. the source of the WSF of crude oil. Incubator 1-16 were in the 3 ℃ regime and the remaining incubators from 17-32 were in the 0 ℃ regime. For each temperature regime, the oiled gravel columns delivered their water to the replicate incubators (4) according to a randomized scheme, represented by the colors in the figure.*

2.4 In Vitro Fertilization

The in vitro fertilization and exposure experiment started on the 28th of January 2018. 49 females and 21 males were anaesthetized in a Finquel bath and stripped. 20 mL of eggs were collected from each female. The quality of the eggs was visually inspected by subsamples before the experiment. We looked at physical properties e.g. shape, transparency and size, which are practical criteria often used in evaluating egg quality (Brooks, Tyler, & Sumpter, 1997). The eggs were then pooled at a ratio of 1:1 and split equally into two separate glass bowls for dry fertilization at their respective incubation temperature (0 and 3 °C) The temperature was maintained at the desired temperature by using a water bath (one container with ice water; and one with a temperature of 3 °C water). Each temperature had thus the same representation from the donors. The milt was also pooled at a ratio of 1:1 and fertilization commenced with adding milt and incubation water from the control treatments to activate gametes. Fertilization was stopped after 10 minutes by rinsing eggs with incubation control water. The fertilized embryos were then incubated in each of the 32 incubators at an estimated concentration of 8000 per incubator.

2.5 Exposure and Sampling

Samplings for the water chemistry were done only during the embryonic developmental phase at 0, 4, 10, 18, 28 and 56 days post-fertilization(dpf). Collection of water samples were done by briefly tapping 500mL from the gravel columns and preserved by adding 0.5 mL 15 % HCl per 100mL water. The samples were stored at
3 °C and sheltered from light until analysis (section 3.6).

Samplings for body burden were done at 4 and 18 dpf. For each treatment and temperature group, 25 embryos of two of the four replicate incubators were sampled and pooled. Sampled embryos were stored at -20 °C for further analysis.

During the experiment mortality was recorded on a daily basis. Dead embryos and larvae were removed from the incubators. Healthy polar cod embryos were distinguished by their positive buoyancy. Dead embryos and larvae sank to the bottom of the incubators; where they were collected and later counted.

Hatching started 26 dpf. for the 3 °C embryos and at 35 dpf. for the 0 °C embryos. The hatching periode was between 26 and 30 dpf. for the 3 °C temperature regime, and between 44 and 64 dpf. for the 0 °C regime.

Sampling for cardiotoxicity analyses was done just after hatch for comparison, i.e. at different timepoints for the 0 and 3 °C groups respectively. The sampling for cardiac activity, deformations and yolk-sak alterations was done 29 dpf. for 3 °C and 47 dpf. for 0°C. Polar cod larvae are small and poorly developed at hatching i.e. altricial larvae. It is therefore difficult to assess the degree of deformities at hatch, which is why cardiotoxic and malformations (jaws ad spinal) were sampled at a later timepoint. The additional cardiotoxic and malformation samples were taken 79 dpf. Methods for cardiac activity, deformities and yolk-sac alterations are detailed in section 3.7 and 3.8.

2.6 Chemical analysis and water samples

Analysis of the sum of 44 PAHs in water samples and embryo samples were conducted by SINTEF. Samples of water were extracted and analysed with gas chromatography and spectrometry using an Agilent 7890 gas chromatography coupled with Agilent 7010B triple quadrupole mass spectrometry. Determinations of PAHs was based on initial studies by Sørensen, Meier, and Mjøs (2016). All embryo samples were extracted as described by Sørensen, Silva, Booth, and Meier (2016)

2.7 Cardiac Activity (heart rate and arrhythmia analyses)

Cardiac activity was recorded using a Leica stereomicroscope. Every specimen was taken from its respective incubator and placed under the microscope in a watch glass containing 500 µl of sea water. The watch glass was placed on an aluminum stage, with a see-through hole and connected to a circulating cooling bath. Ethylene glycol were used as the antifreeze agent in the bath and the temperature was controlled by manual measurements over time with a thermometer until it matched the temperature for the different temperature regimes. Equipment used for recording is shown in **Figure 5**. Video records were recorded for at least 1 minute. Anesthetizing agents were not used because of the possibility of affecting the heart rate of specimens.

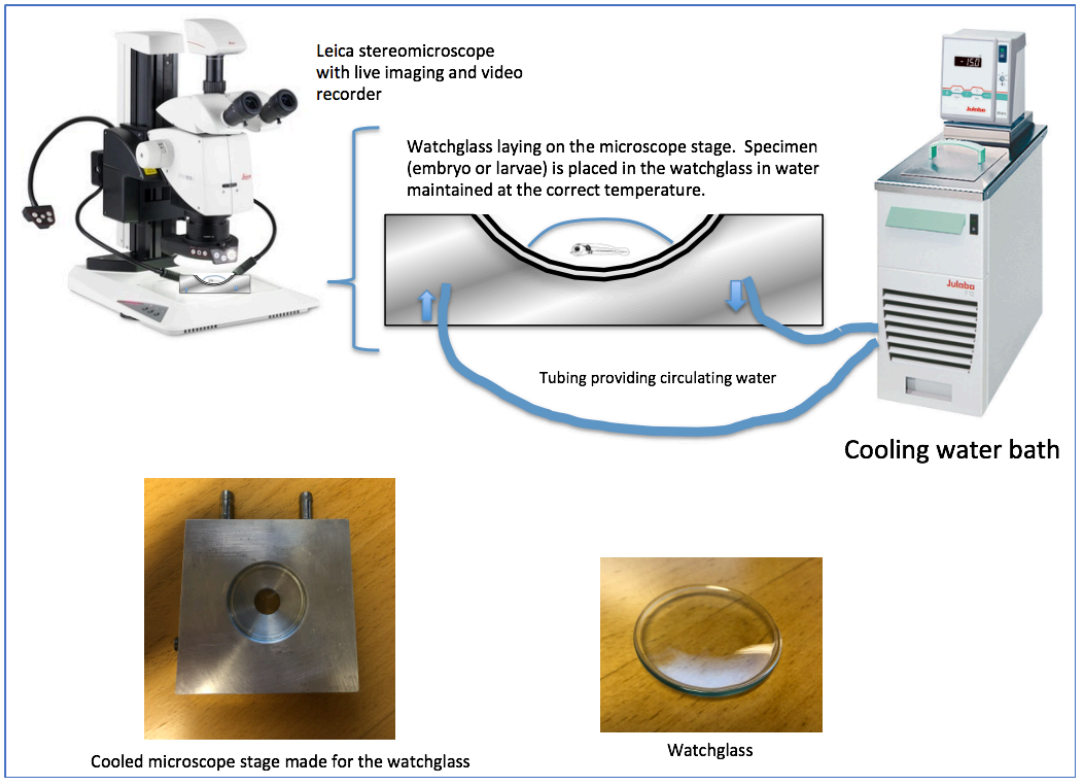

Figure 5. *Equipment and setup to record and take pictures of polar cod larvae. The different equipment is described in the figure.*

The extraction of cardiac activities from the records was done with a QuickTimePlayer 7 (version 10.5). The heart rate (heart beat frequency) was determined based on 30s video segments and expressed as beats per min. Arrhythmia was defined as the variation in length between heart beats, i.e. the standard deviation of length for each beat in seconds (J. P. Incardona et al., 2009). A starting point and ending point of every beat was set. Thereafter the number of frames for each heart beat was determined and converted into seconds, for a total recording time of 20 seconds per individual larvae.

2.8 Pericardial Edema

Pericardial edema was measured as a ratio between the area around the heart and the true yolk, which is shown in **Figure 6**. It was done on the same larvae that were assessed for cardiac activity in the experiment.

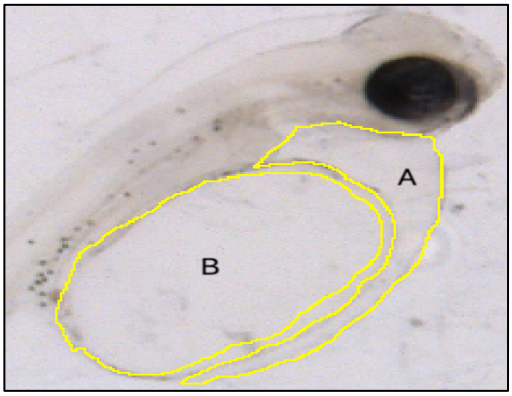

Figure 6. *Pericardial edema as defined in this study. Area A i.e. pericardial edema; was the space surrounding the heart and the true yolk, but still part of the yolk-sac; Area B is the true yolk. The ratio was calculated as follow: A/(A+B) and multiplied with 100 to find the percentage of edema.*

2.9 Malformations and Morphometrics

Malformations in the jaws was categorized as presence/absence of deformities. 10 larvae from M with malformed jaws, without malformed jaws and 10 from C were inspected for cardiac activity (same method as described in section 3.7). This was done to see if cardiac activity differed within the same treatment regardless of the degree of deformities on the exposed larvae. **Figure 7**. shows how larvae were classified to deformed or not deformed for this purpose. This selection was based on a personal opinion so the degree of deformed jaws was selected as a factor where it was impossible to argue whether or not the jaws were deformed or not. Note that this was done under the 0 °C temperature regime being closest to what ELS of polar cod experience in nature now.

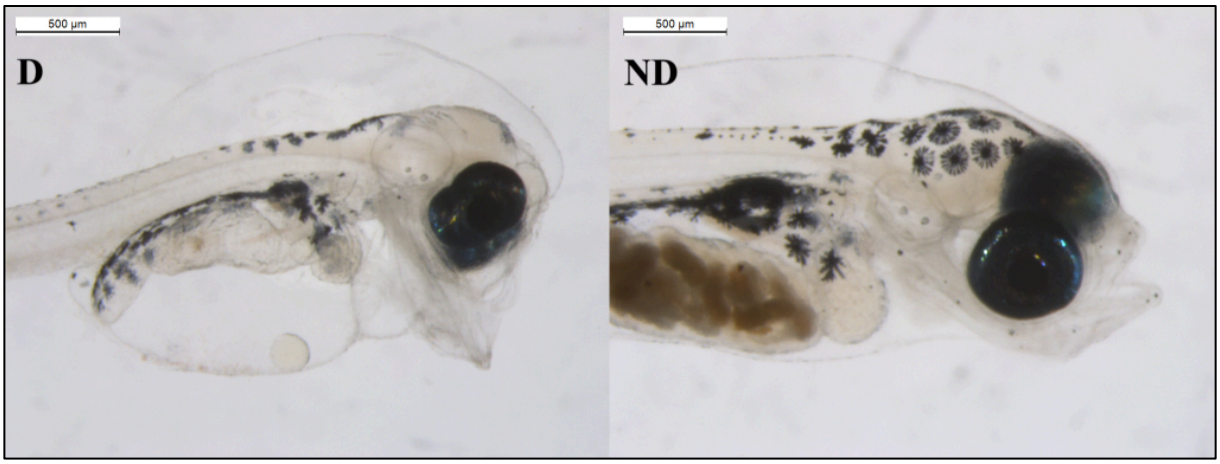

Figure 7. *A picture of two larvae categorized as deformed (D) and not-deformed (ND). 10 specimens similar to D and ND were selected and recorded to look at cardiac activity (heart rate and arrhythmia). Pictures of all larvae were also taken. In addition, 10 larvae from control were also selected for comparison.*

2.10 Statistical analysis

For the statistical analysis a linear mixed effect model was used. This model fits the data and take into consideration that all larvae tested came from different incubators – i.e. avoiding pseudo-replicates. All data were normally distributed, and a one-way ANOVA were used to check for differences between treatments and if temperature were interacting in this regard.

All graphs, figures and analyses were done with Rstudio (version 1.1.463), and the package ggplot were used when creating the figures (except for **Figure 9**. were excel was used; version 16.23).

3 Results

3.1 Water Chemistry

3.1.1 tPAH Concentration in Water Samples

Over time the total aqueous concentration of PAH declined for both temperature regimes. The concentrations over time are shown in **Figure 8**. The initial levels of tPAHs in 0 °C regime were 139.7 ng/L [H]; 8.8 ng/L [M]; 5.99 ng/L [L] and levels for the 3 °C regime were 2.6 ng/L [C] and 237.2 ng/L [H]; 15.2 ng/L [M]; 5.6 ng/L [L] and 3.3 ng/L [C].

For the 0 °C regime the tPAHs for H declines by 77 % within 4 days, and continued until 56 dpf (3 % of initial concentration left). For M the tPAHs concentration declines with about 90 % within 4 days. For C and L the concentrations were low. L had an initial concentration of 6 ng/L and by 56 dpf concentrations were at 1 ng/L. C ranged between 2.6 and 0.065 ng/L.

At 56 dpf most of the PAHs had weathered and the concentrations were slowly stabilising at a low level, but the concentrations H and M were still slightly higher compared to L and C for the 3 °C temperature regime (4.2 ng/L; 1.259 ng/L; 1 ng/L; and 0.065 ng/L, order from H to C).

For the 3 °C regime the aqueous concentration of tPAHs for H was almost twice the initial concentration compared with H in the 0 °C regime. From 0 to 4 dpf, the tPAHs declines with 60 %. The initial concentration for M is 65 ng/L lower compared with the 0 °C temperature regime; with an initial concentration of 15.2 ng/L for 3 °C and 80.7 ng/L for 0 °C. Furthermore, the tPAHs concentrations stabilised faster at a low level for the 3 °C regime.

In general, what's important to deduct from **Figure 8**, is the initial values and how they weather over time. For M and L there is an increase in concentrations at T18 and T4. The increases seen in M (0 °C at T18) and L (3 °C at T4) are most likely because of the uncertainty with measurements.

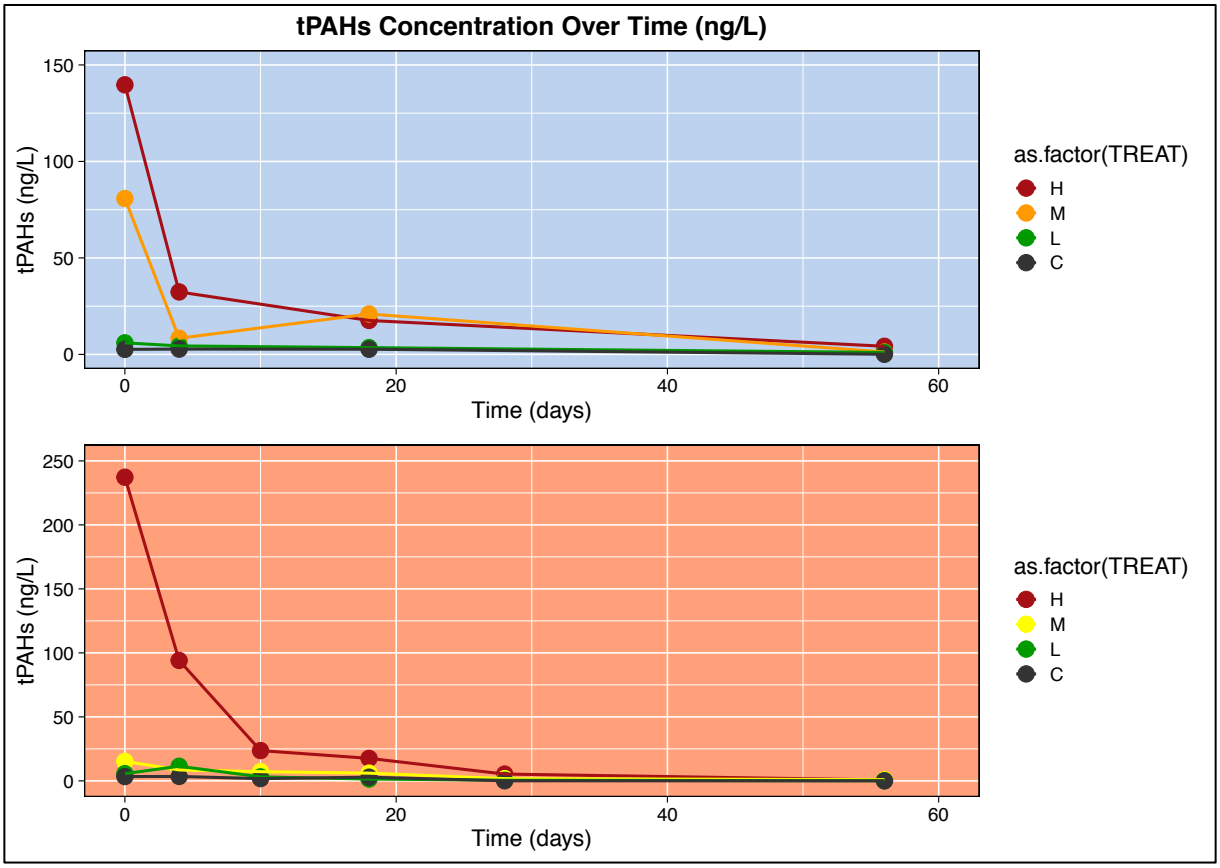

Figure 8. *Total concentration of polycyclic aromatic hydrocarbons (tPAH) in water samples (ng/L) for all treatment groups (H, M, L and C) and temperature regimes (0 and 3 ℃) The figure shows how concentrations of tPAH decreases over time in the crude oil WSF generated from the oiled rock columns.*

Over time, the composition of PAHs also changed (Figure 9.). Figure 9. exemplifies the proportions of tPAHs for H in the 3 °C regime at 0, 4 and 18 dpf. The smaller more soluble naphthalene (NAPs) represents 57.7 % of dissolved PAHs at the initiation of the experiment. This proportion decreases to 49.9 % at 18 dpf. The proportions of other PAHs changes in repsect to this; and the 2-3 ringed PAHs represents the larger portion of this with an increase from 36.8 % to 42.8 % (**Figure 9**.). In general, the more soluble PAHs are released from the system more rapidly, while larger more lipophilic compounds will increase in proportion at a later time, when the most soluble ones are washed out. However, the tPAHs concentrations declines over time regardless of the proportions and solubility of different PAHs (**Figure 8**.)

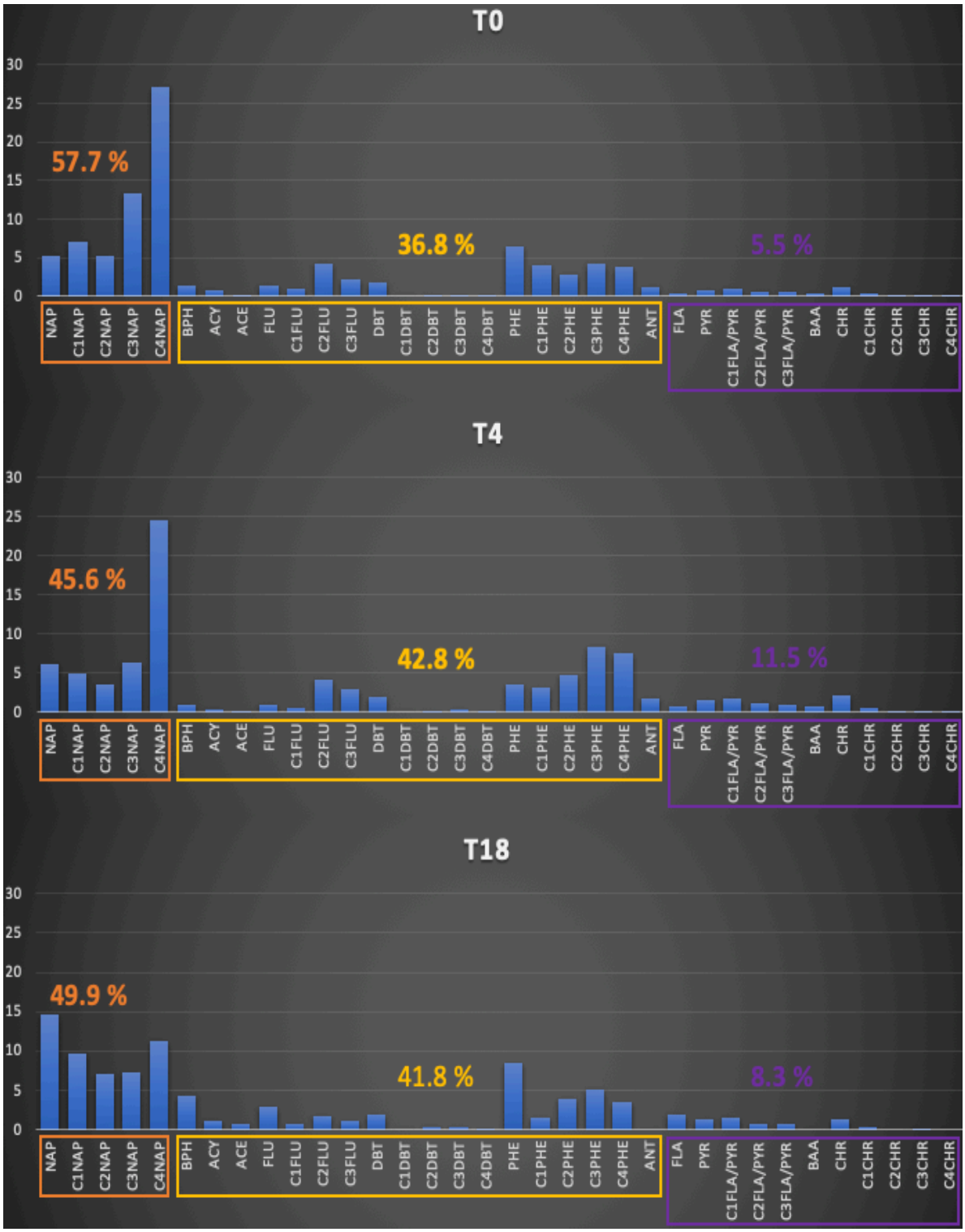

Figure 9. *Proportions of measured PAHs at different time points for H in the 3 °C regime. T0: initiation of experiment; T4: 4 dpf. and T18: 18 dpf. The orange box represents the naphthalene's, the yellow box represents 2-3 ringed PAHs and the purple box represents 4 ringed PAHs. All individual proportions are given with the blue bars, in addition the percentage of each group of compounds is given above the boxes in the same color.*

3.1.2 tPAH Concentration in Embryos – Body Burden

The total concentrations of PAHs in the eggs ranged from 31 to over 1100 ng/g wet weight (**Figure 10**.). The highest concentration measured was 1116.54 ng/g (ww.) in H (3 °C) at 4 dpf and the lowest was 31.45 ng/g (ww.) in C (0 °C) at 18 dpf. In general, embryonic concentrations of tPAH declines between the time increments. This is true for all treatments, except for L (0 °C) and H (0 °C) were there was a slight increase between 4 and 18 dpf (**Figure 10**.). However, this may be because of uncertainties with measurements.

The concentrations found in eggs was a lot higher compared to surrounding water; i.e. the embryos accumulate PAHs from the surrounding waters. 1000 ng/g wet weight is comparable to 1000 µg/L, which is over 4000 times higher compared to water concentrations. This is not a bioaccumulation factor but illustrates that concentrations in the eggs compared to the surrounding waters are clearly different. Thus, we see that eggs to a large extent accumulate WSF from Crude oil.

Over-all the 3 ringed PAHs (e.g. fluorene, phenanthrene, anthracene, dibenzothiophene) are the PAHs that accumulated most. At 4 dpf 57 % of PAHs measured in the eggs were 2-3 ringed. It is slightly lower at 18 dpf with 55 % for 0 °C embryos and 49 % for 3 °C embryos.

The lower molecular weighted naphthalene's have fairly stable proportions between 4-18 dpf for 0 °C embryos; with a slight decrease in the proportions from 33 % to 32 % 18 dpf. Naphthalene's went from 28 % to 44 % between the time increments for 3 °C embryos.

The larger PAHs have the lowest concentrations in the embryos. With a slight increase in the proportions for 0 °C embryos (8 % to 12 %) and a slight decrease for 0 °C embryos (13 % - 5 %).

Percentage described are not actual values of tPAHs in embryos, but the proportions of the PAHs bioaccumulated. Proportions are derived from treatments that actually were contaminated with crude oil i.e. L, M and H treatments.

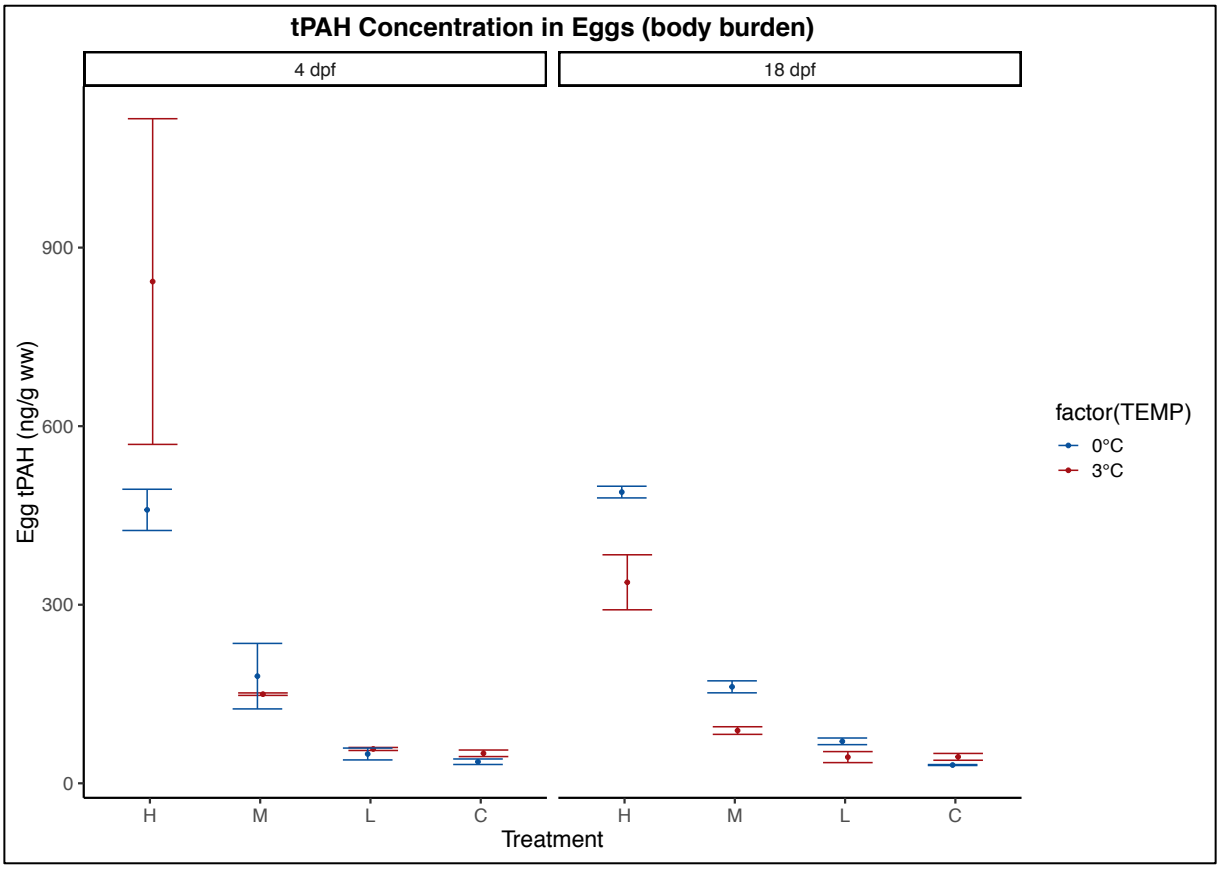

Figure 10. *Total concentration of polycyclic aromatic hydrocarbons (tPAH) in embryos (ng/g ww.) for all treatment groups (H, M, L and C) and temperature regimes (0 ℃ and 3 ℃). The figure shows concentrations at two specific time increments; 4 dpf and 18 dpf.*

3.2 Mortality

Mortality in the experiment was high, which is reflected in **Figure 11**. By the last measurements done in regard to cardiotoxicity; almost every larvae from H in the 0 °C regime had perished, and all larvae from H in the 3 °C regime were deceased. C, L and M treatments are fairly similar over the 80 days period and follows specific events related to the development of ELS of marine fish.

For both temperature regimes we saw an increase in mortality after fertilization. As time passes mortality stabilized around 25 % at 10 dpf. A similar increase in mortality is seen 30 dpf for the three-degree-embryos and and 40 dpf for zero-degree embryos, at the onset of hatching. From hatching we had a steady increase in mortality over time. When start feeding commenced, at approximately 60 dpf for the three-degree larvae; mortality once again increases. Mortality related to start-feeding were not seen for zero-degree larvae since they were not developed that far yet. Zero-degree larvae had started feeding, but there was a delay between start-feeding and observed mortality because larvae still have remnants of their yolk-sac when they start feeding – i.e. for surviving the transition between yolk dependence and feeding for survival.

For showing dose-dependent differences in mortality, 3 time points in the

accumulative mortality curves were chosen. The time points for checking differences in mortality were chosen based on the developmental stages polar cod was at depending on the incubation temperature. 20 dpf was for both regimes during organogenesis (3 °C a bit more developed at this times point compared to 0 °C embryos); 29-47 dpf was after the initiation of hatching and when measurements for cardiotoxicity were done; 50-70 dpf were the initiation the preflexion (first feeding and red blood cells was observed). H treatment showed a significantly higher mortality compared to other treatments (ANOVA; $p>0.0001$).

The stages in embryonic development for polar cod is similar to Atlantic cod (*Gadus morhua*), which is thoroughly reviewed by Hall, Smith, and Johnston (2004). Stages observed in polar cod in this regard was; i: formation of blastodisc; ii: cleavage stages; iii: epiboly; iv: different somite stages and organogenesis (including first observed heart beats at 22 dpf for 3 °C and 33 dpf. for 0 °C); v: hatching further organogenesis, formation of blood cells and flexion stages. This is mentioned because different stages in mortality are related to natural mortality seen in ELS marine fish. Fertilization, hatching and start-feeding are three points of crucial interest in this regard and mortality related to these events are shown in **Figure 11** (i.e. for the 3 °C regime mortality increase at fertilization (T0), at hatching (T25-T33) and at initiation of feeding (around T50)).

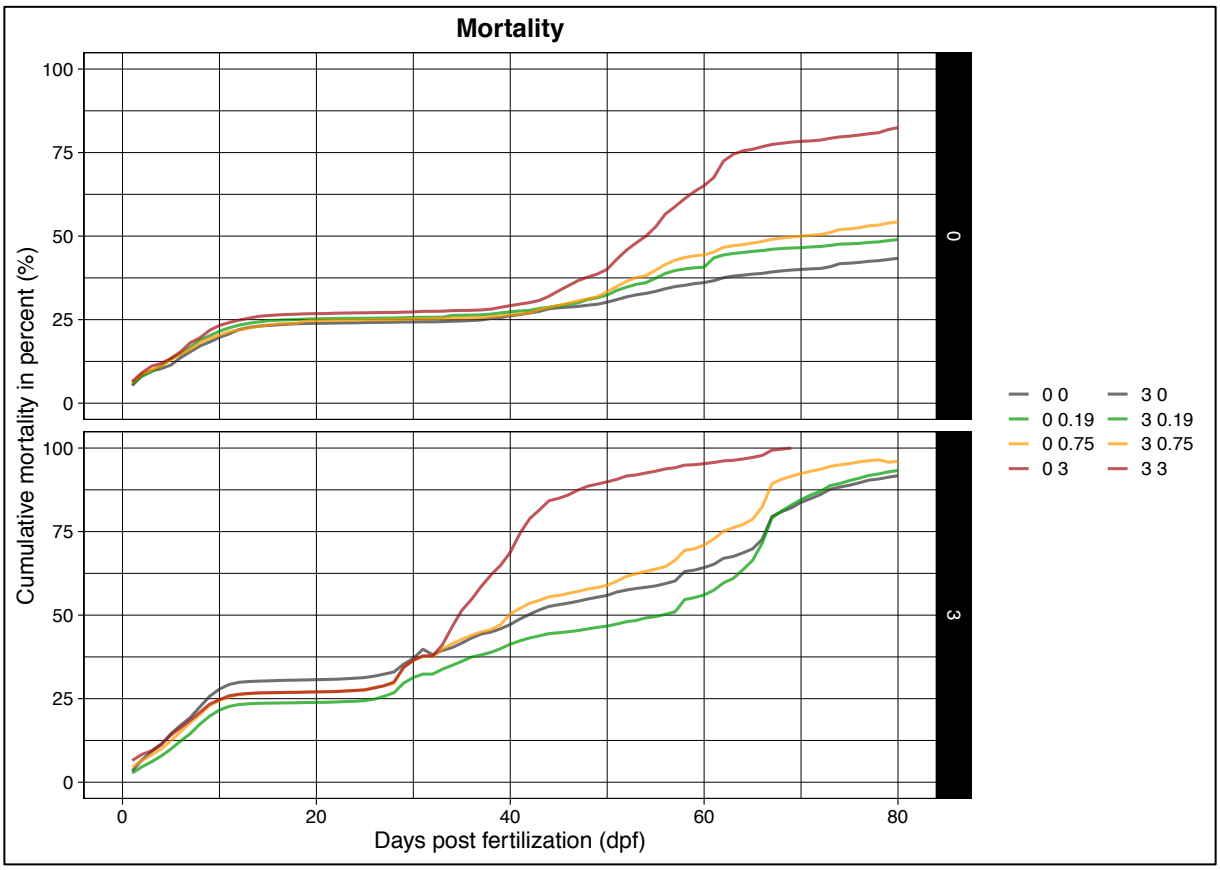

Figure 11. *Accumulated mortality from start to 80 dpf. for both temperature regimes and crude oil treatments. Facets in the figure separates temperature regimes (0 ℃ top, 3 ℃ bottom). Red line = H, yellow = M, Green = L and Grey = C (With true values for crude oil in g/kg gravel shown on the right side of the figure for each temperature regime and treatment).*

3.3 Cardiac Activity (29 and 47 dpf)

3.3.1 Heart rate

Figure 12. illustrates beats per minutes (BPM) of larvae for both temperature regimes. According to this data there is a dose dependent trend of bradycardia for all incubators and treatments (ANOVA, $p<0.003$), and temperature is not interacting with BPM ($p = 0.0945$). However there are differences in the trends between treatments and these results are elaborated in the next paragraphs.

For the 0 °C regime the mean BPM for C was 33.6 ± 8 BPM, L and M had heart rates indistinguishable from the control group (L: 30.5 ± 7.5 and M: 28.4 ± 8 BPM) and H had a significantly (derived from Summary of LME model; $p<0.05$) different heart rate compared to C; with a mean BPM of 26.5 ± 7.43.

However, when we look at the significance level for all different treatments there is no difference between them, but a dose dependent trend of bradycardia with large variation (0 °C). This is because we can't distinguish H from M and L with an ANOVA for the effect of treatment on BPM. Therefore, no significant difference is observed in the 0 °C regime

(ANOVA, p = 0.20).

For the three degrees temperature regime the larvae within H had a mean heart rate of 21.5 ± 5.7 (ANOVA, p<0.003) , which is distinctive from the other treatments; control, low and medium respectively (C (29.8 ± 5.2 BPM); L (29.7 ± 5.14 BPM); M (28.48 ± 4.45 BPM).

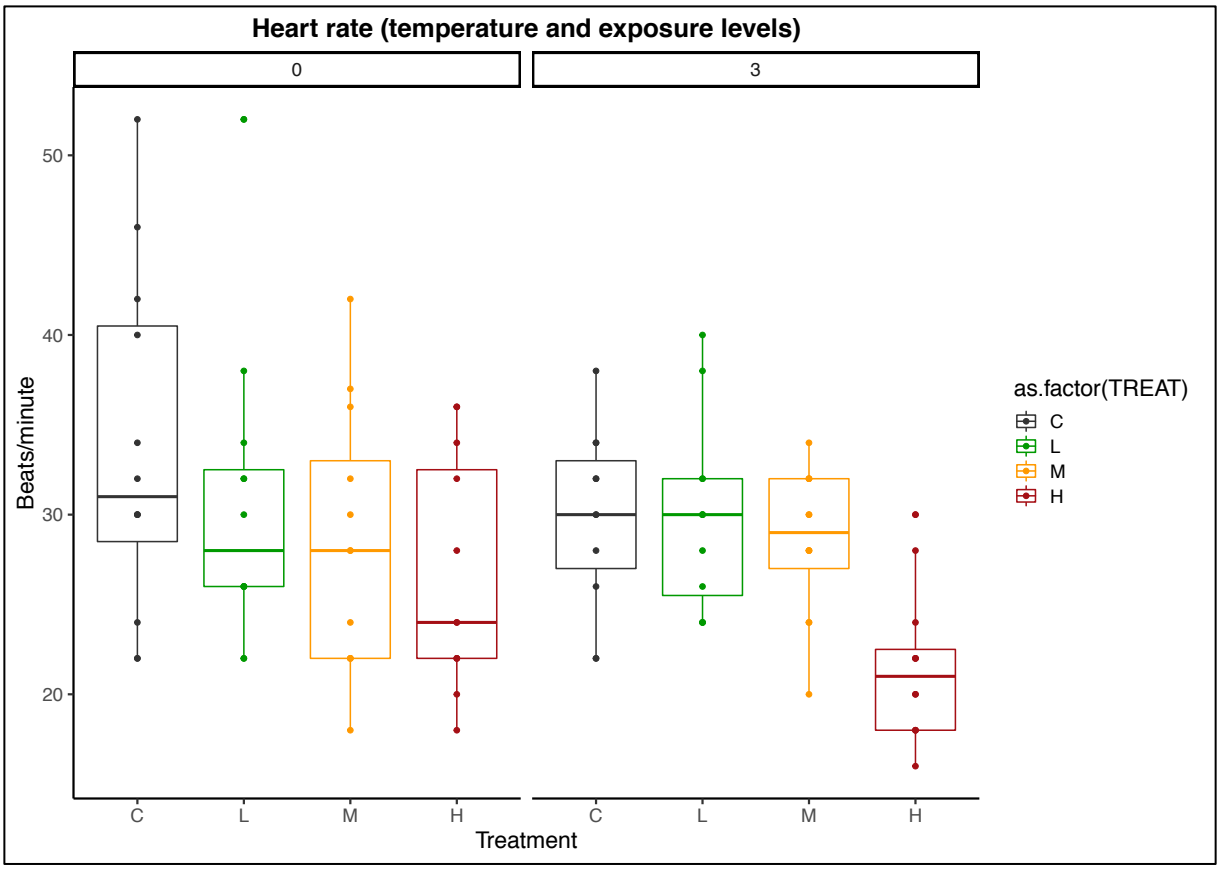

Figure 12. *Heart rate (beats/min) of larvae at hatching (29 dpf for 3 ℃ and 47 dpf for 0 ℃) for exposure levels C: 0g crude oil/kg gravel; L: 0.19 g crude oil/kg gravel; M: 0.75g crude oil/kg gravel & H: 3g crude oil/kg gravel. Facet grids divide larvae into temperature regimes with zero-degree larvae in the first box and three-degree larvae in the second (indicated by titles in facets).*

3.3.2 Arrhythmia

Inter beat variability i.e. arrhythmia, has been documented as a symptom for fish embryos exposed to PAHs from crude oil (J. P. Incardona et al., 2009). For both temperature regimes arrhythmia is similar for C, L and M; with H showing a significantly higher inter beat variability (p>0,0005, ANOVA) regardless of temperature (**Figure 13**.).

The 3 °C H larvae had a mean inter beat variation of 0.26s ± 0.30s. There was an outlier in the data for H, and it was due to the heart stopping for several seconds. However, the outlier didn't affect the results and therefore is part of **Figure 13**. When we compare the other treatment groups in this temperature (3 °C) regime with H, we have less variation, and a steadier beat (C: 0.07s ± 0.023s; L: 0.0815s ± 0.049s; M: 0.0737s ± 0.032s), see **Figure 13**.

The 0 °C larvae show the same trend. The H treatment had a mean inter beat variation

of 0.165s ± 0.042s, which is different from the other treatment groups (C: 0.088s ± 0.025s; L: 0.076s ± 0.022s; M: 0.077s ± 0.024s), also shown in **Figure 13**.

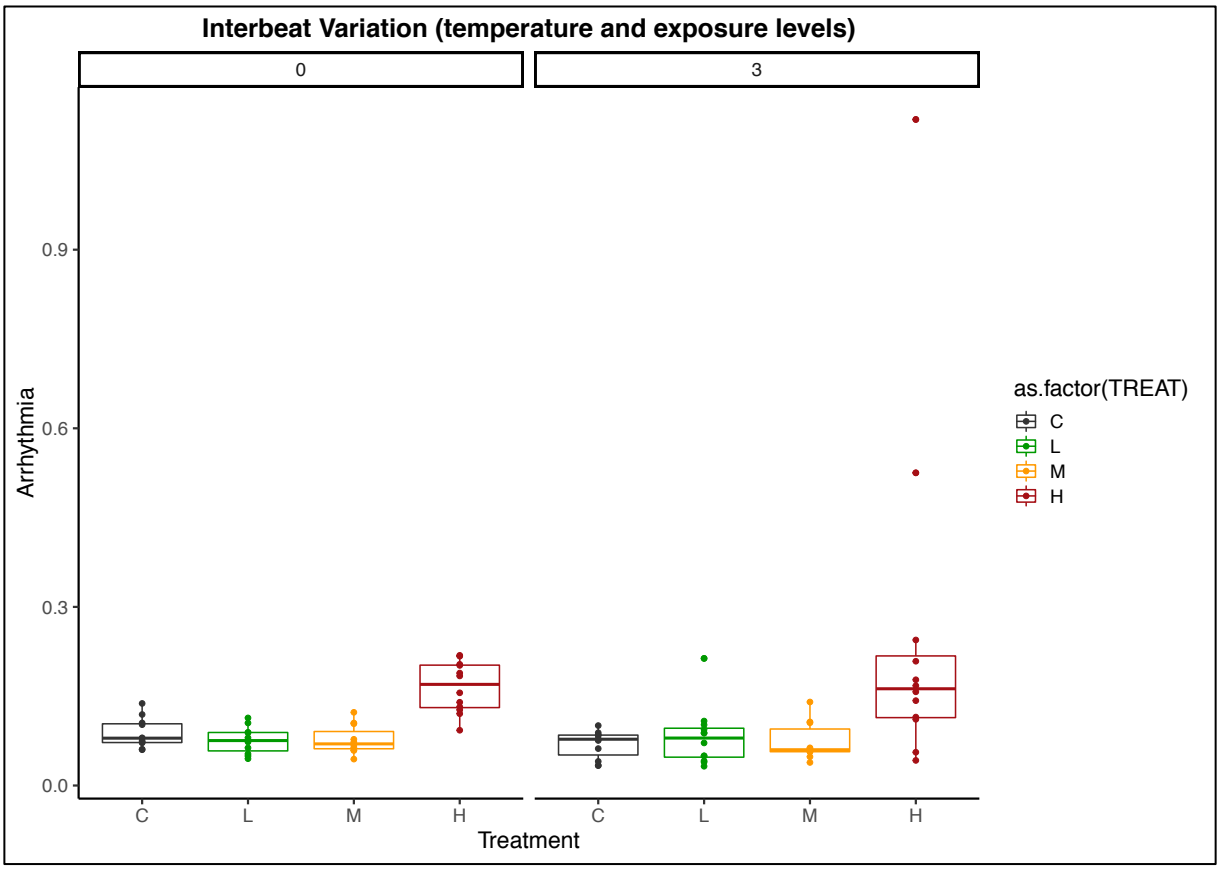

Figure 13. *Interbeat variation (in seconds) for larvae at hatching (29 dpf for 3 ℃ and 47 dpf for 0 ℃) for exposure levels C: 0g crude oil/kg gravel; L: 0.19 g crude oil/kg gravel; M: 0.75g crude oil/kg gravel & H: 3g crude oil/kg gravel. Facet grids divide larvae into temperature regimes with zero-degree larvae in the first box and three-degree larvae in the second (indicated by titles in facets).*

3.3.3 Pericardial Edema

Pericardial edema had a dose-dependent increase from C to H (ANOVA, $p < 0.01$) for the whole data set. The trend of increasing edema was more pronounced for the 0 °C regime. This is clearly seen in **Figure 14**, but we also see a similar trend for the 3 °C larvae.

Mean percentage of pericardial edema (defined in methods, section 2.8) for 0 °C were at 7.34 % ± 1.4 % for C with a steady increase of 2 % ± 1.97 % (L); 5.7 % ± 1.97 % (M) and 8.34 % ± 1.97 % (H), ending on 15.7 % ± 1.97 % for H (**Figure 14**.), which makes H and M different in this temperature regime ($p<0.05$ for M and $p< 0.01$ for H (summary of LME-model)).

For 3 °C larvae there is a trend of increasing pericardial edema with increased exposure levels, however for this temperature regime there is not a significant difference. The mean percentage of pericardial edem for 3 °C was 8.47 % ± 1.4 % for the control group with a steady increase of 0.15 % ± 1.92 % (L); 0.81 % ± 1.92 % (M) and 3.07 % ± 1.96 % (H) from the control, ending on 11.54 % ± 1.96 % for H (**Figure 14**.).

In addition to look at differences between treatment groups the interactions between temperature and treatments were also investigated; no interaction of the kind was found. There were no significant difference between the temperature regimes in regards to edema ($p > 0.05$). However, differences between temperature regimes in regard to pericardial edema was close to significant (ANOVA, p-value of 0.059).

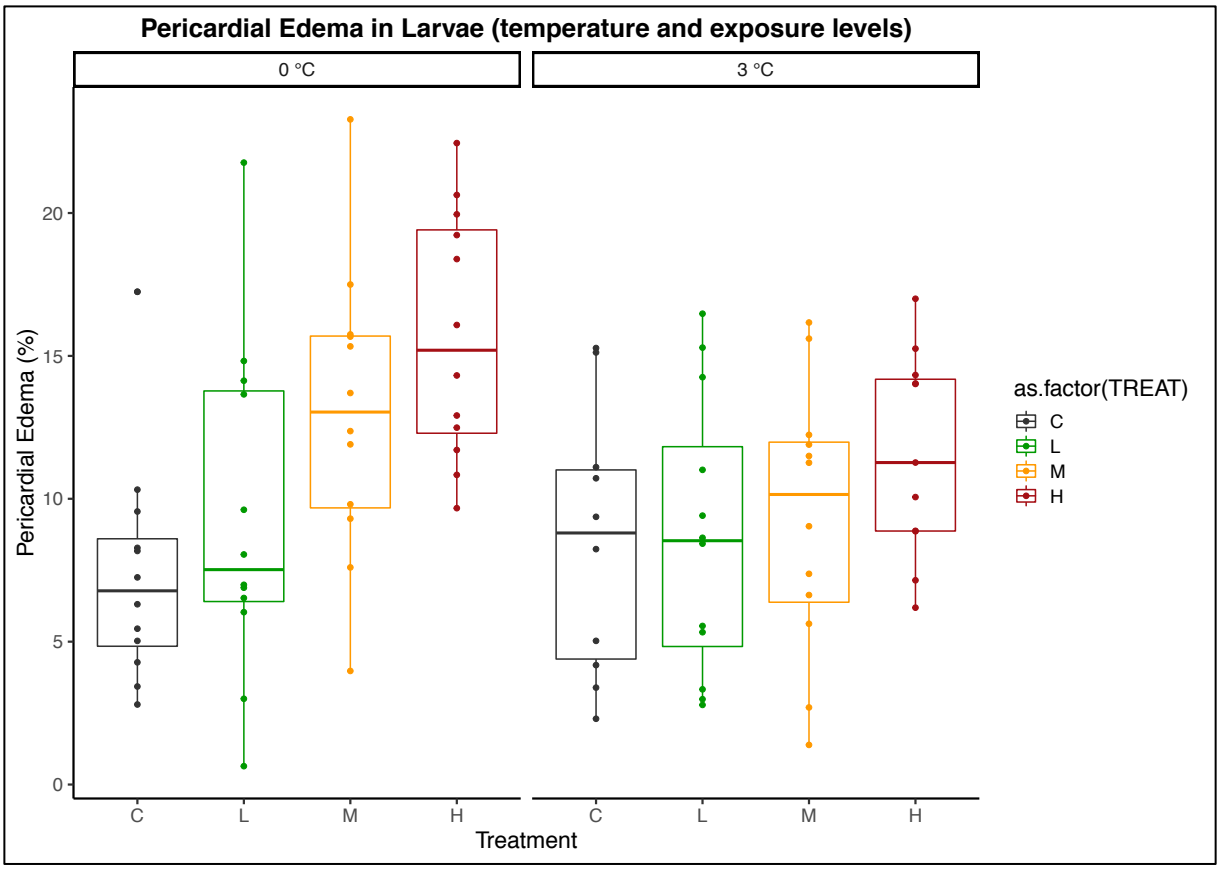

Figure 14. *Pericardial edema (percent) in larvae at hatching (29 dpf for 3 ℃ and 47 dpf for 0 ℃) for exposure levels C: 0g crude oil/kg gravel; L: 0,19 g crude oil/kg gravel; M: 0,75g crude oil/kg gravel & H: 3g crude oil/kg gravel. Facet grids divide larvae into temperature regimes with zero-degree larvae in the first box and three-degree larvae in the second (indicated by titles in facets). Pericardial edema was calculated as a ratio between the amount of space/vascular assemblage around the heart and the whole yolk-sac area (lateral view, see materials and methods).*

3.4 Spinal and Jaw Malformation

For jaw malformations there is a clear dose-dependent effect (ANOVA, p<0.0001) and 100 % of the larvae exposed to H had deformed jaws. Deformities in jaws are shown in **Figure 7**.

Spinal malformation didn't show the same degree of effect in exposed larvae and the treatments were statically indistinguishable (p=0.0574, ANOVA). This is statically close to an observed difference and M has a larger difference from the intercept 40.9 ± 9.4. This is 23.5 % higher ratio compared with C. Furthermore, H differs from control with 17.6 with a mean of 34.9 ± 9.4 % (**Figure 15**.).

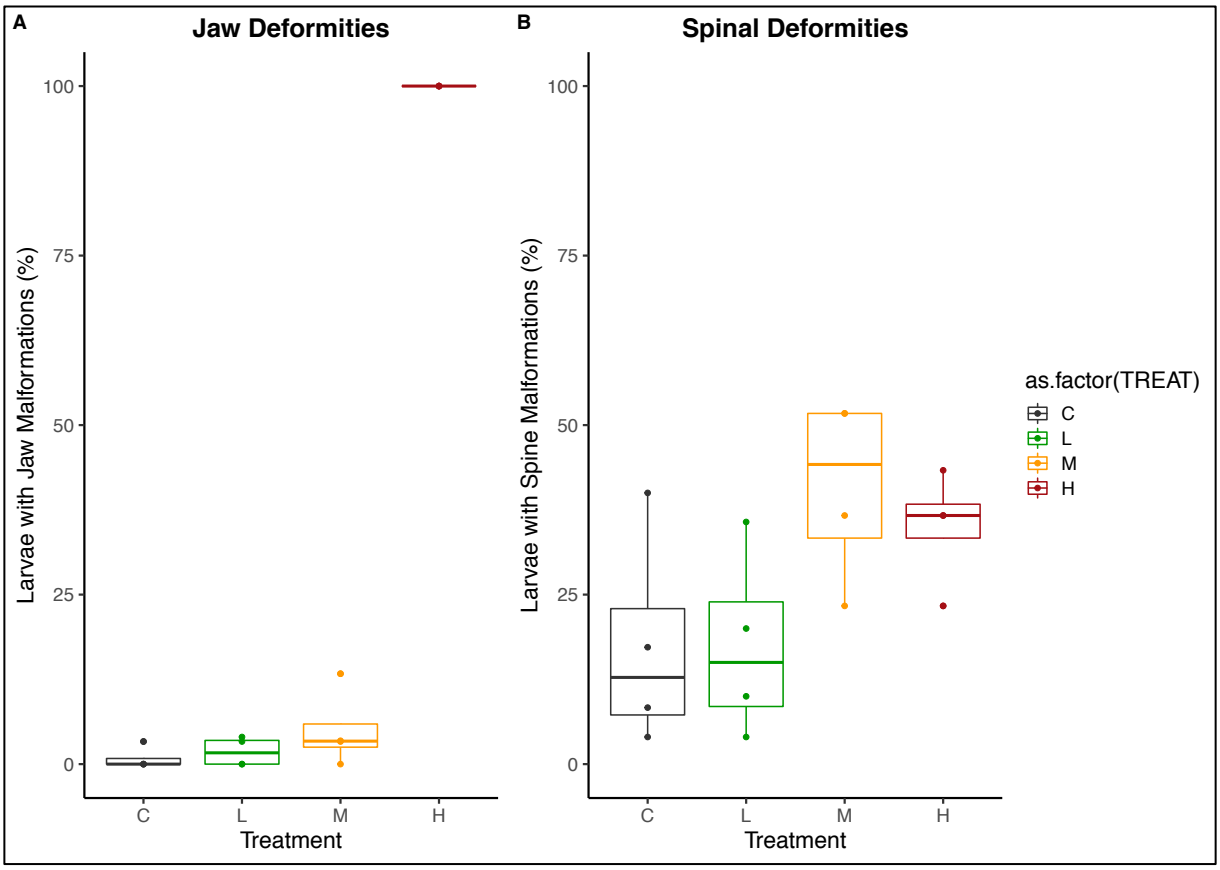

Figure 15. *Frequency of malformations in jaws and spines at T76 in the 0 ℃ temperature regime. There is a clear prevalence of dose-dependent jaw malformations in H. Spine malformations may show a trend of deformities towards higher treatments, but not clear.*

3.5 Cardiac Activity (deformed vs not-deformed; same treatment)

4.5.1 Heart rate (Deformed vs not-deformed; same treatment)

Heart rate between M treatment and C were not different, with mean heart rates of 45.2 ± 1.97 (C) and 43.2 ± 2.7 (M) BPM. For the highly deformed larvae (D) a difference in heart rate was found ($p < 0.01$) compared to C and M. Mean heart rate for D was 35.8 ± 2.7 BPM, see **Figure 16**.

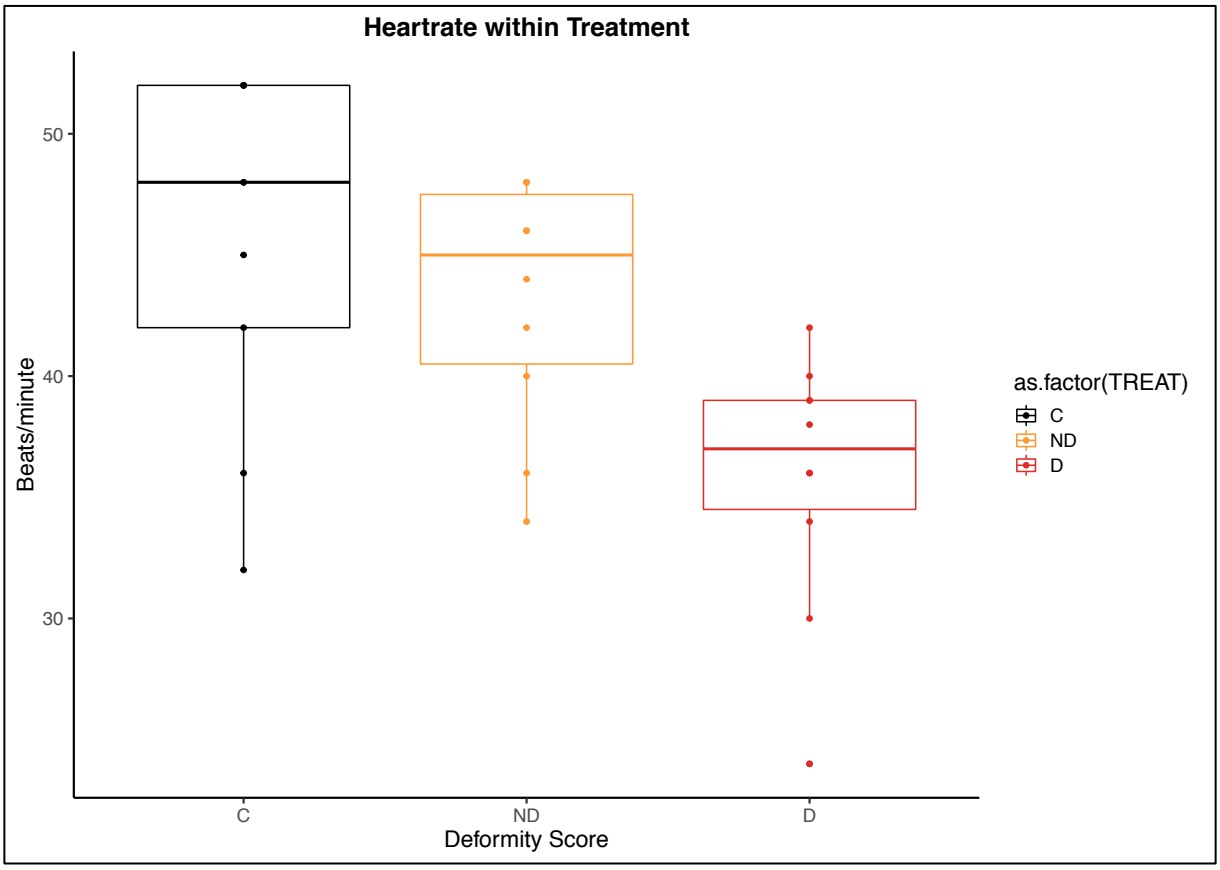

Figure 16. *Heart rate (beats/min) of larvae from C and M. Deformity score is not-deformed and deformed jaws (ND and D) and control is not deformed larvae from C. Deformed larvae have a significantly ($p<0.05$) lower heart rate compared to not deformed and control.*

3.5.2 Arrhythmia (Deformed vs not deformed; same treatment)

Arrhythmia between C, ND and D were indistinguishable. There was a slight trend of increase in arrhythmia from C to DF, with mean inter beat variations of C: 0.0511 ± 0.0082 s; ND: 0.575 ± 0.117 s and D: 0.0679 ± 0.0113 s (**Figure 17**.). The increase is very small, but in all of tests for cardiac activity we see the same trend of slow heart rates and more arrhythmia; thus, it might be of importance and will be discussed.

For both heart rate and arrhythmia in regard to section 4.4.1 and 4.4.2, the number of specimens were lower than previously desired. This was due to complications during recordings with larvae moving too much at this stage of development.

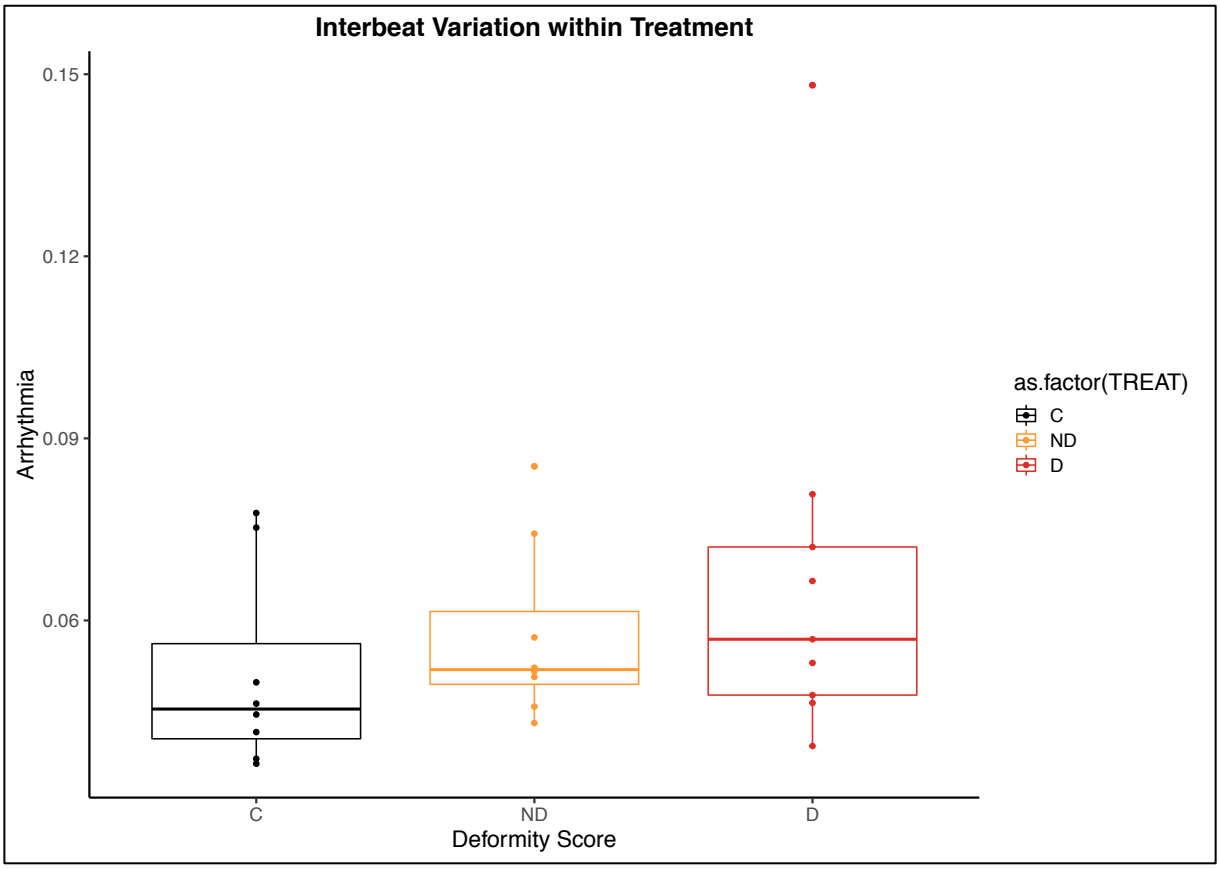

Figure 17. *Interbeat variation (in seconds) for control (C), not-deformed (ND) and deformed larvae (D).*

4 Discussion

4.1 Chemistry in Water and Embryos

The experiment conducted and described with WSF of crude oil had implications for cardiac activity and malformations in ELS of the Arctic species polar cod. Embryos and larvae were exposed to environmentally realistic concentrations of PAHs from crude oil. The highest value of tPAHs measured in the experiment were at 237 ng/L; i.e. a small fraction compared to water concentrations of what was measured after e.g. Exon Valdez oil spill or other comparable experiments (Hodson, 2017).

We saw a dose dependent relation between the concentrations and the response of embryos and larvae for bradycardia, arrhythmia, pericardial edema and jaw malformations. Based on exposure levels reported from other studies with EC50s ranging between 0.3 to 2500 μg/L this study indicates that polar cod is very sensitive to exposure from crude oil (Hodson, 2017; Nahrgang et al., 2016).

Cause-effect relationships are difficult to assess in crude oil WSF studies WSF studies since crude oil houses thousands of different chemicals. Moreover, many of the congeners of the PAH in crude oil are unknown, which further complicates the evaluation of mixture toxicity (as earlier introduced). Single compound studies have shown that specific compounds like pyrene, phenanthrene, fluorene and dibenzothiophene cause severe effects, both morphologically and physiologically (J. P. Incardona et al., 2005; J. P. Incardona et al., 2004) – effects similar to the present study. Although nominal water concentrations of specific PAHs in these single compound studies mentioned above were at/or above their solubility limit, i.e. several orders of magnitude above concentrations measured of these PAHs within the crude oil WSF.

From observed data it is certain that the embryos in the present study were bioaccumulating PAHs from the surrounding waters. The embryonic concentrations were several times higher in the eggs compared to the water masses, and they had a higher proportion of the more lipophilic PAHs compared to the water (e.g. phenanthrene, dibenzothiophene and anthracene), suggesting a higher bioaccumulation rate for more lipophilic PAHs. Embryos in H had bioaccumulated concentrations between roughly 500-1100 ng tPAHS per g wet weight. These bioaccumulated concentrations of tPAHs were similar to the lower embryonic concentrations observed on pacific herring by J. P. Incardona et al. (2009) where cardiotoxic effects were documented. Thus, the sensitivity of polar cod embryos to crude oil WSF seemed to be similar to what has been documented for pacific herring in relation to cardiotoxic effects.

4.2 Effects of Crude Oil WSF on Cardiac Activity and Malformations

This is the first study to document dose-dependent effects on cardiac activity – i.e. arrhythmia and heart rate in polar cod larvae. There was a steady decline in heart rate and a steady increase of arrhythmia in all larvae measured with increasing exposure to the crude oil WSF. However, with some differences between temperature regimes in relation to level of

significance. The the dose-dependent effect were expected and previously well documented in the literature for different fish species, but not polar cod (J. P. Incardona et al., 2009; J. P. Incardona et al., 2015; J. P. Incardona et al., 2014; Nahrgang et al., 2016).

The heart is believed to be a specific target for toxic effects related to PAHs and symptoms observed in this study have been well-documented with single compound studies (J. P. Incardona et al., 2005; J. P. Incardona et al., 2004; Zhang et al., 2013). The toxicological effects observed in single compound studies are credited especially to the tricyclic PAHs, which are found to bioaccumulated in the present study as well. The tricyclic PAHs most likely plays a role in the toxicity observed, but it can't be concluded as the only cause of cardiotoxicity, since this has not been documented at environmentally realistic doses in single compound studies. Single compound studies have been used as screening tools to understand mechanisms at very high doses of single PAHs. The studies do not fully reflect the complex mixture toxicity from the WSF of crude oil where effects have been observed down to 0.3 μg/L (Hodson, 2017; J. Incardona, 2017). Furthermore, the PAHs are not the only chemicals that are present in crude oil and with thousands of different isomers and congeners we cannot be sure if interplay between chemicals or other constituents not measured is toxic for the larvae in the present study (Hodson, 2017).

The malformations assessed in the current study (absence/presence of jaw and spinal curvature and pericardial edema) also showed dose-dependent increase in occurrence of deformities and area of pericardial edema. These malformations are believed to be closely related to cardiotoxicity (J. P. Incardona et al., 2004). Hicken et al. (2011) documented change in the morphology of the heart at low concentrations of tPAH from the WSF. Cardiac failure during the early developmental stages may explain why fluid accumulates around the heart due to a change in ion-balance and thus affecting the osmotic pressure in surrounding tissue (J. Incardona, 2017). A failing heart and fluid accumulation may also cause a nutritional deficit to peripheral areas in the embryo since the form and function of the heart during development of early life stages is important for normal development (Glickman & Yelon, 2002). However, malformations are also thought to be caused by downregulation of specific genes that plays a role in development of skeletal features (J. Incardona, 2017).

The trends observed in this study of malformations correlating with observed cardiotoxic effects (bradycardia and arrhythmia) supports the hypothesis that cardiotoxicity can be an initial vector for malformations. For larvae measured just post hatching this correlation was clear for pericardial edema. At T76 (assed from growth pictures from the 0 °C regime) there is also a clear effect of jaw malformations (100 % of larvae from H had deformed jaws) and a trend of spinal curvature, and this correlated with measurements for cardiac activity measured at T79, which were the natural comparison to malformations in jaws and spine.

The cardiotoxic measurements at T79 were done to specifically see if malformations were linked to cardiotoxicity. Highly malformed larvae did show a lower heart rate compared to normal larvae from the same treatment and control; but for arrhythmia the trend wasn't as transparent (seen in **Figure 17**.). Several single compound studies and WSF-studies have documented malformations in relation to toxicity from PAHs (Carls et al., 2008; Carls et al., 1999; Hicken et al., 2011; Jung et al., 2013; Nahrgang et al., 2016). Spinal curvature is often described as one of the main symptoms of malformations in ELS of fish, and J. P. Incardona

and Scholz (2016) generally described jaw malformations as a secondary feature of malformation. However, this study showed that this wasn't necessarily true – at least not for polar cod, where the prevalence of jaw malformation is much more dominating compared to spinal curvature. However, this was late in the experiment and maybe the larvae with highly deformed spines were deceased.

The observed dose-dependent effects for malformations and cardiac activity is thus affecting ELS of polar cod on the organismal level and the three first hypothesizes in the objectives section 1.6, were confirmed in this thesis. Environmentally realistic doses of PAHs from the WSF of crude oil were cardiotoxic and malformations are most likely a secondary symptom initiated by cardiac disruption.

4.3 Effects of Temperature

In general the present study can't document clear differences regarding temperature. None of the measured variables reveal that temperature is affecting toxicity in any manner. BPM is also very low compared to similar studies with polar cod (Nahrgang et al., 2016). Therefore, the observed variation regarding BPM can be a conduct of the calibration of the cooling stage. Maybe the observed low heart rate was because the cooling stage was too effective and therefore lowered the presumably normal heart rate of the larvae. The platform was manually calibrated by measuring 500 µL of water with a thermometer. Getting accurate and preferred temperatures in a small watchglass with a thermometer that barely breached the surface of 500 µL of water was difficult, but appropriate temperatures were presumably acquired. Although the method can be questioned due to this, it is certain that all larvae were handled in the same manner and thus observed differences in BPM reflects a dose-dependent relationship for toxicological effects.

Furthermore, there is also no clear trend regarding temperature. The heart rate for the 3 °C regime is lower compared to the 0 °C regime; and for pericardial edema the 0 °C regime seems to have a larger observed pericardial edema. Temperature were not affecting arrhythmia either, but maybe the temperature increase in the present study wasn't sufficient enough to add additional physiological stress to the embryos. Kunz et al. (2016) revealed that the upper temperature tolerance limit for mature polar cod was at 8 °C, optimal feed efficiency were at 0 °C and optimum growth performance were at 6 °C. This shows that mature polar cod at least have some tolerance to temperatures between 0-8 °C. However, polar cod eggs do not choose their preferred temperature in the environment. The eggs are also closely related to timing of the spring bloom in the Arctic ecosystems and thus eggs are adapted to hatch when food is available (Ponomarenko, 2000).

The only trend that was observed regarding temperature is a higher mortality for the 3 °C regime. This study were not specifically investigating mortality, but recognize that even if temperature wasn't interacting with dose-dependent toxicity in an adverse way all the larvae in the experiment were dead for the 3 °C temperature regime at T79, which is a strong indicator that larvae from the 3 °C regime may have had some underlying physiological stressors. Further research is needed to understand if a higher temperature may affect toxicological response in polar cod.

This shows that the last hypothesis in section 1.6 can't be disproven by this study, but

either can it provide evidence that an additional stressor (i.e. temperature) increases toxicological potential for ELS of polar cod.

5 Conclusion

The observed effects on the organismal level in the present study show that polar cod is highly sensitive to the WSF of crude oil. We can conclude that there is a dose-dependent relation to progressively increasing doses from the WSF of crude oil.

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