Effects of road runoff on tadpoles of the common frog (*Rana temporaria*)

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Master thesis in toxicology

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Statens vegvesen

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Last, but certainly not least, I want to give a round of applause to the 1200 tadpoles I managed to unintentionally kill. I'm terribly sorry.

Abstract

Road contamination is an issue in and around cities worldwide, with concerns for human and environmental exposure. Mitigation actions are implemented along highways to reduce the quantity of contaminants entering the surrounding ecosystems. A sedimentation pond is established to prevent the contaminants in road runoff from entering surrounding aquatic ecosystems. These ponds are often open areas and thus habitat to a number of organisms, including amphibians. Amphibians are regarded as an especially vulnerable group when exposed to contaminants, because of their highly permeable skin and aquatic life stages. Road runoff contains a mixture of contaminants, and organic contaminants are viewed as especially important for inducing negative effects in organisms. Polycyclic aromatic hydrocarbons are abundant in road runoff and sedimentation ponds, with sources from oil and the wear of cars and roads.

The present study aimed to investigate the effects of organic contaminants on early life stages of the common frog (*Rana temporaria*) living in sedimentation ponds. To assess this aim sampling of tadpoles was performed in three different ponds, one sedimentation pond receiving road runoff, one sedimentation pond receiving tunnel wash water and road runoff and one naturally occurring pond with no known source of local contaminants. In sediment samples from the sedimentation ponds the concentration of several contaminants were classified with "poor quality", which may cause toxic effects in aquatic organisms. Analysis of the tadpole tissues provided information about several parameters. Tadpoles from sedimentation ponds were generally at a later stage and larger in size compared to tadpoles from the naturally occurring pond. Contaminants present in the ponds may increase the growth rate of the tadpoles. Concentrations of PAH metabolites were higher in the tadpoles from sedimentation ponds, indicating that the tadpoles do metabolize PAHs and that PAHs are present for uptake in the water-column and/or through diet. There was an induction of CYP1A in the tadpoles from sedimentation ponds compared to tadpoles in the naturally occurring pond, and a higher level of induction at later sampling times indicating increasing biotransformation at later stages in tadpole development. There was an increase in DNA damage in tadpoles living in sedimentation ponds, especially ponds that receive both tunnel wash water and road runoff. This indicates that tunnel wash has an additional genotoxic effect to tadpoles (in addition to road runoff).

The overall result suggests that tadpoles living in sedimentation ponds are affected negatively by the contaminants from road runoff. With regard to the mitigation purpose the ponds have, this study points at the importance of including effects on organisms when deciding on the mitigation action during construction of roads and tunnels, as well as the importance of filter mechanisms in already existing ponds.

Abbreviations

AADT	Annual average daily traffic
Abs	Absorbance
B[a]P	Benzo[a]pyrene
CYP	Cytochrome P450
CYP1A	Cytochrome P450 1A
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
em	Emission
EQS	Environmental quality standards
EROD	7-ethoxyresorufin *O*-deethylase
ex	Excitation
GC	Gas chromatography
HPLC	High performance liquid chromatography
masl	Meter above sea level
MS	Mass spectrometry
NIVA	Norwegian institute of water research
NPRA	Norwegian Public Roads Administration
NVE	Norwegian Water Resources and Energy Directorate
PAH	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
ROS	Reactive oxygen species
SUDS	Sustainable urban drainage systems
UiO	University of Oslo
WSP	Wet sedimentation pond

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1 Introduction

Road pollution has a large environmental impact on nearby ecosystems due to the mixture of contaminants released by road-related activities. The amount of contaminants derived from roads is correlated with a continuous increase in traffic especially evident in and around cities (Statens Vegvesen, 2017b). Traffic is an essential part of modern society and the increase in traffic in some areas appears inevitable. Such increases do however increase the environmental exposure to road-associated contaminants. The contaminants from roads can be airborne, such as exhaust, or it may stay at the surface and be washed away with runoff water. The runoff contains high levels of contaminants that poses a threat to the surrounding waterbodies (Meland, 2010; Åstebøl et al., 2011). According to the Water Framework Directive, implemented in the Norwegian law in 2007, all water bodies should have an acceptable chemical and ecological quality by 2021 (European Parliament, 2008). For surface waters this means that the level of pollutants should follow the environmental quality standards (EQS) set by the Norwegian Environment Agency (2016). The waters should have a healthy ecosystem including availability of nutrients, biodiversity and an abundance of species. This emphasizes the importance of mitigating pollution from sources such as highway runoff to ensure better quality of the water entering aquatic systems. When new roads are built, the Norwegian Public Roads Administration (NPRA) performs an environmental risk assessment to assess whether there should be measures implemented to filter the runoff. This assessment is based upon the amount of annual average daily traffic (AADT) and the vulnerability of the recipient (Meland, 2016) and if measures are needed, there is a requirement for a discharge permit from the county administration (Statens Vegvesen, 2017a). A measure often implemented in Norway is the construction of Sustainable Urban Drainage Systems (SUDS). These systems reduce the risk of contamination of environments in close proximity to roads and treat road runoff by sedimentation of particle bound contaminants as well as breakdown and dilution of the contaminants. One such drainage system is a wet sedimentation pond (WSP), which is found around roads with a high vehicle density, such as European route E6 in the southern part of Norway.

The primary function of WSP is to manage the quantity and quality of water from roads. The ponds retain contaminants bound to particles and dilutes the concentration of water-soluble contaminants in runoff (Bækken 2005). The ponds are therefore exposed to a range of toxic

contaminants including organic contaminants as polycyclic aromatic hydrocarbons (PAHs), trace metals as Cu, Zn and Pb, as well as NaCl from de-icing of the roads (Marsalek, 1997; Barbosa & Hvitved-Jacobsen, 1999; Meland, Borgstrom, et al., 2010; Ranneklev et al., 2016). The amount of contaminants in road runoff is affected by many factors, including the amount of traffic, type of traffic, speed of vehicles, kind of tires and tunnel wash events (Meland et al., 2010b; Åstebøl & Hvitved-Jacobsen, 2014).

Most sedimentation ponds in Norway are constructed with a pre-sedimentation pond and a main pond (Figure 1). Road runoff will move from the inlet into the pre-sedimentation pond where a large part of the particles, and particle-bound contaminants, will sediment. For PAHs the different molecular size of the contaminant corresponds to the grain size of particles (Krein & Schorer, 2000). This results in PAHs with high molecular weight to sediment near the inlet of the pond, while PAHs with smaller molecular weight will follow smaller grains and sediment near the outlet (Krein & Schorer, 2000; Simon & Sobieraj, 2006). The inlet and outlets are usually constructed under water to ensure proper function during winter and also works as an oil separator (Meland, 2016). A WSP can have up to 86% of an overall treatment efficiency of total PAHs (Vollertsen et al., 2007).

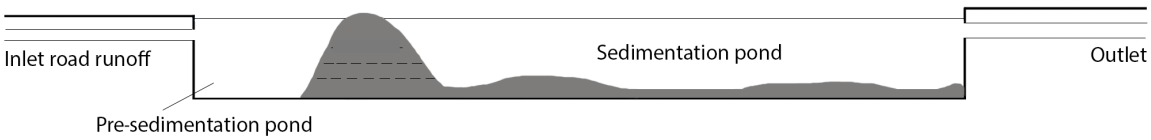

Figure 1: Schematic drawing of a WSP. The inlet of road runoff leads directly to a pre-sedimentation pond followed by the main pond and the outlet at the far end from the inlet. The inlet and outlet are submerged in water.

Contaminants found in sedimentation ponds may also derive from tunnel wash water. The tunnels are not subject to precipitation and an accumulation of contaminants can be found inside tunnels. To keep the good friction of the roads and to remove the contaminants the tunnels are washed at regular intervals throughout the year. The washing events depends on amount of traffic, and results in a flux of chemicals entering the water system (Paruch & Roseth, 2008). The washing event may include detergents to remove the contaminants from the tunnels and these detergents can in turn increase the toxicity of the tunnel wash water for instance by increasing the desorption rates of PAHs with heavier molecular weight (Allan et al., 2016).

The sedimentation ponds are however colonized by and the habitat for a wide range of organisms. The abundance of wetland areas and pond systems are on a decline worldwide (Zedler & Kercher, 2005), increasing the possibility of the sedimentation ponds being used as habitat for species located near and in freshwater (Brand & Snodgrass, 2010). This results in the ponds increasing and maintaining high biodiversity and biomass on a local and a regional scale (Le Viol et al., 2012; Scher & Thièry, 2005; Sun et al., 2018). This high biodiversity can be a result of good food access or it may indicate that some organisms thrive in a slightly contaminated area. Moreover, this biodiversity can lead to contaminants being transported into other ecosystems. Birds such as sea gulls can predate amphibians living in these ponds (Kilpi & Byholm, 1995), resulting in contaminants being further transported into terrestrial or marine environments.

1.1 Amphibians in sedimentation ponds

The sedimentation ponds are often thriving habitats for amphibians that depend on water to fulfill their life cycle (Le Viol et al., 2012; Scher & Thièry, 2005; Snodgrass et al., 2008). Amphibians are susceptible to contamination from the environment through water, air and their diet. This is partly due to their gills as tadpoles and their permeable skin as adults (Sparling, 2010). On a global scale there is a decrease in amphibians (Alford & Richards, 1999; Blaustein & Wake, 1990; Houlahan et al., 2000) and several species have been on the worldwide red list for several years (IUCN, 2018). It is estimated that 48% of the amphibian species are declining and 7% are critically endangered (Stuart et al., 2004). This is mainly due to habitat loss and overutilization, but other processes and sources, such as environmental contaminants, are also large contributors (Blaustein & Kiesecker, 2002; Collins et al., 2009; Stuart et al., 2004). It is documented that contaminants may cause lethal and sublethal effects on amphibians including behavioral changes, endocrine disruption, reduced growth, increased susceptibility to diseases and reduced locomotion (Boone & James, 2003; Bridges, 1997; Bryer et al., 2006; Diana et al., 2000; Hayes et al., 2006; Malcolm & Shore, 2003; Relyea, 2004, 2009; Rohr et al., 2008). Road-associated contaminants are reported to increase mortality in newly hatched gray treefrogs (*Hyla versicolor*) (Brand et al., 2010), reduce size at metamorphosis for the American toad (*Bufo americanus*), and induce 100% mortality for tadpoles of the wood frog (*Rana sylvatica*) (Snodgrass et al., 2008).

The common frog (*Rana temporaria*) is the most abundant amphibian in Norway (Dolmen et al., 2004). It is found throughout temperate lakes and ponds and is a highly adaptive species (Dolmen, 2008), hence this frog is found in many sedimentation ponds. Previous studies from sedimentation ponds in Norway have shown lethal and sublethal effects on the tadpoles of the common frog. Tadpoles from Skullerud and Vassum sedimentation ponds have accumulated higher concentrations of PAHs compared to dragonfly (Odonata) species (Grung et al., 2016) and tadpoles from the same ponds have shown to accumulate various metals (Johansen, 2013; Meland et al., 2013). A tunnel wash event at Vassum was found to cause a very high percentage of mortality in the tadpole population (Johansen, 2013). These studies have shown that it is of interest to further investigate the effects on the common frog living in sedimentation ponds.

1.2 Polycyclic aromatic hydrocarbons, their metabolites and effects

Chemically polycyclic aromatic hydrocarbons (PAHs) are formed by the fusion of two or more aromatic rings during natural events or human activities. It is created during processes generating high heat, such as volcano outbursts and combustion processes. Some PAHs are listed as a priority substance for environmental quality standards (EQS) in the EU legislation of the Water Framework Directive from 2008 (European Parliament, 2008). Some of these are implemented in the Norwegian law with a set allowable concentration in freshwater (Table 1.1). Other PAHs are not implemented in the EU legislation, but are set as especially important for Norwegian waters and thus regulated (Norwegian Environment Agency, 2016). The PAHs are available for uptake by aquatic organisms through gills and food consumption because of the lipophilicity of the chemical. When entering an organism, the PAHs can be converted into more water-soluble structures. This transformation produces structures that can be excreted by the organism. During this transformation harmful products may be created, including reactive metabolite species and intermediate structures such as reactive oxygen species (ROS) (Penning et al., 1999), which may in turn alter cellular function, causing toxicity and initiating carcinogenicity (Miller & Ramos, 2001). PAHs have shown to cause a variety of negative effects on organisms including mortality in all life stages, growth reduction, lesions, tumors, deformities, reduced condition factor, estrogenic effects and cardiac dysfunctions (Logan, 2007). When organisms are subject to PAHs, an induction of changes in gene expression can be found (Barranco et al., 2017).

Table 1.1: EQS for selected PAHs in freshwater, sediment and biota from the report M-608 by the Norwegian environment agency on basis of the EQS from the EU water framework directive and in regard to conditions in Norwegian waters.

Substance	**Annual average (µg/L)[1]**	**Maximum allowable concentration (µg/L)[1]**	**EQS sediment (mg/kg dry weight)[2]**	**EQS Biota (µg/kg wet weight)[3]**
Anthracene	0.1	0.1	0.0046	2400
Fluoranthene	0.0063	0.12	0.40	30
Naphtalene	2	130	0.027	2400
Benzo[a]pyrene	$1.7x10^{-4}$	0.27	0.18	5
Acenaphtylene[4]	1.28	33	0.033	-
Acenaphtene[4]	3.8	3.8	0.10	-
Fluorene[4]	1.5	33.9	0.15	-
Phenantrene[4]	0.5	6.7	0.78	-
Pyrene[4]	0.023	-	0.084	-
Benzo[a]anthracene[4]	0.012	0.018	0.06	304
Chrysene[4]	0.07	0.07	0.28	-
Dibenzo[ah]anthracene[4]	0.0006	0.14	0.027	-

1. The annual average and maximum allowable concentration are set for freshwater.

2. The standards are set for coastal waters

3. The standards for biota is based on marine organisms

4. PAHs especially regulated in Norwegian waters

PAHs can be found in high densities in highway runoff and tunnel wash water (Grung et al., 2017; Grung et al., 2016; Meland & Rødland, 2018; Napier et al., 2008; Petersen et al., 2016). The source of the PAHs found in runoff water may be weathering of road pavement asphalt or road dust (Kose et al., 2008). The road dust is a mixture of exhaust, weathered asphalt, lubricating oils, gasoline, tire particles, atmospheric fallout and soil (Takada et al., 1990). Oil and petroleum leaking from vehicles also contribute to the total PAHs in the runoff (Carls et al., 2008).

1.3 Biomarkers

Biomarkers reflect an interaction between a biological system and an environmental hazard (Van der Oost et al., 2003). Nordberg et al. (2009) defined it as "Quantifiable behavioral, physiological, histological, biochemical, or genetic property that is used to measure response to an environmental change". This can be measure of chemicals, group of chemicals, metabolites or effects of pollutants at a molecular level to an ecosystem level. The literature is not consistent on the definition but in the present study the biological response at the individual level will be used.

Common biomarkers used for ecotoxicology include responses to exposure of PAHs and the induction of enzymes related to cellular defense. This includes measurements of PAH metabolites created during biotransformation, measuring enzyme groups that are important for biotransformation of PAHs and investigating genotoxic effects of PAHs and their metabolites. The result might show high concentrations but it may not necessarily imply adverse effects on endpoints such as reproduction or survival. The biomarkers in the present study indicate exposure to a specific type of contaminant and can provide a warning of exposure to environmental contaminants. When studying biomarkers in amphibians there are several parameters that greatly affect the activity of a biomarker, including developmental stage, sex, temperature and season (Sparling, 2010).

1.3.1 PAH metabolites

For some organisms, such as mammals, fish and amphibians, PAHs are readily metabolized and will not directly accumulate in the organism (Whyte et al., 2000). Other organisms such as blue mussels and plants do not metabolize PAHs to the same extent and can be used directly for measuring the PAHs in the environment. For organisms that do metabolize PAHs the biotransformation products, i.e. PAH metabolites, can be measured to determine the level of PAHs the organism has been exposed to. These metabolites are found in bile or other transformation organs as well as in excretion organs. Measuring PAH metabolites is an established biomarker for assessing the PAH exposure in fish (e.g. Grung et al., 2009), mammals (e.g. Strickland et al., 1994), and to some extent in amphibians (e.g. Ueda et al., 2011). It can be measured by using an analytical method, such as separation by high-performance liquid chromatography (HPLC) followed by detection by fluorescence (Ariese et al., 2005).

1.3.2 Cytochrome P-450

During phase 1 metabolism of PAHs a superfamily of heme-containing proteins called cytochrome P-450 (CYP) are activated. Families of this group of enzymes, particularly CYP1A, work to break down the ring structure of PAHs and create substances that can be excreted by the organism. The concentration and activity of these enzymes increases with an increase in exposure to PAHs (Whyte et al., 2000), meaning that a high level of exposure to PAHs results in higher levels of certain families in this enzyme group. CYP is well preserved and can be found in all organism groups that have an active metabolism. It is found in all tissues with the main activity in the liver. The measuring of CYP activity is a widely used biomarker and is well documented in organisms such as fish (Whyte et al., 2000) and has been performed on tadpoles of several frog species (Doherty & Khan, 1981; Johansen, 2013; Jung et al., 2004).

1.3.3 Genotoxicity

Exposure to contaminants with genotoxic properties can cause modifications in cellular components such as the deoxyribonucleic acid (DNA) (Shugart, 2000). During cell replication and metabolism, the DNA is transitioning from being a stable double-stranded molecule to an intermediate more unstable structure. Genotoxic contaminants may directly or in the form of intermediate structures interact with cellular processes or cell components. These intermediate structures may be free radicals or reactive oxygen species (ROS). These substances can interfere with the DNA backbone which may result in strand breaks (Shugart, 2000). By measuring the amount of strand breaks it is possible to determine how much contaminants have affected the organism. Comet assay is a commonly applied method that enables small levels of DNA damage to be detected such as single and double strand breaks (Ostling & Johanson, 1984; Singh et al., 1988).

1.4 Aims and hypotheses

The objective of this thesis was to clarify whether tadpoles living in highly polluted ponds were affected by road-associated contaminants. This was achieved by sampling tadpoles from two sedimentation ponds with different levels of exposure and from one natural occurring pond. Quantification of morphological changes and different biomarkers, including measurement of PAH metabolites, CYP1A concentration and activity, as well as DNA

damage was performed to examine the effects of contaminants. The study addressed the aim by five objectives and by testing seven hypotheses:

Objective 1: To clarify whether living in sedimentation ponds disrupts the normal development of tadpoles.
H_1 = Developmental stages are different in tadpoles living in sedimentation ponds compared to reference pond.
H_2 = Tadpole fitness decreases in tadpoles living in sedimentation ponds compared to reference pond.

Objective 2: To clarify whether PAH metabolite concentrations are elevated in tadpoles living in sedimentation ponds.
H_3 = The level of PAH metabolites found in tadpoles from sedimentation ponds is higher compared to tadpoles from the reference pond.

Objective 3: To clarify whether the concentration and activity of CYP1A increases when tadpoles are living in sedimentation ponds.
H_4 = CYP1A concentration is higher in tadpoles living in sedimentation ponds compared to a reference pond.
H_5 = CYP1A activity is higher in tadpoles living in sedimentation ponds compared to a reference pond.

Objective 4: To clarify whether there is an increase in DNA damage in tadpoles living in sedimentation ponds.
H_6 = DNA fragmentation is higher in tadpoles living in sedimentation ponds compared to a reference pond.

Objective 5: To clarify whether tadpoles are more affected by sediments polluted with contaminants from road runoff compared to sediments not containing any known source of contaminants.
H_7 = Tadpoles exposed to sediment from sedimentation ponds are more affected than tadpoles exposed to sediment from a reference pond.

2 Materials and methods

2.1 The study sites

Figure 2.1: Locations of ponds were sampling took place. The red dots represent the location of the sedimentation ponds (Vassum and Skullerud) while the green represents the reference pond (Svartadammen).

2.1.1 Vassum sedimentation pond

Vassum sedimentation pond is situated south of Oslo along highway E6 in Akershus county, Norway. It receives tunnel wash water from Nordbytunnelen as well as road runoff from the highway E6 with a heavy amount of traffic. The tunnel is cleaned 4-6 times each year and the coarse material and debris from the runoff is removed through gully pots before entering the sedimentation pond (Meland et al., 2010a). The pond also receives tunnel runoff from two smaller tunnels, Smihagen and Vassum. The amount of road runoff the pond receives comes from 17 000 m^2 of open road areas (Meland et al., 2010a). According to the Norwegian Public Roads Administration (NPRA) the average annual daily traffic (AADT) in this area is 42620 vehicles per day. The pond has a pre-sedimentation pond and a main pond. It is in close proximity to lake Årungen and treated effluents from the sedimentation pond are discharged to the river Årungselva.

Figure 2.1.1: Vassum sedimentation pond with Norbytunnelen in the background. The pre-sedimentation pond is seen in front and the main pond in the back. Photo: Kjersti Wike Kronvall

2.1.2 Skullerud sedimentation pond

This is a sedimentation pond situated in the south of Oslo along the highway E6 in Oslo county, Norway. It receives road runoff from the highway E6, and the effluents from the sedimentation pond is released to the river Ljanselva (Figure 2.1.2). The pond has a closed pre-sedimentation tank and an open main pond (Figure 2.1.2). According to the NPRA the AADT in this area is 70936 vehicles per day.

Figure 2.1.2: Left: The pond at Skullerud during summer, with a semi-trailer at E6 in the background. Right: Ljanselva, in close proximity to the pond at Skullerud.

2.1.3 Svartadammen

Svartadammen is a pond found in Brannfjellskogen at Ekeberg in southern Norway. This pond has a high biodiversity and is habitat of several amphibian species. Svartadammen was used as the reference pond in this study as there was no known source of contamination of the pond. It is listed as an A-value area by the Norwegian environmental agency, which means that this area is a very important area for biodiversity. It has been assigned this value on the basis of the high occurrence of amphibians (Norwegian Environment Agency, 2008).

Figure 2.1.3: Left: Svartadammen seen from northern end. May 2017. Right: Large cluster of eggs from the common frog found at Svartadammen April 2017.

2.2 The study species

The common frog (*Rana temporaria*) is distributed throughout Norway at altitudes up to approximately 1000 meter above sea level (Figure 2.2a). The species mates and spawns in small lakes and ponds between April and June depending on the altitude and latitude (Dolmen, 2008).

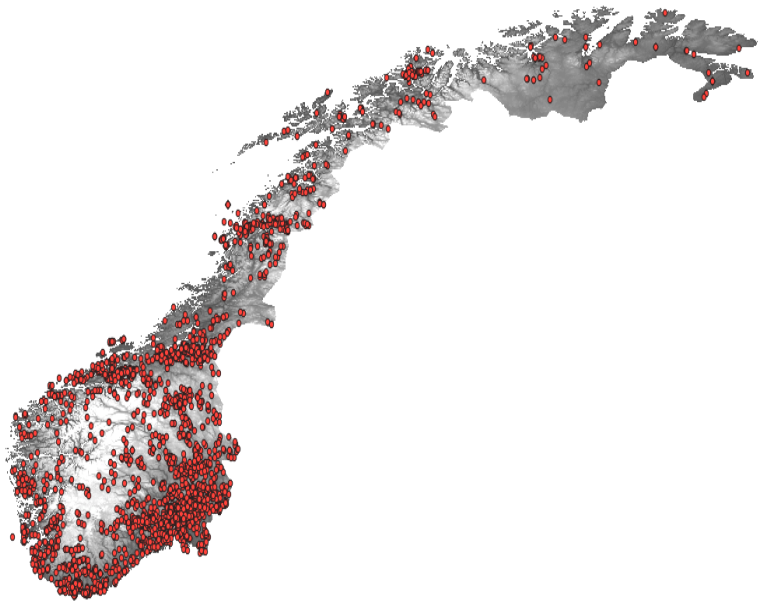

Figure 2.2a: Locations of *R. temporaria* in Norway. A clustering is seen in the southernmost part (map made with QGIS with altitude data from The Norwegian water resources and energy directorate (NVE) and specie observations from the Global Biodiversity Information Facility (GBIF)).

There are two frog species in the southern region of Norway, the common frog and the moor frog (*Rana arvalis*). Determination of the specie based on the egg clusters is possible. The moor frog eggs will be located at the bottom of ponds at the start of embryogenesis while the common frog eggs will be located in more shallow waters and float up to the surface (Dolmen, 2008). The best way to determine the species at larvae stage is to examine the mouth region and the labial tooth rows (Figure 2.2b). The common frog has four lower labial tooth rows, while the moor frog in contrast has three (Dolmen, 2008).

Figure 2.2b: Mouth region of the common frog where four labial tooth rows can be spotted.

2.2.1 Life cycle

Frogs have differing habitats during their life cycle but are always to some extent dependent on water. There are three main stages in the life cycle, this include egg, larvae (tadpole) and adult (frog). During these life stages the animal is subject to contaminants in different ways. The frogs return to their native pond to mate and spawn and eggs are layed and hatched in the water column (Figure 2.2.1). The eggs and tadpoles are exposed mainly through the water column while adult frogs are subject to contaminants from both water, air and through their diet. The egg has a protective gelatinous coating, which mainly consists of water and salts (Sive et al., 2000). The tadpole is dependent on freshwater and eats deposits and small plant material (McDiarmid & Altig, 1999). The development of a tadpole can be divided into several under stages (Gosner, 1960). During its development it will loose its tail, develop internal gills and legs. When the tadpole starts to emerge from the water the metamorphosis into an adult frog continues and the frog develops lungs, a semi-permeable skin and there are changes in the organ system (McDiarmid & Altig, 1999). The frogs are philopatric, meaning that they return to their birthplace for mating and spawning.

Figure 2.2.1: Mating of the common frog, spring 2017.

2.3 Sampling

Locations where samples were taken were Skullerud and Vassum sedimentation ponds, and Svartadammen as the reference pond. There were five subsequent samplings between weeks 18-24 of 2017. The sampling consisted of sediment and biota based on the development of the tadpoles. Temperature loggers (HOBO Pendant data loggers, Onset®) were placed in the

water column in each pond and detected the temperature during the whole sampling period (Appendix B).

2.3.1 Sediment

Composite bed sediment samples consisting of five to ten subsamples was collected by use of a Van Veen grab. The upper 5 cm of the sediments were sampled. In Skullerud and Vassum the samples were taken at depths from 0.5 to 0.9 meter. The sediment was sifted through a 5mm sift and stored in pre-incinerated glass jars (with aluminium foil between sample and lid) and lid at -30°C.

2.3.2 Biota

Tadpoles of the common frog were sampled with a pond net (95 µm) at different developmental stages in a total of five sessions. After collection they were brought into the laboratory and dependent on the developmental stage either directly killed and put in liquid nitrogen or kept in a glass aquarium with water from the ponds until dissection. The stages were determined based on Gosner (1960) and divided into four groups (Table 2.3). All stage groups are not found in all ponds and therefore the sampling times will be used further in the analysis.

Table 2.3: Developmental stages of tadpoles divided into four groups based on important characteristics of development. The time of sampling and in which ponds these stage groups are found is implied.

Stage group	Stage	Characteristics	Sampling time	Ponds
Stage 1	< 25	Transition from external to internal gills	Sampling 1 - 3	Svartadammen and Skullerud
Stage 2	25 – 31	Development of back limbs	Sampling 3 - 4	Svartadammen, Skullerud and Vassum
Stage 3	32 – 37	Development of back and front limbs	Sampling 4 - 5	Svartadammen, Skullerud and Vassum
Stage 4	> 37	Reduction of tail and metamorphosis	Sampling 4 - 5	Skullerud and Vassum

Dissection

To get a better picture of how the embryos were exposed, the gelatinous outside coating was removed. As no method to the author's knowledge has been developed for removing the coating the process was tried out in several different ways. First centrifugation of the samples was performed at 1500 rpm for 20 minutes (Eppenford Centrifuge 5810 R), then subsequently with a higher level of centrifugation, 3000 rpm for 20 minutes. Second, cutting each coating out with a scalpel. Third cutting an incision in the coating using a scalpel followed by using the suction of a plastic pipette. The third method was chosen as the most precise, and used further on in the analysis.

To reduce risk of cross-contamination all equipment was washed in 96 % ethanol and rinsed in distilled water between different tadpoles and tissues. All samples were snap frozen in Nunc® Cryo tubes (Sigma-Aldrich) in liquid nitrogen and stored at -80°C if no other method is specified.

From the first field sampling eggs were collected. The gelatinous outside coating was removed as described above, by a cut in the coating with a scalpel and further removed by using the suction of a plastic pipette. The remaining embryo was pooled by location, snap frozen in liquid nitrogen and stored at -80°C. From the second field sampling the tadpoles had just started developing organs and hence was too small for organs to be dissected. The tadpoles were rinsed in distilled water, pooled with ten tadpoles in each Cryo tube, snap frozen in liquid nitrogen and stored at -80°C.

From field sampling 3-5 individual tadpoles were anaesthetized in a solution of 1:2000 MS-222 (Ethyl methanesulfate, Sigma-Aldrich) and distilled water for 5 minutes. The tadpoles were rinsed in distilled water to stop further induction of anesthesia, which may lead to stop of blood flow. Weight (g), length (cm) and stadium (Gosner 1960) was measured. Under a stereo microscope (60x magnification) the tadpole was opened with a scalpel from mouth to gut opening, exposing the ventral view with heart, gills, intestines and abdomen in view (Figure 2.3). Excess water was dried using a clean tissue. Blood samples were immediately taken with a heparinized glass capillary tube (1.15 ± 0.05mm, Brand) by cardiac puncture of the heart. The samples were transferred into an Eppendorf tube with 50 µL PBS-EDTA and kept on ice. The liver was immediately snap frozen in Cryo tubes. The rest of the intestine and stomach content, gills and the remaining parts of the tadpole were put in separate Cryo

tubes and snap frozen in liquid nitrogen. All samples, except blood samples, were stored at -80°C until further analysis.

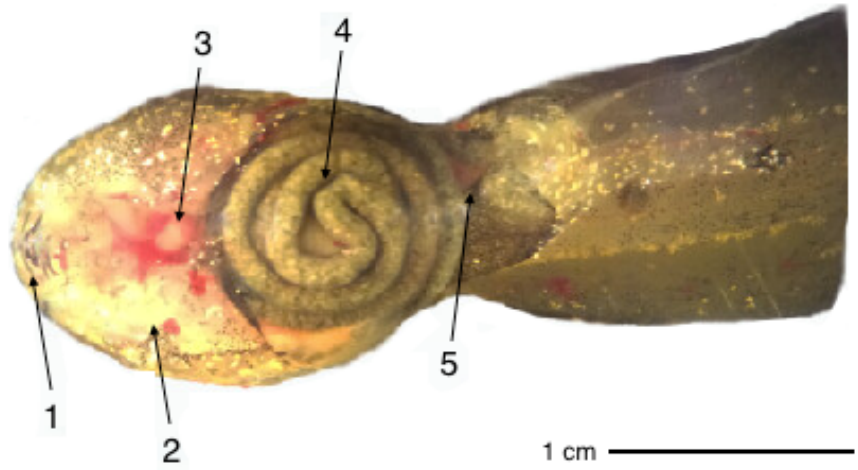

Figure 2.3: Exposed tadpole from the ventral view. 1. Mouth opening. 2. Gills. 3. Heart. 4. Intestines. 5. Rectum.

2.4 Biometrics and morphology

Biometrics parameters in the form of length and weight measurements, was investigated to examine the difference from each pond. This was measured in all tadpoles from sampling 3-5. Measurements of body condition indices were investigated to determine the fitness of the tadpoles from each pond.

2.4.1 Condition index

The condition index (CI) was measured in all tadpoles from sampling 3-5. The CI was calculated using the following equation:

$$CI = \frac{Body\ weight\ (g)}{Length\ (cm)\wedge 3} \times 100 \qquad \text{(Equation 1)}$$

A high condition index can indicate high fitness. The weight and length measurements as well as all condition indices can be found in appendix C.

2.4.2 Liver somatic index

Average liver somatic index (LSI) was measured in all tadpoles at sampling 3-5. The LSI was calculated using the following equation:

$$LSI = \frac{Liver\ weight\ (g)}{Body\ weight\ (g)} \times 100 \qquad \text{(Equation 2)}$$

When the liver somatic index is low in an organism compared to others of the same specie, there is an indication of lower fitness. The weight measurements and all liver somatic indices can be found in appendix C.

2.5 PAH concentration

The chemical analysis of sediment and biota was performed at the NIVA lab using a gas chromatography (GC) linked to a mass spectrometry (MS). The analysis is explained in detail in Ruus et al. (2005) and briefly explained here.

2.5.1 Sediment

The samples were homogenized and added internal standards (200ng of naphthalene d8, acenapthene d8, phenantrene d10, chrysene d12, perylene d12 and anthracene d10). Samples were extracted with dichloromethane and extracts was cleaned by gel permeation chromatography and solvent exchanged to cyclohexane. The extracts were analysed by GC and MS (Agilent GC 6890 with MSD 5973; Agilent Technologies, Wilmington, DE, USA). The MS detector was operated in selected ion monitoring mode. The GC was equipped with a DB-5MS column (30m, 0.25 mm i.d. and 0.25 um film thickness) and operated in splitless mode. The gas flow rate was set to 1.2 mL/min. The initial GC temperature was 60°C increased in steps to 310°C. The injector temperature was set to 300°C, transfer line temperature 280°C and MS source temperature 230°C. The quantification of individual components was done using the internal standard method.

To classify the PAH levels found in this study an environmental standard developed by the environmental agency was used (Table 3.1a) (Agency, 2016).

Table 2.5: Limit values from the Norwegian environmental agency, based on previous tests on organisms, risk assessment and toxicity on organisms (PNEC values).

Background	Good	Moderate	Poor	Very poor
Background level	No toxic effect	Chronic effect: long exposure time	Acute toxic effects: short exposure time	Comprehensive toxic effects

2.5.2 Biota

PAH concentration was measured in eggs and small tadpoles from sampling 1 and 2. The samples were homogenized with an ultra Turrax and added internal standards (as described in 2.5.1). Extraction of the PAHs was done with *n*-pentane and dried over sodium sulphate. The samples were analyzed by gas chromatography (GC) coupled with a mass spectrometry (MS) as described in section 2.5.1 for sediment.

2.6 Biomarkers

2.6.1 PAH metabolites

To establish the amount of PAH metabolites high-performance liquid chromatography (HPLC) with fluorescence detection was used on intestine samples of the tadpoles. This method uses a PAH C18 column, were the metabolites are separated. A fluorescence detector at the end measures the amount of individual metabolites present.

Preparation of samples

To help extract PAH metabolites from the intestine samples an ultrasound bath (Branson Ultrasonic corporation) was used. This separates the solid to a liquid, as the means of centrifugation is not strong enough to break the cell membranes in the sample. Ultrasound only requires some liquid to transfer its energy (Priego-Capote & Luque de Castro, 2004), hence 50 µL distilled water was added to the samples. The ultrasound bath was run for 8 minutes at 70 % intensity. 25 µL of sample was added to 50 µL distilled water. 10 µL internal standard (triphenylamine) was added to the samples and 20 µL of the active enzyme β-glucuronidase/aryl sulfatase was added, this will deconjugate phase 2 glucuronides/sulfates of PAHs to OH-PAHs. The samples were incubated at 37°C for 1 hour. 200 µL methanol was added and the mixture was centrifuged at 13 000 rpm for 10 min. The supernatant was transferred to HPLC vials and kept at -20°C until HPLC analysis.

HPLC analysis

The sample was added to a solvent and pumped through a column filled with silica particles coated with alkyl chains (C18). Separation is done by the means of two mobile phases (A: ammonium acetate and B: acetonitril). This will separate the compounds based on chemical property, in this case polarity. The PAHs have a benzene ring structure that each has a different fluorescence wavelength depending on the amount of double bonds (Table 1). The

fluorescence was used as a detector and the concentration is measured of each PAH-metabolite depending on amount of fluorescence emitted at each wavelength.
The preparation of the samples was done by the authors and the HPLC analysis was performed at NIVA by Merete Grung.

Table 2.6: The fluorescence detection by the HPLC C18 column with excited and emitted wavelengths.

Chemical	Excitation/emission (nm)	Retention time (min)
1-OH-Phenanthrene	256/380	10.22
1-OH-Pyrene	346/384	13.53
ISD triphenylamine	300/360	19.30
β-OH-Benzo[a]pyrene	350/430	22.58

2.6.2 Cytochrome P-450 protein and activity in liver

Liver samples were used, as this is the main organ for biotransformation.

Isolation of hepatic microsomes

The microsomal fraction (S100) of the liver samples was isolated from liver tissue, as described by Dignam (1990). Each sample was thawed on ice and added to Precellys tubes with 20 ceramic beads (Precellys 24 Soft Tissue homogenizing 1.4 mm ceramic beads, Bertin Technologies). An ice-cold homogenization buffer (2.5 mL 0.1 M potassium-phosphate buffer (pH 7.8) containing KCl (0.15 M), 1 mM dithiothreitol (DTT) and glycerol (5% v/v)) was added to the sample and the samples were homogenized using the Precellys homogenizer (Bertin Technologies) at 6000 rpm; 3x10 sec; 5sec pauses with Cryolys 5 bar system pressure (70 L/min) at 4°C filled with liquid nitrogen. The homogenate was transferred into Eppendorf tubes on ice and centrifuged at 10 000 x g for 30 min at 4°C (Heraeus Multifuge 3 S-R, Kendo Laboratory Products). The supernatant was transferred to centrifugation tubes on ice and centrifuged at 100 000 x g for 60 min at 4°C (Sorvall MTX150 Micro-Ultracentrifuge, Thermo Fischer Scientific). 200 µL microsomal buffer (0.1 M potassium-phosphate buffer (pH 7.8) containing KCl (0.15 M), DTT (1 mM), EDTA (1 mM) and glycerol (20% w/w)) was added to the pellet in two rounds. The samples were transferred to Eppendorf tubes and homogenized for minimum 3 x 5 seconds on ice using a motorized pistil for Eppendorf tubes (VWR). The microsomal fraction was then transferred to three aliquots in Eppendorf tubes:

20 µL for protein content, 20 µL for ELISA and the rest for EROD. The samples were immediately frozen at -80°C.

Protein content

Twenty µL of the microsomal fraction was used to find the concentration of protein using a modified Lowrys method (Lowry et al., 1951). This is a two-step colorimetric assay based on the reaction of proteins with an alkaline copper tartrate solution and Folin reagent. A dilution series of bovine serum albumin (BSA) protein standards in 0.1 M Tris buffer (pH 8): 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL. Each sample of homogenized liver from S100 fraction was diluted 1:5 in 0.1 M Tris buffer (pH 8). To each well in a clear Nunc 96-well microtiter plate 10 µL of each sample was added, as well as each dilution of the standard and blanks that are only 0.1 M Tris buffer. To each of the wells 25µL of alkaline copper tartrate solution (BioRad) and 200 µL of Folin reagent (BioRad) is added and the plate is gently moved to mix the reagents. The plates are incubated for 15 minutes at room temperature and absorbance was read with SynergyNX BioTek plate reader at 750 nm. A standard curve was made from the BSA dilution series to calculate protein concentration based on fluorescence.

CYP1A protein - Enzyme-linked Immunosorbent Assay (ELISA)

This is an indirect enzyme-linked immunosorbent assay (ELISA) that measured the amount of anti-CYP1A antibodies that binds to cytochrome P4501A protein. This gives an idea of the relative levels of CYP1A protein in the samples. Samples of hepatic microsomal fraction were thawed on ice and diluted into 10 µg/mL protein using a coating buffer (1 capsule of carbonate-bicarbonate buffer (Sigma) in 100mL distilled water. One reference sample was prepared for each plate (0.5 mL per plate). 100 µL coating buffer was added in eight replicates of a 96 titer plate (Nunc-Immuno™ Microwell™ MaxiSorp™). 100 µL of the prediluted sample was added in triplicates to the wells, including the reference sample. The plate was incubated over night at 4°C.

The plates were washed three times (2 x 30 sec + 90 sec) using TTBS (10 L distilled water with 8.84 g Trizma-HCl, 17.44 g Trizma base, 292 g NaCl, 5 mL Tween-20 (Sigma P1379)) using an automatic plate washer (ScanWasher 300). 300 µL blocking buffer (1% w/w bovine serum albumin (BSA) in TTBS) was added to all wells and incubated for 60 minutes. The plates were then washed in TTBS three times. 100 µL of primary antibody (CP226) diluted

1:1000 in antibody buffer (TTBS with 0.1% BSA) was added to the wells. The plates were incubated overnight at 4°C.

The plates were washed three times in TTBS (Scanwasher 300). 100 µL of the secondary antibody (GAR-HRP) diluted 1:3000 in antibody buffer was added to all wells. The plates were incubated for 6 hours at 4°C. The plates were washed five times (4x 30 sec + 90 sec) using TTBS. 100 µL color development buffer, TMB Plus (Kem-EN-Tech), was added to all wells using a multipipette. The plates were incubated in the dark for 5-10 minutes. 50 µL stop solution (1.5 M H_2SO_4) was added to each well. Absorbance was red using a SynergyNX BioTek plate reader at 450 nm. Samples were standardized against reference samples between all plates.

7-Ethoxyresorufin-*O*-deetylase (EROD) activity

CYP1A1 catalyzes the oxidative deethylation of 7-ethoxyresorufin to resorufin. Induction of CYP1A enzyme can be measured in samples by reading the fluorescence emitted by resorufin. The assay is described by Burke and Mayer (1974) and modified by Eggens and Galgani (1992). Samples of hepatic microsomal fraction were thawed on ice following a dilution to a total of 1 mg/mL protein in phosphate buffer (0.1 M potassium-phosphate buffer, pH = 8.0). 180 µL 5mM 7-ethoxyresorufin and 30 mL phosphate buffer was mixed and stored at 4°C as the reaction mix. A 2x dilution series was prepared using phosphate buffer and a stock solution of resorufin sodium salt (1mM resorufin standard in DMSO). The dilution series ranged from 5 nM to 640 nM. β-NADP was thawed on ice and diluted from 50 mM to 2.4 mM (120 µL 50 mM β-NADP stock solution in 2.38 mL phosphate buffer). 275 µL of standard series including reaction mix as blank was added in duplicates, 50 µL of phosphate buffer (blank) was added in eight replicates, 50 µL diluted samples and reference samples were added in triplicates to a Nunc™ 96-well plate. 200 µL of reaction mix and 25 µL the 2.4 mM NADP-solution was added to all wells with blank, samples and reference samples.

Fluorescence was read immediately with a Synergy MX platereader in 8 steps at 530/590 nm excitation/emission for a total of approximately 4 minutes.

2.6.3 DNA damage

To establish the potential DNA damage in tadpoles, blood samples were taken from each individual tadpole. The single cell gel electrophoresis method, commonly called comet assay, can determine double and single stranded breaks in the DNA (Ostling & Johanson, 1984; Singh et al., 1988). This assay uses an electrophoresis current to extract DNA fragments out from the cell nuclei. Smaller fragments will migrate further than larger fragments since size affects their mobility (Olive & Banáth, 2006). The fragments migrated out from the nuclei is called the "tail", and the nuclei with the intact DNA is called the "head". The result is measured in % tail intensity.

The blood was diluted in 50 µL PBS-EDTA (pH 7.5). While the rest of the tadpoles were dissected the diluted samples were kept on ice. 75 mg low melting point (LMP) agarose was dissolved in 10 mL buffer and heated until a clear liquid with no crystals. 90 µL of the agarose solution was transferred to Eppendorf tubes and stored in a heating block at 37°C. 10 µL of the diluted blood samples were carefully mixed with 90 µL LMP agarose and mixed by gently stirring. 20 µL of the sample and agarose solution was embedded on the hydrophilic side of a GelBond® film placed on an aluminum block pre-cooled to 4°C. At least two replicates of each sample were embedded the films. To stop the cellular activity and ensure that DNA was released but intact, the films were transferred into lysis buffer (89 ml Lysis stock solution, 10 mL DMSO and 1 mL Triton X-100) and stored in darkness over night at 4°C.

The following day the films were embedded in electrophoresis buffer (pH = 12) for 5 minutes before placed in an electrophoresis chamber with electrophoresis buffer. The electrophoresis was run at 4°C for 25 minutes at 24V. The electrophoresis electric current makes the negatively charged DNA strands migrate in the direction of the positive pole. After electrophoresis the films were neutralized by rinsing for 5 minutes in neutralizing buffer before being transferred to fresh neutralizing buffer for 10 minutes. This was done to ensure there is no more electrophoresis buffer present. The films were rinsed further in distilled water for 5 minutes before being placed in a container with 96 % ethanol to be fixated and dehydrated in the dark over-night.

The samples were stained with a gel stain that binds to DNA and emits fluorescence. 50 µL prediluted Sybr GOLD (in DMSO) was diluted in 50 mL Tris-EDTA buffer and films were placed in the solution on a rocking table for 20 minutes, covered to keep in the dark. The subsequent scoring was performed in the dark as the stain is light sensitive. The films were mounted on a plexiglass using distilled water and fit with a cover glass. The slide was placed under a fluorescence microscope (Carl Zeiss Axio Scope A1, ex/em 520/610) with blue light transilluminators applied with a real time camera (Allied Vision Technologies). The scoring of the fragmented DNA in cells was conducted using Comet Assay IV (Perceptive Instruments, version 4.2). The same person did scoring of the cells, and cells out of focus or overlapping cells were avoided. If the computer software analyzed incorrectly because of debris or contrast, the scoring was edited manually. A total of 50 cells were scored per gel.

2.7 Experimental study

An exposure study was set up to expose tadpoles to contaminated sediment from sedimentation ponds. Sediment was collected from each pond with a Van Veen grab and sifted through a 5 mm sift. The sediment was homogenized by thoroughly stirring and stored in separate steel buckets. Water from the reference pond (Svartadammen) was collected in plastic containers and filtered with GF/C filters (47 mm Ø, Whatman). Fully moulded 20 L glass aquariums (VWR; 20 L) were placed in a randomized set up in two rows in a temperature regulated room at 8°C with 12:12 hour light:dark periods. Each aquarium was filled with homogenized sediment from the respective pond and filled with 15 L filtered water from Svartadammen. The aquarium was left for 48 hours to let the sediment settle. In each aquarium an air diffuser connected to an air circulation pump (Marina model 50) was added to ensure aerated water and circulation. In total 1200 tadpole eggs were collected from Svartadammen and 100 eggs was added to each aquarium.

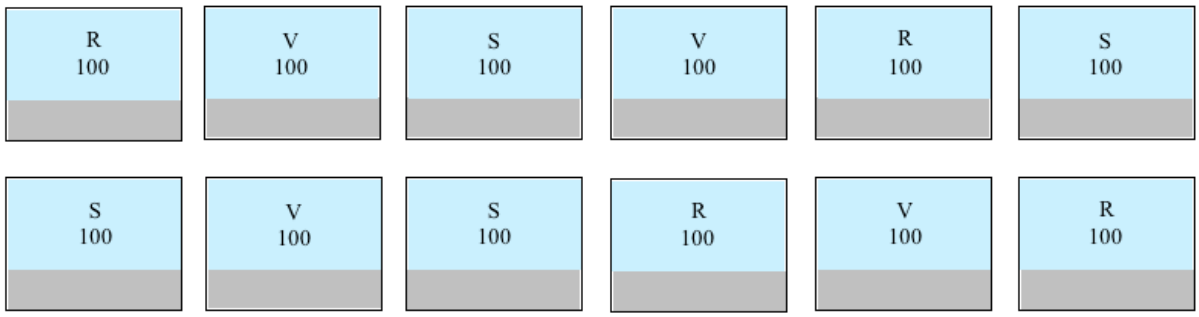

Figure 2.7: Experimental setup with randomized placement of aquaria, sediment from the pond indicated with R = Reference (Svartadammen), V = Vassum and S = Skullerud. Filtered water and 100 eggs from Svartadammen were added to each aquarium.

2.8 Statistical analysis

Raw data was processed in Microsoft Excel for Mac. All statistical analyses were conducted using the software RStudio (RStudio, 2009-2016, version 1.0.136). The packages used in R are shown in table 2.8.

Table 2.8: Overview of packages used in R.

R-package	Reference	Use
ggplot2	Wickham (2009)	Plot presentation
car	Fox and Weisberg (2011)	Stats
vegan	Oksanen et al. (2017)	Multivariate analysis
dunnTest	Dinno (2017)	Post-hoc testing

2.8.1 Univariate statistics

Parametric tests have the assumptions of normal distribution and homogeneity of variance. Parametric tests are viewed as more robust with more statistical power compared to non-parametric test and were used in the analysis if possible. To test whether each group of samples met the assumption of normality the Shapiro Wilk's test was performed (Shapiro & Wilk, 1965). In order to test whether the assumption of homogeneity of variances between two comparing groups were met the Levene's test was performed (Levene, 1960). If both assumptions were met parametric tests were performed with no transformation. If the assumptions were not met log-10 transformation was performed and used if the assumptions now were met. If the assumptions still were not met, non-parametric tests were used. Non-parametric tests are useful for unadjusted between-group comparisons, when there are outliers that must be retained for substantive reasons or skewed variables (Vittinghoff et al., 2011).

The parametric test used was a one-way ANOVA. This test determines whether the mean value of the outcome is the same over all *populations* or if the mean value differ in at least two of the *populations* (Vittinghoff et al., 2011). If a significant p-value was found using the one-way ANOVA a Tukey's honest significant difference (HSD) post-hoc test was applied to establish which groups differ (Tukey, 1949). The non-parametric tests being used were the Wilcoxon rank-test, also known as Mann-Whitney U test (Wilcoxon, 1945) or the Kruskal-Wallis one-way analysis of variance (Kruskal & Wallis, 1952). Wilcoxon rank-test, used for comparing two groups, is based on the assumption that the distributions being compared differ in mean or median, but not in variance (Vittinghoff et al., 2011). The Kruskal-Wallis test is a non-parametric one-way ANOVA on ranks and is thus useful to test significance on two or more groups. If significant p-value was found in the Kruskal-Wallis test, a Dunns post hoc test with Bonferroni correction was applied to determine which groups differ (Dunn, 1961).

In all statistical tests the significance level α was set to $p = 0.05$.

2.8.2 Multivariate statistics

Principal Component Analysis (PCA) was conducted to seek structure and explore correlations, similarities and differences between the measured variables at the reference pond, Skullerud and Vassum. PCA is an eigen-valued based gradient analysis method, based on a linear statistical model. Meaning that it measures covariation between variables. There are several gradient analysis methods and PCA is used because it let us use variables with measurements of different units without constraining the data. The PCA was applied to biomarkers and morphology data to gain an overview as well as to detect any unnoticed patterns in the data. The response variables used for PCA was CI, LSI, the PAH metabolites (1-OH-pyrene and 1-OH-phenantrene) and the DNA damage (from comet analysis). The explanatory variables were location and sampling time. The CYP1A results were not included because they had too small sample size to be reliable variables as well as PCA being sensitive to missing values.

3 Results

3.1 PAH concentrations

3.1.1 Sediment

The concentration of PAHs detected in sediment samples was higher cocentration in Skullerud compared to Svartadammen (Table 3.1.1). A suspision of mix-up of the sediment samples from Vassum resulted in concentrations from previous studies were included in Table 3.1.1. Based on the classification from the Norwegian Environment Agency (2016) the contaminants that were described with poor environmental quality in both sedimentation ponds were fluoranthene, pyrene, benzo[b,j]fluoranthene, indeno[1,2,3-dc]pyrene, dibenzo[ac/ah]anthracene and benzo[g,h,i]perylene. In Vassum anthracene was also classified with a poor quality level. Many of the PAHs were classified with a moderate impact level, and the rest of the PAHs were classified with good or background levels. The latter includes all sediment samples from Svartadammen.

Table 3.1.1: Concentration of PAHs and alkylated PAHs found in sediment samples. The units are all in µg/kg dry weight. Classification is shown in different colors. The first row represents total PAH16 and the last row represents the concentration of alkylated PAHs. Ref = Svartadammen, Sku = Skullerud, Vas = Vassum

Location	LOQ	Ref	Ref	Sku	Sku	Sku[3]	Sku[4]	Vas[5]	Vas[6]	Vas[4]	EQS
Sampling		1	3	1	3	-	-	-	-	-	
ΣPAH16		<282	<293	2784	2687	4550	1200	2118	3050	6200	-
Naphtalene	10	<LOQ	<LOQ	110	150	<100	30	180	<50	<100	0.027
Acenaphtylene	5	<LOQ	<LOQ	18	20	<10	<10	30	<50	<25	0.003[2]
Acenaphtene	6	<LOQ	<LOQ	46	50	<100	<10	5	<50	<25	0.10[2]
Fluorene	1	<LOQ	<LOQ	38	32	100	30	50	216	<100	0.15[2]
Phenanthrene	1	2.1	2.8	190	170	630	140	170	602	1200	0.78[2]
Anthracene	1	<LOQ	<LOQ	29	28	<10	<10	22	119	160	4.6
Fluoranthene	1	3.1	5.3	330	320	660	210	200	386	1000	400
Pyrene	1	2.6	4.0	640	680	940	340	480	648	1800	0.084[2]
Benzo[a]anthracene	1	1.3	2.0	110	86	350	30	40	75	160	0.06[2]
Chrysene	1	1.7	3.0	180	170	160	60	190	374	240	0.28[2]
Benzo[b,j]fluoranthene	1	4.2	5.5	230	210	430	60	94	153	200	140
Benzo[k]fluoranthene	1	1.5	2.0	52	47	120	<10	51	75	<25	140
Benzo[a]pyrene	1	1.5	2.4	120	110	280	30	50	81	140	180
Indeno[1,2,3-cd]pyrene	1	1.5	2.6	130	87	180	40	56	8	180	63
Dibenzo[ac/ah]anthracene	1	<LOQ	<LOQ	43	34	230	<10	22	<50	<25	0.027[2]
Benzo[g,h,i]perylene	1	<LOQ	<LOQ	95	73	480	100	220	237	510	84
Dibenzothiophene	1	<LOQ	<LOQ	13	10	-	-	8	-	-	-
Benzo[e]pyrene	1	1.8	2.6	290	280	-	-	190	-	-	-
Perylene	5	<LOQ	<LOQ	120	130	-	-	60	-	-	-
C1-Naphtalene[1]	10	<LOQ	<LOQ	110	110	-	-	-	-	-	-
C2-Naphtalene[1]	60	<LOQ	<LOQ	260	260	-	-	-	-	-	-
C3-Naphtalene[1]	100	<LOQ	<LOQ	1100	850	-	-	-	-	-	-
C1-Phenanthrene[1]	10	<LOQ	<LOQ	330	270	-	-	-	-	--	-
C2-Phenanthrene[1]	10	<LOQ	<LOQ	1400	1300	-	-	-	-	-	-
C3-Phenanthrene[1]	10	<LOQ	<LOQ	990	970	-	-	-	-	-	-
C1-Dibenzothiophene[1]	10	<LOQ	<LOQ	85	76	-	-	-	-	-	-
C2-Dibenzothiophene[1]	10	<LOQ	<LOQ	740	630	-	-	-	-	-	-
C3-Dibenzothiophene[1]	10	<LOQ	<LOQ	2040	1800	-	-	-	-	-	-
% alkylated PAHs		<82%	<78%	72%	69%						

1. Alkylated PAHs.
2. These EQS are based on Norwegian levels and not implemented in the EU list.
3. Skullerud main pond by Leistad (2007).
4. Samples from Skullerud and Vassum, unpublished report by the NPRA (2014).
5. Vassum main pond quantified by Grung et al. (2016).
6. Vassum pre-sedimentation pond by Meland (2012).

3.1.2 Biota

PAH measurements of biota were performed in embryos and newly hatched tadpoles at sampling 1 and 2 (Table 3.1.2). There were low levels of PAHs in all samples and at sampling 1 at Vassum there was a higher amount for both pyrene and fluoranthene. All samples from sampling 2 were under the detection limit.

Table 3.1.2: Detection of fluoranthene and pyrene in embryos from sampling 1. All units are represented in ng/g dry weight. The LOD is the lowest observed detection value.

Location	**Reference**	**Reference**	**Skullerud**	**Skullerud**	**Vassum**	**Vassum**	**EQS**
Sampling	1	2	1	2	1	2	
Fluoranthene	6.5	<LOD	6.4	<LOD	8.0	<LOD	30
Pyrene	5.6	<LOD	5.1	<LOD	6.6	<LOD	-
LOD	4	11	4	7	3	5	-

3.2 Morphology

3.2.1 Biometrics

The length and weight of the tadpoles increased linearly with the increased stages (Figure 3.2.1). Larger tadpoles both in weight and length were at later stages of development. When comparing the locations with the length measurements there was a significant difference between Svartadammen and Vassum at stage 3 (Tukey's HSD, p = 0.007) and Skullerud and Vassum at stage 4 (Tukey's HSD, p = 0.02). The weight measurement shows significant difference between Svartadammen and Skullerud at stage 3 (Tukey's HSD, p = 0.03), Svartadammen and Vassum at stage 3 (Tukey's HSD, p = 0.03) and between Skullerud and Vassum at stage 4 (Tukey's HSD, p = 0.002). The tadpoles from Vassum sedimentation pond was larger compared to the other ponds throughout the whole sampling period.

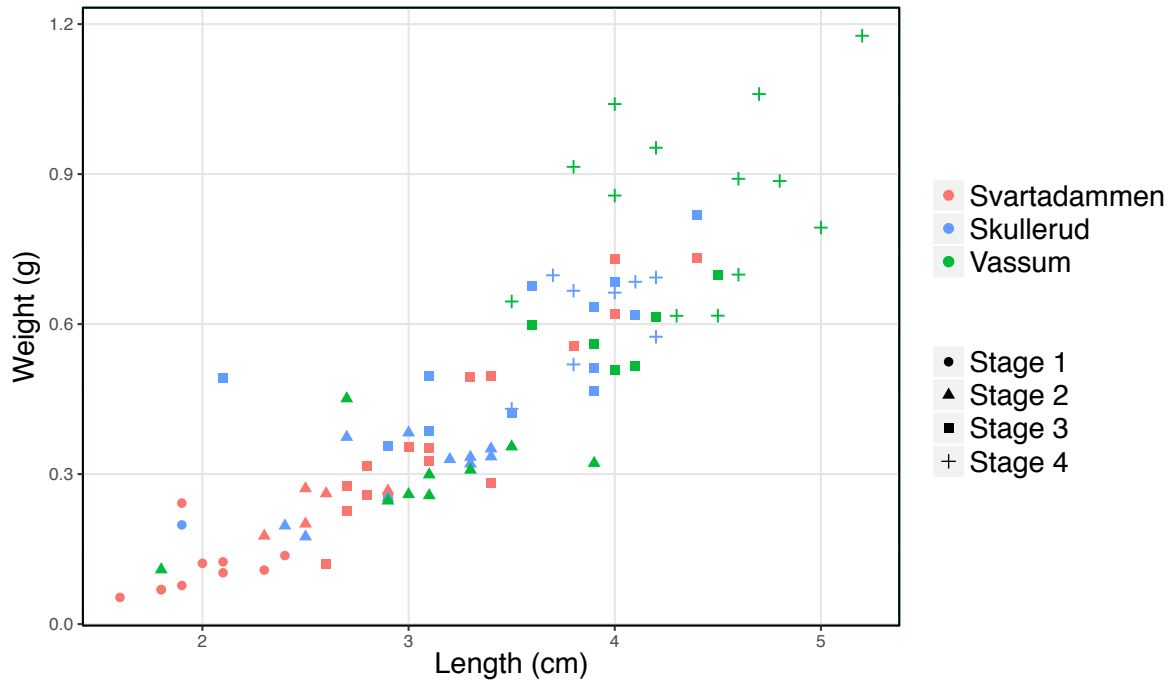

Figure 3.2.1: The length and weight of tadpoles. Reference pond is shown in red, Skullerud in green and Vassum in blue. The shape of the points represents the different stages. The largest tadpoles are found at the latest stage in Vassum sedimentation pond. The smallest tadpoles are found in the reference pond at the first stage.

3.2.2 Condition index

The condition index was consistent with both sampling time and location (Figure 3.2.2). There was a slight decrease for the tadpoles that were sampled at a later time period as well as for those sampled from sedimentation ponds (Vassum and Skullerud) compared to the reference pond. In the tadpole samples at Vassum the decrease was evident from sampling 3 to 4 although not statistically significant.

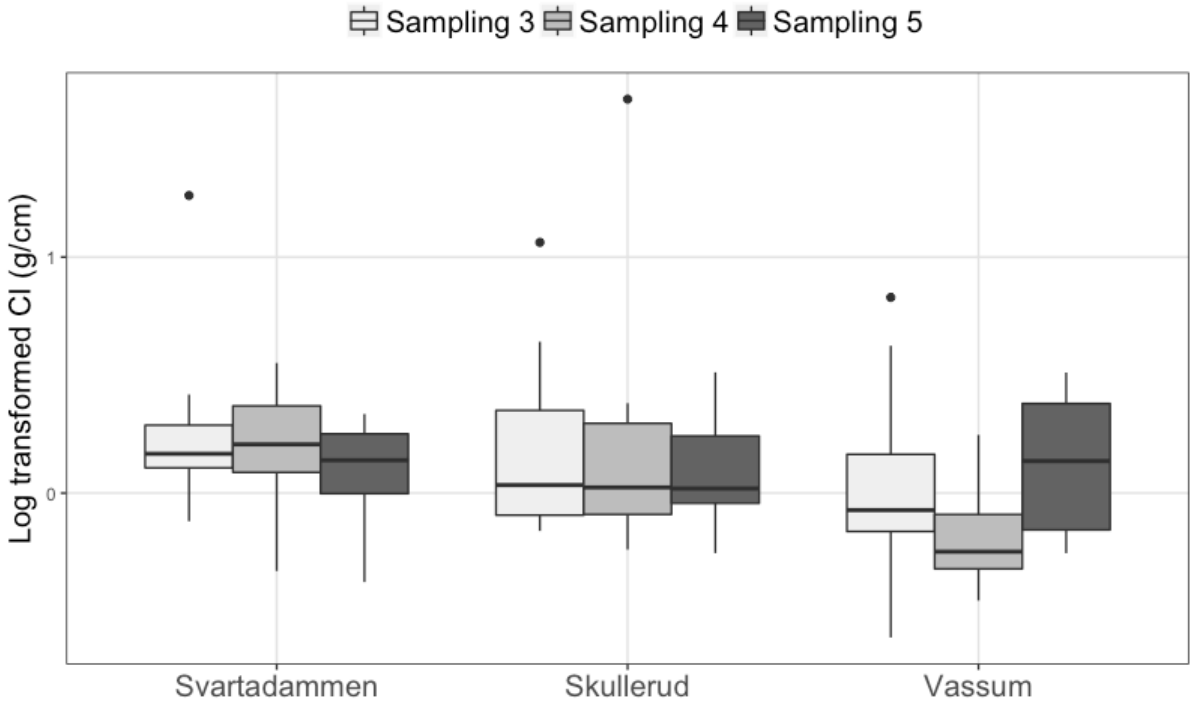

Figure 3.2.2: Log transformed condition index from each location at sampling time 3, 4 and 5. The box is defined by the 1st and 3rd quartiles, the line represents median and the whiskers extend to the most extreme data points that are no more than 1.5 times the interquartile range (IQR). The filled dots are observations that exceeds or fall below the range of the whiskers. n = 9-11 in each group.

3.2.3 Liver somatic index

The liver somatic index decreases when the tadpoles grow (Figure 3.2.3). The LSI was generally low in the tadpoles living in the sedimentation ponds (Vassum and Skullerud) compared to the reference pond for each sampling. A significant difference was found between sampling periods in Svartadammen at sampling 3 and 5 (Dunn's Test, p = 0.0003) and sampling 4 and 5 (Dunn's Test, p = 0.04). In Skullerud there was a significant difference between sampling 3 and 4 (Dunn's Test, p = 0.003). In Vassum there was a significant difference between sampling 3 and 4 (Dunn's Test p = 0.005). The only significant difference between locations was between Svartadammen and Skullerud at sampling 4 (Tukey's HSD, p = 0.0004).

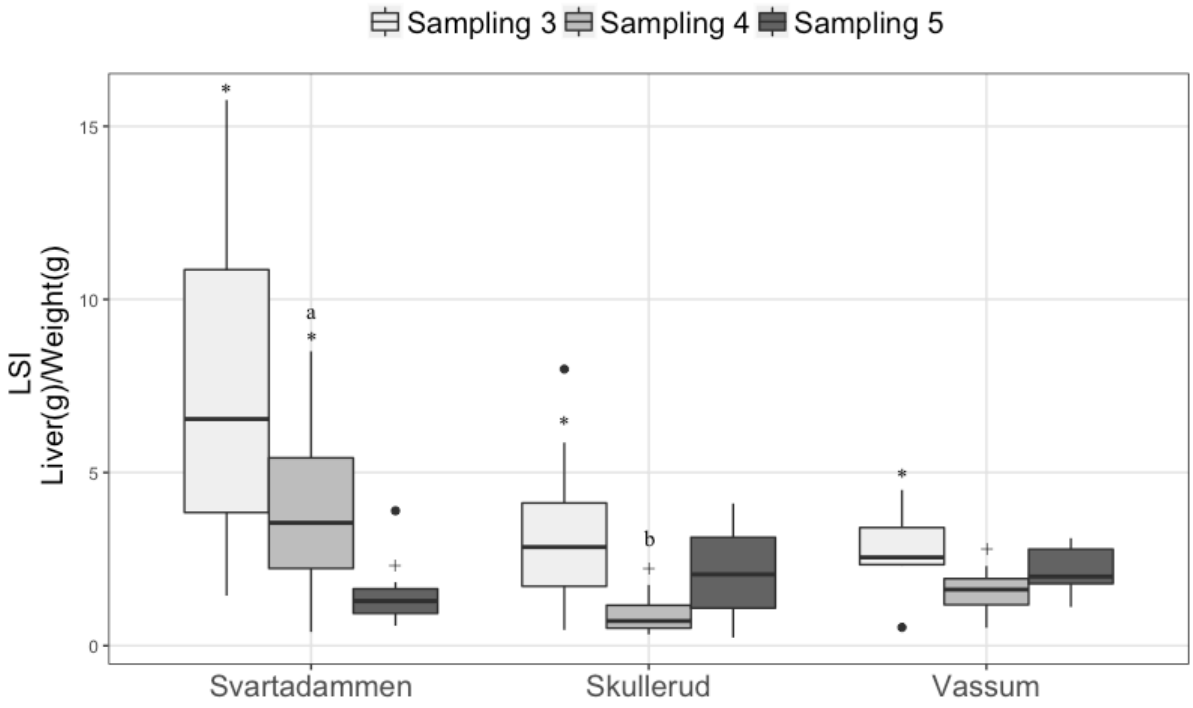

Figure 3.2.3: Liver somatic index of tadpoles from each location at sampling time 3,4 and 5. The box is defined by the 1st and 3rd quartiles, the line represents median and the whiskers extend to the most extreme data points that are no more than 1.5 times the IQR. The filled dots are observations that exceeds or fall below the range of the whiskers. The asterisk and the + sign represents a significant value in one location at different sampling times. The letter a and b represents a significant value between locations at the same sampling time. n = 9-11 in each group.

3.3 Biomarkers

3.3.1 PAH metabolites

The concentration of 1-OH-pyrene was higher in the tadpoles from Vassum compared to the other locations and the samples from Vassum were at all times above the detection limit (Figure 3.3.1a). The highest mean concentration in the Vassum tadpoles was detected at sampling time 4. In the tadpole samples from Skullerud there was a significant difference between sampling 3 and 5 (Tukey's HSD, p = 3.20e-05) and 4 and 5 (Tukey's HSD, p = 9.0e-06) and at Vassum a significant difference between sampling 3 and 4 (Tukey's HSD, p = 1.9e-04). At sampling 3 there was a significant difference between Svartadammen and Skullerud (Dunn's test, p = 0.02) and between Svartadammen and Vassum (Dunn's test, p = 0.009). A significant difference was found at sampling 4 between Svartadammen and Skullerud (Tukey's HSD, p = 1.3e-06), Svartadammen and Vassum (Tukey's HSD, p = 1.0e-09) and Skullerud and Vassum (Tukey's HSD, p = 4.0e-07). At sampling 5 Svartadammen was significantly different from both Skullerud and Vassum (Dunn's test; p = 0.007, p = 0.0002).

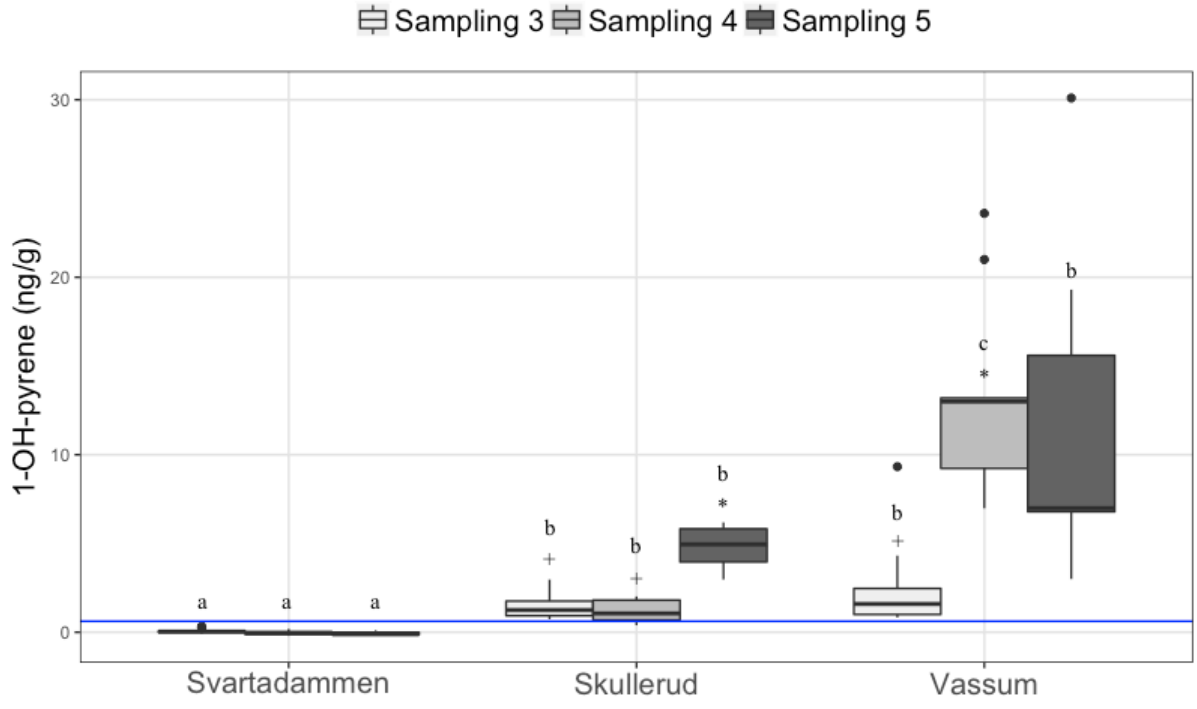

Figure 3.3.1a: 1-OH-pyrene in tadpole intestine from the 3 locations and sampling time 3,4 and 5. The box is defined by the 1st and 3rd quartiles, the line represents median and the whiskers extend to the most extreme data points that are no more than 1.5 times the IQR. The filled dots are observations that exceeds or fall below the range of the whiskers. The blue line represents the detection limit. The asterisk and the + sign represents significant difference in one location at different sampling times. The letters a, b and c represents significant difference between locations at the same sampling time. n = 9-11 in each group.

There were higher concentrations of 1-OH-phenantrene in Skullerud samples and at all sampling times the level was above the detection limit (Figure 3.3.1b). There was a significant difference between all sampling times in Skullerud (Tukey's HSD; 3 and 4 p = 0.003, 3 and 5 p = 1.0e-09 and 4 and 5 p = 7.0e-05) and between sampling 3 and 5 at Vassum (Tukey's HSD, p =0.03). There was significant difference between Svartadammen and Skullerud at all sampling times (Dunn's test; 3 p = 0.004, 4 p = 0.008 and 5 p = 0.0008) and between Skullerud and Vassum at all sampling times (Dunn's test; 3 p = 0.0002, 4 p = 0.0001, 4 and 5 p = 0.002).

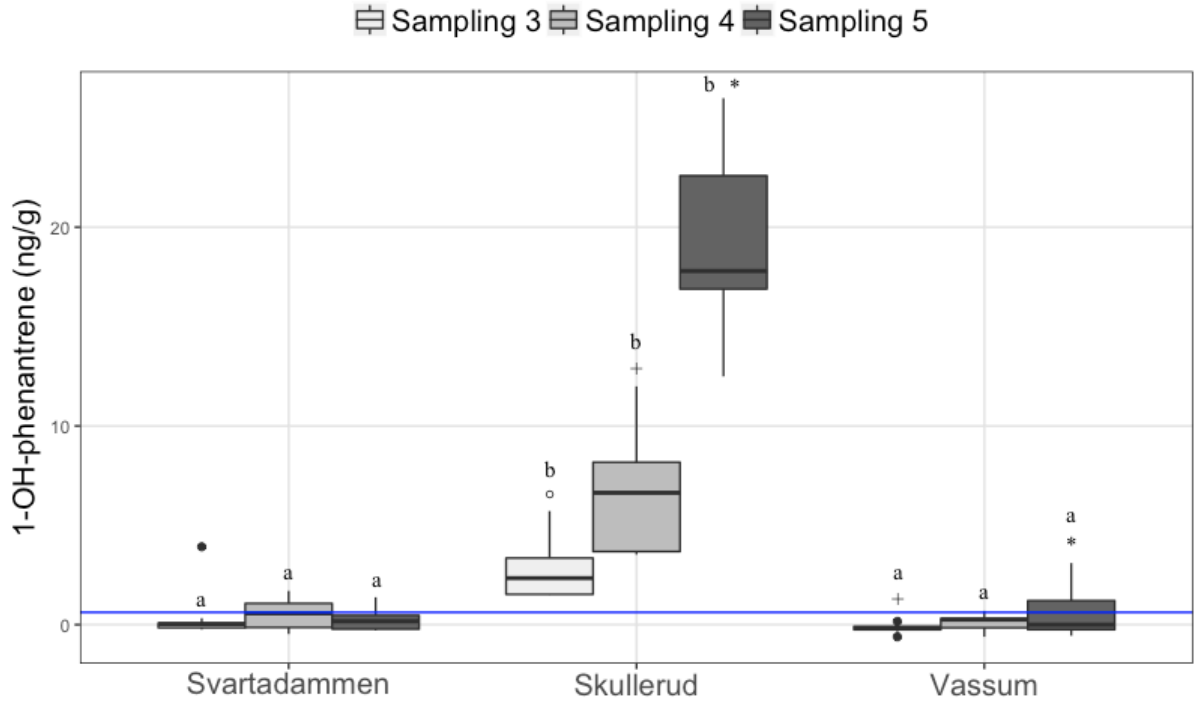

Figure 3.3.1b: 1-OH-phenantrene in tadpole intestine from the 3 locations and sampling period 3,4 and 5. The box is defined by the 1st and 3rd quartiles, the line represents median and the whiskers extend to the most extreme data points that are no more than 1.5 times the IQR. The filled dots are observations that exceeds or fall below the range of the whiskers. The asterisk, the + and ° sign represents significant difference in one location at different sampling times. The letters a and b represents significant difference between locations at the same sampling time. n = 9-11 in each group.

The concentration of 3-OH-benzo[a]pyrene was under the detection limit for all tadpole samples from all ponds.

3.3.2 CYP1A concentration

CYP1A protein was higher in the tadpoles from sedimentation ponds compared to the tadpoles from the reference pond (Figure 3.3.2). The highest observed levels were at the last sampling time in Skullerud, while the highest mean value was found at sampling 3 in Vassum. Significant difference was found at sampling 5 between Svartadammen and Skullerud (Tukey's HSD, p = 0.0005) and Svartadammen and Vassum (Tukey's HSD, p = 0.03). There was also significant difference between sampling 3 and 5 at Skullerud (Tukey's HSD, p = 0.03).

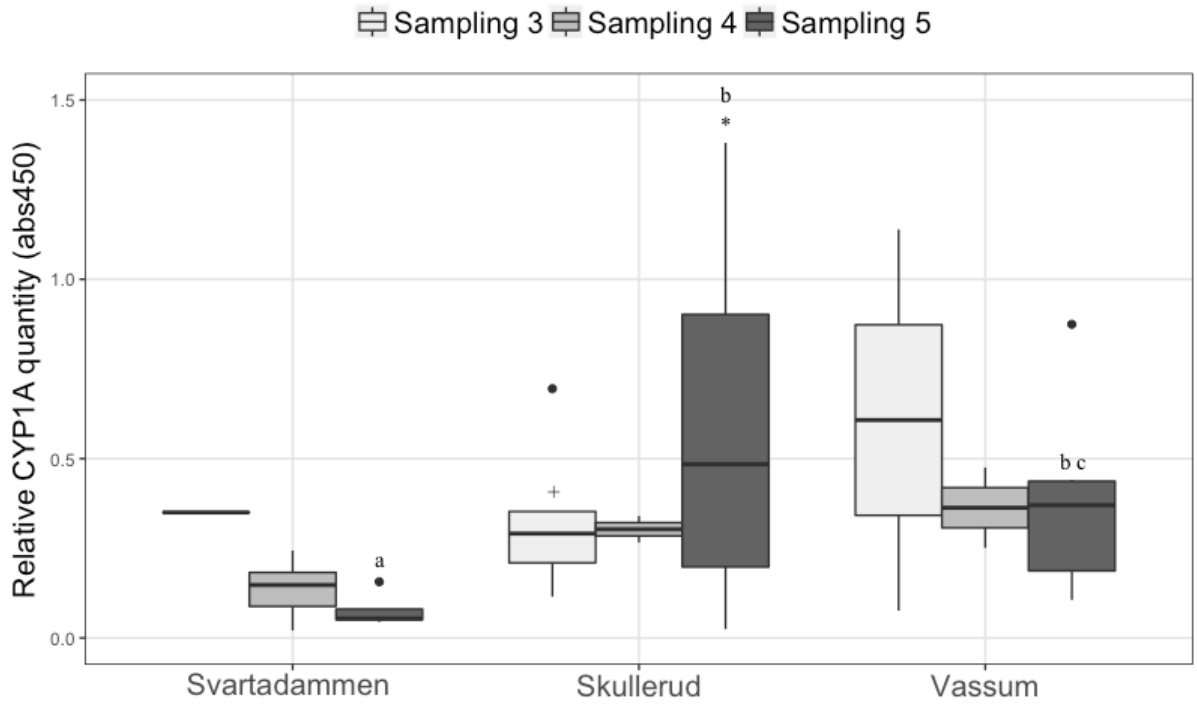

Figure 3.3.2: Relative CYP1A quantity in tadpole liver from the 3 locations and sampling time 3, 4 and 5. The box is defined by the 1st and 3rd quartiles, the line represents median and the whiskers extend to the most extreme data points that are no more than 1.5 times the IQR. The filled dots are observations that exceeds or fall below the range of the whiskers. The asterisk and + sign represents significant value between different sampling times at the same location. The letters a, b and c represents significant value between locations at the same sampling time. n = 4-10 in each group.

3.3.3 CYP1A activity

The CYP1A activity was measured in pmol resorufin/min/mg by EROD activity (Figure 3.3.3). There were high levels of CYP1A in sampling 4 and 5 in the sedimentation ponds, and an increase through the sampling periods. No statistical analysis was run because there were too many values below 0 and a statistical analysis of such small sample size would not provide reliable results.

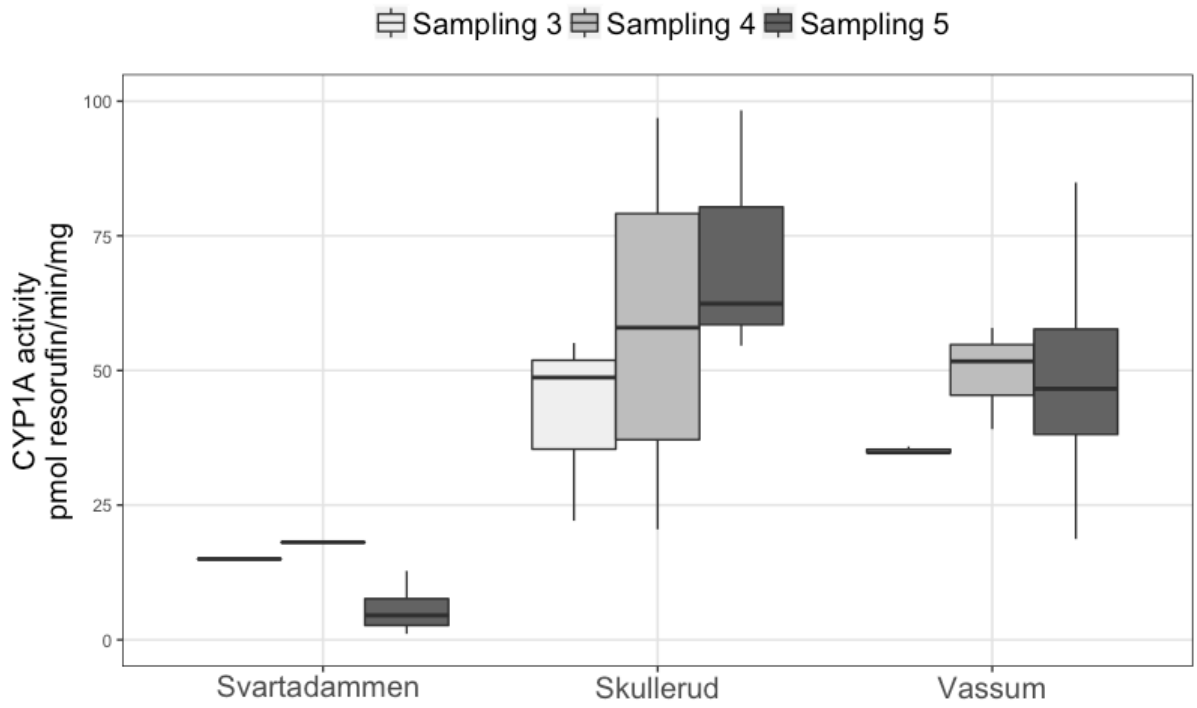

Figure 3.3.3: EROD activity in tadpole liver from the 3 locations and sampling time 3, 4 and 5. The box is defined by the 1st and 3rd quartiles, the line represents median and the whiskers extend to the most extreme data points that are no more than 1.5 times the IQR. n = 1-8 in each group.

3.3.4 DNA damage

DNA damage in tadpoles was higher in the last sampling from Vassum compared to the other locations and sampling times (Figure 3.3.4). The Vassum samples had an increasing level of damage, with some tadpoles having high levels of damage (> 40 %) in the last sampling. The damage was low in Svartadammen through the whole sampling period. There was a significance difference within Skullerud at sampling 3 and 5 (Dunn's test, p = 0.03) and at Vassum between all sampling times (Dunn's test; 3 and 4 p = 0.03, 3 and 5 p < 1.0e-09, 4 and 5 p = 0.03). At sampling 5 Vassum was significantly different from both Svartadammen (Dunn's test, p < 1.0e-09) and Skullerud (Dunn's test, p = 0.008).

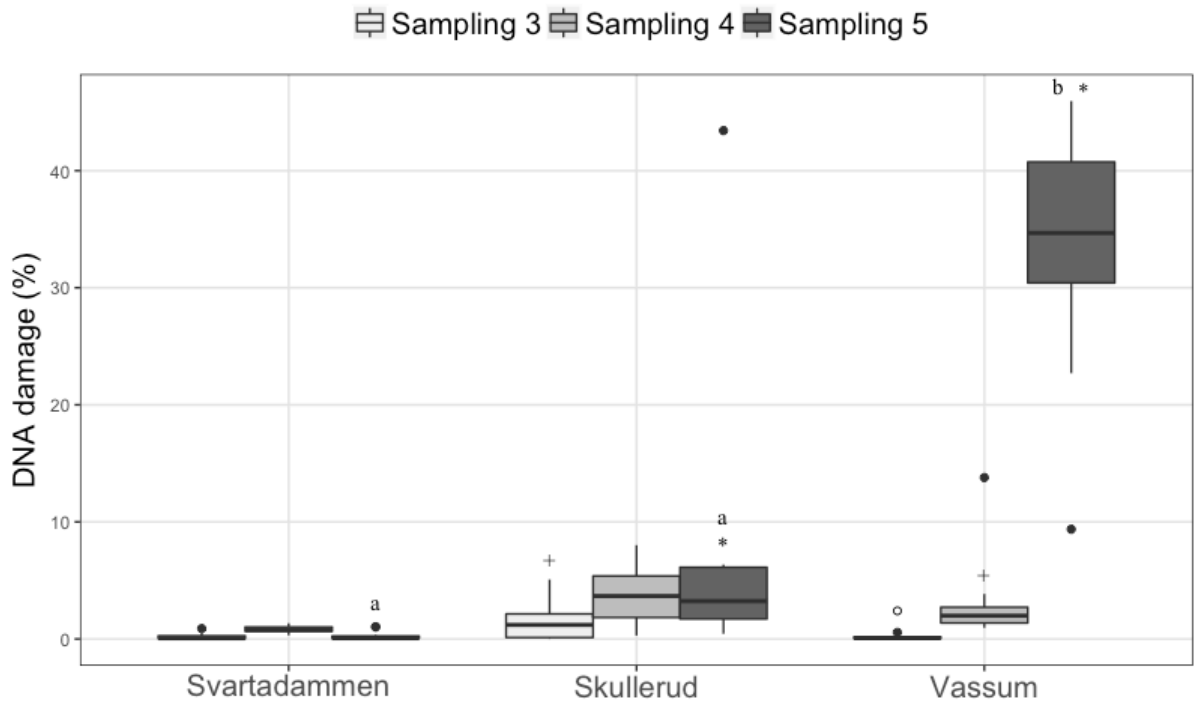

Figure 3.3.4: Percentage of DNA damage detected in tadpole blood from the 3 locations and sampling time 3, 4 and 5. The box is defined by the 1st and 3rd quartiles, the line represents median and the whiskers extend to the most extreme data points that are no more than 1.5 times the IQR. The filled dots are observations that exceeds or fall below the range of the whiskers. The asterisk, + and ° represents significant value between sampling times at one location. The letters a and b represents significant value between locations at the same sampling time. n = 9-11 in each group.

3.4 Principal component analysis

A clustering of tadpoles from Svartadammen was seen at all sampling times, moving toward the LSI variable where the samples from sampling 3 were closely related to LSI. Clustered near the center were samples from Skullerud and Vassum at sampling 3 and to some extent sampling 4 in Skullerud. A positive correlation between 1-OH-pyrene and comet was seen and a clustering in the Vassum samples at sampling 4 and 5 toward 1-OH-pyrene and comet. There was a negative correlation between 1-OH-pyrene and LSI as well as comet and LSI, meaning that when the LSI decreases 1-OH-pyrene and DNA damage increases. This very same trend was seen between 1-OH-phenantrene and LSI, where LSI decreases when 1-OH-phenantrene increases. 1-OH-phenantrene clusters with Skullerud samples at sampling time 5 and 1-OH-phenanthrene and CI was slightly positively correlated.

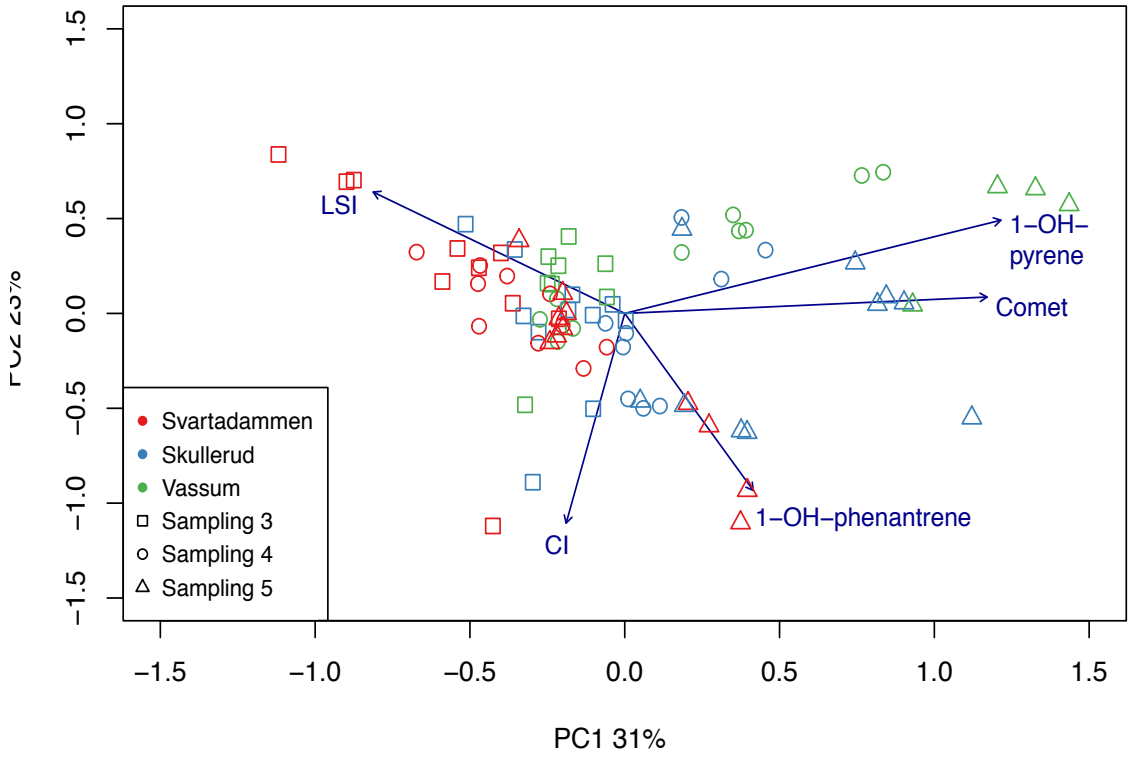

Figure 3.4: PCA biplot with PC axis 1 at the x-axis and PC axis 2 at the y-axis. The locations are shown in different colors, the sampling periods in different symbols. PC1 accounts for 31% of the variation in the dataset, PC2 accounts for 23%. The biomarker best correspondent with PC1 was LSI, while Comet and CI best corresponds with PC2. Points found in the center of the axis (0,0) will have a predicted value close to the mean value. A point close to the extremity of an arrow has an above mean predicted value of that biomarker. Points close to each other have a similar response to the biomarkers. The direction of the arrows indicate increasing value and the length of the arrows indicates increasing variation. Arrows pointing in the same direction are positively correlated and arrows pointing in opposite directions are negatively correlated. The correlation is indicated by the angle between arrows, with high correlation if small angle.

3.5 Experimental study

The data from a planned experimental study are not presented, due to 100% mortality of all tadpoles before or during hatching, presumably due to keeping the eggs/tadpoles for too long at the initial (sampling) temperature.

4 Discussion

The number of studies performed on *Rana temporaria* frogs and tadpoles are scarce in relation to organic contaminants. However, frogs from different species and families, e.g. *Xenopus* and *Rana*, show similar effects (Carlsson 2014). Comparisons in this discussion therefore includes other frog species, if possible *Rana* species, but extends to other species when necessary.

4.1 PAH concentrations

The sediment samples from the sedimentation ponds show that many of the PAHs were above the environmental quality standards (EQS) outlined by the Norwegian Environment Agency (2016). The result includes historical data of sediment samples taken from both Skullerud and Vassum, due to inconclusive results from Vassum from this study. Several of the contaminants in the sediment samples from Skullerud and Vassum were classified with poor quality. Aquatic organisms may show negative effects when exposed to such concentrations of contaminants over a short time. Many of the PAHs in the sediment samples were classified as moderate, indicating that exposures over a longer period of time could have negative effects on organisms. In a final risk assessment it is essential to include EQS for biota and water, as these are important factors when evaluating the overall effects of the contaminants. However, the observed concentrations in sediment give reason to believe that negative effects in tadpoles could occur. High concentrations of alkylated PAHs were found in the sediment samples from Vassum and Skullerud. Even though effects of alkylated PAHs were not tested directly in this study, there is no reason to believe that they do not affect organisms.

When comparing the total PAH16 found in the sediment samples from Vassum, the samples from the pre-sedimentation pond shows that this pond has a higher concentration of total PAH16 than the main pond, with a level of 5100 ng/g and 2118 ng/g respectively (Grung et al 2016). This indicates that the level of contaminants the tadpoles are subject to at Vassum is lower when living in the main pond and that the pre-sedimentation pond is limiting the amount of exposure. The high concentration found in the report by NPRA (Statens Vegvesen, 2014) indicates that the pond at Vassum has a varying degree of contamination and the concentration depends on factors such as precipitation, storms and tunnel wash events.

Fluoranthene and pyrene were the only non-metabolized PAHs quantified in biota. The highest levels were found in the Vassum sedimentation pond for both substances. Fluoranthene has an EQS in biota set at 30 µg/kg and the samples analyzed herein had a level of 0.008 µg/kg. There is no EQS set for pyrene. The values in all biota samples were very close to the detection limit where the analytical uncertainty can be up to 100%, which indicates unreliability of these results. The embryo samples from sampling 1 had similar concentrations in all ponds, suggesting that the coating outside the egg is useful in protection of the embryos. Studies have shown that the coating does contain contaminants (Marquis et al., 2006), but it does not necessarily mean that they enter the embryo. The low concentrations of PAHs found in this study thus supports the fact that the coating protects the embryo against organic contaminants (Marquis et al., 2006). The tadpoles from the second sampling show very low levels of PAHs. Before the second sampling there had been no precipitation events above 2 mm (Appendix B), which reduce the amount of contaminants entering the sedimentation ponds. Tadpoles have been previously sampled at later stages from Vassum and Skullerud, where the level of total PAH16 in tadpoles were 310 µg/kg and 100 µg/kg from Vassum and Skullerud respectively (Grung et al., 2016). This indicates that the level of contaminants in tadpoles increases with the length of time it stays in the sedimentation pond, and with the tadpoles increase in size. This is also seen for metal accumulation in tadpoles from the sedimentation ponds, the longer period the tadpoles stay in the ponds the more accumulation is observed (Johansen, 2013; Meland et al., 2013). There is reason to believe that the longer the tadpoles stay in the ponds the more affected they are by contaminants, seeing as the concentration of some of the PAHs found in sediment were classified as poor and indicate effects when exposed to the contaminants over a period of time.

Other contaminants can cause negative effects in tadpoles and previous studies have found metals and metalloids in both sediment and water samples from Skullerud and Vassum (Johansen, 2013; Meland et al., 2013). Metals can be acutely toxic to tadpoles and disrupt normal development (Mouchet et al., 2006a). De-icing of the roads is performed throughout the winter season and leads to elevated NaCl concentrations in the ponds. NaCl have been found to affect tadpoles negatively by decreasing growth and increasing mortality before metamorphosis (Brand et al., 2010; Gallagher et al., 2014; Squires et al., 2010).

4.2 Morphology and fitness

There are several parameters that determine and affect the growth rate and development of tadpoles. These parameters include presence of food sources, inter- and intra-specific competition, predators, temperature and contaminants. With regard to contaminant levels in the ponds, some of these parameters were examined further.

Morphology

In line with hypothesis 1 the tadpoles from sedimentation ponds were at different developmental stage compared to the tadpoles from the reference pond. The tadpoles from sedimentation ponds were at a later stage and were larger in size compared to tadpoles from the reference pond. This result is consistent with the finding of Brand et al. (2010) who observed that tadpoles exposed to sediment containing road runoff metamorphose earlier than non-exposed tadpoles. Other studies have found that tadpoles of several species of frogs metamorphose at a larger body size when living in areas affected by stormwater (Scheffers & Paszkowski, 2016; Sievers et al., 2018). Sievers et al. (2018) discovered that when the level of PAHs increased, the larger the tadpoles were at metamorphosis. In addition, exposed tadpoles showed a lower survival rate and lower olfactory response to predators. The decrease in the latter effect may increase the risk of predation when tadpoles are subject to contaminants. The presence of predators may also change life history traits of the tadpoles and potentially increase or decrease the rate of metamorphosis (Gazzola et al., 2017; Tiberti & von Hardenberg, 2012). There are predators present in all ponds investigated in this study, but the predators known to be in Vassum are invertebrates such as Odonata larvae while fish are present in Skullerud, including common minnow (*Phoxinus phoxinus*) and pike (*Esox lucius*). Studies have shown that predation from fish reduces survivorship and increases the risk of decreasing species richness and amphibian communities (e.g. Hamer and Parris 2011; Gaerne 2001). In Svartadammen the salamanders *Tritirus vulgaris* and *Triturus cristatus* are present, and these salamanders predate tadpoles. A decrease in survivorship and size has been shown when tadpoles of *Rana arvalis* were subject to predation from *T. vulgaris* (Henriksen 1990). There are in total four amphibian species present at Svartadammen and this can increase competition and reduce the growth rate in tadpoles (Griffiths et al., 1991).

Tadpoles are also dependent on abiotic factors that affect the growth and developmental rate. At Vassum there is good light conditions, with few trees overshadowing the pond. This in

turn results in a higher growth of nutrients and detritus that acts as food source for tadpoles, which may thus affect their growth rate (McDiarmid & Altig, 1999). The Skullerud pond is partly covered by a crossing bridge and vegetation around the pond. Svartadammen is located in an area with trees surrounding the pond providing shadow. Tadpoles exposed to more light may increase the sensitivity of tadpoles to some contaminants, e.g. PAHs (Hatch & Burton, 2009). The temperature in the water column was quite similar in all ponds, but there was a somewhat higher temperature at Vassum throughout the whole sampling period (Appendix B). The Skullerud pond is larger than the Vassum pond, and heating of the water will take longer in this pond compared to Vassum. The fact that tadpoles in the reference pond is at an earlier developmental stage and has a smaller size than the tadpoles found in the sedimentation ponds may be explained by several factors including the lower temperature in the water at Svartadammen (Merilä et al., 2008). Frogs are very flexible regarding their size at metamorphosis, and even individuals of the same species can metamorphose at different sizes (Wilbur and Collins 1973). Being at different stages can be caused by a plethora of factors, and may not directly indicate an effect of contaminants. An exposure study with specific concentration of contaminants is needed to assess the impact on developmental stages.

Condition

In the present study a decrease in tadpole fitness when the tadpoles were living in sedimentation ponds was expected. The fitness measurements that were included were condition index (CI) and liver somatic index (LSI).

There was no significant difference in the CI between the ponds, but a lower CI in the sedimentation ponds compared to the reference pond was observed. The length of the tadpoles increased more than the weight of the tadpoles, indicating that the tadpoles living in sedimentation ponds had lower fitness. The median weight of the tadpoles from Vassum were close to 0.9 g while the tadpoles from Skullerud were closer to 0.6 g. Earlier studies by Johansen (2013) detected the same pattern in the weight of tadpoles in the same sedimentation ponds. Different predators can affect size of tadpoles and in this study there are different predators present in each pond. Tadpoles subject to invertebrate predators, such as in Vassum, are expected to have a larger body size and tadpoles subject to fish predators, such as in Skullerud, are expected to have smaller body size (Johnson et al., 2015). This was confirmed in this study as tadpoles from Vassum were larger than tadpoles from Skullerud.

A bias to keep in mind when this study was performed is that even though the tadpoles were randomly sampled throughout the ponds the larger tadpoles are usually easier to capture. The smaller tadpoles are hidden between plants and other material in the ponds. This bias exists in all ponds and thus should not interfere with the interpretation of differences in tadpoles between the different ponds.

The LSI was lower at later sampling times, meaning that as the tadpoles grow the LSI is reduced. This was evident for all samples, and in Svartadammen a big change was observed between sampling 3 and 5. The LSI was generally lower in sedimentation ponds, which is consistent with hypothesis 2. In the tadpoles from the sedimentation ponds there was a reduction in LSI in the tadpoles from sampling 3 to 4 and an increase from sampling 4 to 5. Compared to the developmental stages, this drop in sampling 4 is evident at stage group 3 and similar over all locations. A precipitation event prior to sampling 4 increased the influx of chemicals in the ponds (Appendix B). Studies have shown that exposure to contaminants can decrease liver weight (Regnault et al., 2014; Zaya et al., 2011). The reduction of the LSI observed in all ponds can be a case of tadpoles allocating their energy into growth and preparation for metamorphosis (McDiarmid & Altig, 1999). The metamorphosis demands energy resources, and the liver may not be prioritized. This results in reduction of hepatic glycogen and or/lipid content. The increase in LSI in the last sampling, with tadpoles in stage group 4, may be a result of diet change to more lipid rich substances and feeding on larger animals (McDiarmid & Altig, 1999).

4.3 Biomarkers

4.3.1 PAH metabolites

In line with hypothesis 3 the tadpoles in sedimentation ponds metabolize PAHs to a greater extent than tadpoles from the reference pond. The metabolites that were detected in the intestinal samples were 1-OH-pyrene and 1-OH-phenanthrene. These metabolites derive from pyrene and phenanthrene, substances both found in the sedimentation ponds. The levels of both metabolites were above detection limit in the tadpoles from the sedimentation ponds while all the samples from the reference pond were below the detection limit. 1-OH-phenantrene was evident with a higher concentration in the samples from Skullerud where the concentration in sediment samples was between 170-190 μg/kg, and alkylated phenantrenes

were present as well. Previous studies of the water column (Table 4.1) show that in the latest studies the level of phenanthrene from road runoff was higher at 0.11 µg/L, compared to samples from tunnel wash water at 0.03 µg/L. This indicates that there are components of tunnel wash water that dissolves phenanthrene. 1-OH-pyrene was evident in tadpoles from Vassum and almost all of the Skullerud samples were above the detection limit. The highest level of 1-OH-pyrene was found in Vassum at sampling 4, indicating that the tadpoles had been exposed to PAHs. Just prior to sampling 4 there was a precipitation event with 48 mm downfall in 72 hours, after a dry period of 3 weeks, and the road runoff would subsequently have a higher concentration of contaminants. During precipitation episodes and tunnel wash events the concentration of road related contaminants in the ponds peak (Karlsson & Viklander, 2008; Lundberg et al., 1999). The level of pyrene was higher in tunnel wash water (Table 4.1) and might explain why the level of this metabolite was more present in the tadpole samples from Vassum. Both 1-OH-pyrene and 1-OH-phenantrene have been found in fish exposed to the tunnel wash water reaching Vassum (Skarsjø, 2015), and fish from Skullerud have levels of both metabolites exceeding the environmental assessment criteria for marine fish (Grung et al., 2016).

The source of PAHs can be studied by the use of relative amount of individual PAHs (Brown & Peake, 2006). Pyrene can be derived from petrogenic and pyrogenic sources, the former from oil and the latter generated through combustion processes (Neff et al., 2009). In Vassum, subject to tunnel wash water and road runoff, the heavier PAHs can become more available by the use of detergents from washing of the tunnels (Allan et al., 2016). Higher molecular weight PAHs, such as pyrene, may bind to particles in runoff water to a greater extent due to their lipophilicity (Simon & Sobieraj, 2006) and thus sediment upon entering the sedimentation pond. Phenantrene can come from petrogene sources, derived from fossil fuel such as oil. At both Skullerud and Vassum the ratio of certain PAHs in sediment samples are above 0.1 (anthracene / anthracene+phenanthrene) and below 0.5 (fluoranthene / fluoranthene+pyrene), which indicates a mix of pyrogenic/petrogenic and petrogenic sources (Karlsson & Viklander, 2008). In this study the tadpoles do take up some of the PAHs present in the sedimentation ponds, meaning that PAHs must be present and available for uptake in the water column. Water samples were not quantified in this study but samples previously taken at ponds subject to road runoff and tunnel wash water show measurable concentrations of PAHs (Table 4.1). This indicates that the exposure to contaminants is higher for tadpoles living in sedimentation ponds compared to naturally occurring ponds.

Table 4.1: The PAH concentration in water affected by road pollution. (TWW = Tunnel wash water, RR = Road runoff). Concentrations in water are presented in total µg/L.

	Hewitt and Rashed (1992)	**Paruch and Roseth (2008)**	**Zhang et al. (2009)**	**Meland et al. 2010ab**
Water	RR	TWW	RR	TWW
Phenantrene	0.25	0.17	0.11	0.03
Pyrene	0.29	0.61	0.09	0.23
Benzo[a]pyrene	0.01	0.36	0.01	0.02
Σ PAH16	1.86 (8)	-	0.55	0.82

4.3.2 CYP1A

The objective to quantify concentration and activity of CYP1A was performed by the use of two different analyses.

The results from the quantification of CYP1A, by the use of the ELISA assay, were consistent with hypothesis 4, with higher amounts of CYP1A in tadpoles from sedimentation ponds compared to tadpoles from the reference pond. As previously stated, tadpoles in sedimentation ponds are subject to PAHs and these contaminants stimulate the induction of CYP1A. The increase in sampling 3 in the Vassum tadpoles cannot be directly explained, however prior to the sampling there had been a dry period of approximately 3 weeks followed by a precipitation event with approximately 40 mm downfall in 48 hours (Appendix B). The precipitation event contributed to an influx of contaminants prior to the time of sampling. Quantification of CYP1A previously performed on fish from Skullerud were in line with the levels of CYP1A in this study (Grung et al., 2016), and previous studies of fish exposed to tunnel wash water from Vassum indicate an increase in transcription and induction of CYP1A activity in brown trout (*Salmo trutta*) (Meland et al., 2010b).

The results for hepatic CYP1A activity, measured as EROD, were consistent with hypothesis 5 and show that EROD activity was elevated in tadpoles living in sedimentation ponds compared to the reference pond. Although not tested significantly the results show that the activity was higher in the sedimentation pond and lower in the reference pond, suggesting a response to environmental exposure. The result is in line with previous studies in tadpoles exposed to PAHs. Tadpoles of wood frogs (*Lithobates sylvaticus*) exposed to high concentrations of PAHs in oil sand wetlands showed increased EROD activity (Hersikorn &

Smits, 2011). Tadpoles of *Xenopus laevis* showed EROD levels similar to the present study after exposure to water contaminated from industrial activity (Gauthier et al 2004). Jung et al. (2004) hypothesized that tadpoles have less developed CYP450 enzymes compared to adults, with activity of 5 pmol/min/mg in metamorphs and 0.3 pmol/min/mg in tadpoles of the green frog (*Rana clamitans*) and measurements of 238 nmol/min/mg in adult cane toad (Rocha-e-Silva et al., 2004). Johansen (2013) found similar EROD activity as Jung et al. (2004) in common frog tadpoles from Vassum and Skullerud, however that study measured the whole tadpole and was not tissue specific. The present study observed higher EROD activity in tadpoles compared to the study of Jung et al. (2004) and Johansen (2013), with levels up to 85 pmol/min/mg, indicating that the tadpoles in this study have well-functioning CYP1A enzymes. It would be interesting to investigate the level of CYP1A of adult *Rana temporaria*, as the levels for tadpoles are far below the levels detected in adult cane toad with 238 nmol/min/mg (Rocha-e-Silva et al., 2004). Fish exposed to tunnel wash water from Nordbytunnelen, which ends up in Vassum sedimentation pond, had elevated EROD activity with levels of up to 500 pmol/min/mg (Skarsjø, 2015) which was far above the activity found in this study but it supports the fact that there are contaminants present in the pond that induce CYP1A and its activity.

4.3.3 DNA damage

In line with hypothesis 6, DNA damage was higher in tadpoles from sedimentation ponds compared to tadpoles from the reference pond. The comet assay is a picture of the cell at the exact time the samples were taken, therefore the assay will detect both strand breaks caused by exogenous compounds and naturally occurring strand breaks. When a cell replicates and transcribes, it will create temporary strand breaks, thus the level of DNA damage in the tadpoles from the reference pond is thought to be of natural origin and not caused by contaminants. With levels of strand breaks under 5% found in the reference pond compared to over 40% in the sedimentation ponds gives reason to believe that the tadpoles from the sedimentation ponds have been subject to contaminants inducing DNA damage. The level of DNA damage in tadpoles from Skullerud was below 10% in most tadpoles at all sampling times. Previous studies of fish from this pond show levels of DNA damage up to 50%, but with a median of approximately 15% (Grung et al., 2016). The tadpoles from Vassum had a higher percentage of DNA damage in tadpoles at the latest sampling time. These tadpoles spent the longest time in the pond, and were thus subject to contaminants for a longer period

of time. With the higher bioavailability of contaminants in the Vassum pond, due to the use of detergents when maintaining the tunnels (Allan et al., 2016; Andersen & Vethe, 1994) genotoxic compounds may have become more available to the tadpoles. After a tunnel wash event in Nordbytunnelen, a high amount of mortality was observed in tadpoles the following day (Johansen, 2013). This illustrates that tunnel wash water, and the detergents, can be acutely toxic to tadpoles. The comet assay has been carried out in amphibians, both in tadpoles and adults with significantly higher damage in organisms exposed to contaminants (e.g. Mouchet et al., 2005a). Increase in DNA damage has been found in *Xenopus laevis*, after one day of exposure to hydrocarbons in draining water (Mouchet et al., 2005b). Although some studies have shown that DNA damage in tadpoles decreases after a period of exposure to PAHs (Mouchet et al., 2006b), the high levels of DNA damage observed in Vassum samples suggest that negative effects on the population can occur.

Other contaminants, e.g. lead, present in road runoff have shown to increase DNA damage in frogs (Mouchet et al., 2006a). Meaning that the mixture of contaminants present in the ponds may cause the DNA damage seen in this study. In the ponds, several factors need to be taken into account regarding sensitivity to contaminants. Light exposure can increase the sensitivity to contaminants, and studies investigating the effects of sunlight and exposure to low concentrations of fluoranthene showed that tadpoles of *Rana pipiens* and *Xenopus laevis* experienced greater sensitivity (Hatch & Burton, 2009). For this study the tadpoles at Vassum were more exposed to sunlight, as previously stated, and therefore possibly more sensitive to contaminants. This might partly explain why a higher percentage of DNA damage was found in tadpoles from this pond. The increasing sunlight will also lead to more hypoxic waters and degradation of sediment at Vassum has previously been observed (Rambøll, 2016). This may in turn decrease the oxygen availability in the water column and increase stress in tadpoles (Crowder et al., 1998), and in turn increase their sensitivity to contaminants. The fact that there were more contaminants present in the Vassum pond was thought to be the main reason for the increase in DNA damage in the tadpoles from this pond.

Although there are few studies investigating the effect of organic contaminants on DNA strand breaks, several studies have investigated micronucleus formation (Fanali et al., 2018; Gauthier et al., 2004; Matson et al., 2009). The increase in micronucleus formation when subject to similar concentration of contaminants gives reason to believe that genotoxic effects occur in tadpoles living in the sedimentation ponds.

4.4 Multivariate relationships

In general, the PCA plot was consistent with the result from condition and biomarkers in the tadpoles and the correlation between biomarkers that show negative effects in the tadpoles. The PCA had a positive correlation between DNA damage and 1-OH-pyrene with a clustering of tadpoles from Vassum. High levels of DNA damage and higher concentration of the metabolite were observed in the Vassum tadpoles. This suggests that the tadpoles have been exposed to pyrene, possibly causing DNA damage. Biotransformation of contaminants can lead to reactive metabolites, in turn inducing DNA damage. A high level of the pyrene metabolite was found at sampling 4, and the DNA damage was highest in sampling 5 in the tadpoles from Vassum.

The LSI was negatively correlated with the metabolite species, especially 1-OH-phenantrene and a clustering of the samples from Svartadammen close to the LSI were observed. This was consistent with the finding that there were low levels of the metabolites and higher LSI in the samples from Svartadammen. When tadpoles are exposed to contaminants, energy is allocated to producing detoxifying enzymes. This will in turn lead to less energy available for use in physiological processes. Therefore, the decrease found in LSI may indicate that the energy reserve is allocated to detoxifying contaminants.

The PCA plot explains 54% of the total variation in the dataset. The part of the variation not explained in this plot may represent some underlying gradients that might not have been included in the plot or in the study. Including the analysis of CYP1A quantity and EROD activity would have been interesting, to further investigate if these two variables could contribute to explain more of the variation.

4.5 Exposure study

The exposure study would have provided more information about the actual exposure and effects of sediment-bound contaminants to the tadpoles. The reasons for the failure of the experiment are not clear and were probably caused by several factors.

The temperature in the room was kept stable at 8°C while temperature at the actual locations increased over time, hence the tadpoles developed slower than under field conditions. Under normal circumstances tadpoles usually postpone development until conditions are favorable

(McDiarmid & Altig, 1999). However, some studies have shown that when common frog tadpoles have been exposed to low temperatures for a longer period of time developmental rates can come to a point were increased energy expenditure affect body size negatively (Laugen et al., 2003). The tadpoles were to be moved to a room with higher temperature, however the tadpoles had possibly reached a stage were further development was no longer possible. Another explanation is that there could have been an issue with the egg cluster. The tadpoles were all from the same pond and there is always a large degree of mortality during the development. The common frog lay up to 4000 eggs, but only a small amount (up to 50) tadpoles will metamorphose to the juvenile/adult stage (Dolmen, 2008; McDiarmid & Altig, 1999).

A combination of these factors is thought to be the main reason for the death of the tadpoles since no tadpoles survived, not even in the control.

4.6 Biodiversity hotspot or ecological trap?

Sedimentation ponds are found to be a useful measure for reducing the amount of road-associated contaminants entering the aquatic environment. However when deciding on how to mitigate the contaminants, one should consider their effect on organisms. This study shows effects on tadpoles exposed to road related contaminants. Previous studies from the sedimentation ponds have observed that tadpoles accumulate other contaminants such as metals (Johansen, 2013) and other studies have found elevated concentrations of PAHs and other road related contaminants in plants, fish and invertebrates (Grung et al., 2016; Johansen, 2013; Meland et al., 2013). Although many studies emphasize the fact that these ponds are habitat for many organisms, and thus increasing biodiversity in a world were ponds and wetland areas are decreasing (e.g. Brand & Snodgrass, 2010; Le Viol et al., 2012; Sun et al., 2018), it is important to keep in mind the risk of contaminants being harmful to the performance and functionality of organisms (e.g. Mouchet et al., 2005b; Sievers et al., 2018). With a tunnel wash event resulting in close to 100% mortality of frogs in the receiving pond, NPRA concluded that open sedimentation ponds should no longer be constructed when new tunnels are built. A question regarding the already present ponds receiving tunnel wash water needs to be included. There was no tunnel wash event during the sampling of tadpoles from Vassum in this study, but if an influx of tunnel wash water were to happen during the tadpole development a larger impact on the organisms could possibly have occurred. Seeing as the

function of ponds receiving road runoff and especially tunnel wash water could be an ecological trap and help contaminants transport, and possibly accumulate, in the ecosystem, including more measures to filter the water before the open pond should be further investigated. In Vassum, investigations of additional filter mechanisms of the tunnel wash water from the surrounding tunnels resulted in several options that could improve the mitigation of the contaminants (COWI, 2012; Rambøll, 2016). One suggestion was to introduce further filtering of the water from both tunnels and roads, which will reduce the amount of contaminants entering the sedimentation pond. Another suggestion was to construct a separate, closed, water reservoir for tunnel wash water from the three tunnels. This would reduce the amount of contaminants reaching the sedimentation pond, and result in the pond only receiving road runoff. In tadpoles from Skullerud there are less negative effects, for instance in DNA damage, compared to the Vassum pond. To remove the tunnel wash water from the pond could hence be an action that reduces the effect on organisms in the pond and reduce the amount of contaminants entering the surrounding water bodies.

5 Conclusions

The overall aim of this study was to investigate whether tadpoles living in ponds receiving road-associated contaminants were negatively affected. When investigating whether tadpoles in sedimentation ponds had disrupted normal conditions the result showed no apparent difference between tadpoles from sedimentation ponds and from the reference pond. The tadpoles from sedimentation ponds were generally larger, and at later stages, than the tadpoles from the reference pond. The differences that were observed in size, CI and LSI could possibly be caused by contaminants, but other factors may have been involved. This indicates that the fitness of the tadpoles from sedimentation ponds is not necessarily lower than in tadpoles from naturally occurring ponds.

In general the biomarkers showed higher levels compared to the reference pond. There were found elevated concentrations of PAH metabolites, induction of CYP1A, increase in CYP1A activity and more DNA damage in tadpoles from sedimentation ponds compared to the reference pond. This indicates that the tadpoles in the sedimentation ponds are subject to contaminants that are available for uptake. The largest percentage of DNA damage was found in the tadpoles from Vassum, there is reason to believe that tunnel wash water affects the tadpoles to a greater extent compared to exposure to road runoff only. The sediment samples from Vassum had a slightly higher concentration of PAHs, but the difference is not very large. Hence the result does not readily suggest that tadpoles living in sedimentation ponds receiving both road runoff and tunnel wash water are more affected by PAHs than tadpoles only exposed to road runoff. It is of importance to bear in mind that this was a study conducted under field conditions, resulting in variations that cannot be controlled for.

The overall result indicates that the tadpoles living in sedimentation ponds are affected by contaminants. At which level it interferes with their development is uncertain, but nonetheless the results from this study show negative effects in the tadpoles living in ponds affected by road-related contaminants. Thereupon there is reason to conclude that sedimentation ponds are affecting tadpoles' normal function. Construction of sedimentation ponds is a useful measure to protect the surrounding ecosystem, yet it affects organisms living in such ponds. It is of importance to take this into account when deciding on the mitigation process when building and maintaining roads and tunnels.

6 Future perspectives

A lab exposure study would be a good indicator to determine the effects of contaminants in road runoff. This will give a more direct link to the stages and a better foundation to base the results upon. The exposure study should expose tadpoles to sediment from ponds receiving road runoff and from ponds also receiving tunnel wash water and the study should follow the increase in temperature seen in nature. To include a similar study investigating the effects of the water from the ponds would also be beneficial.

To examine the ponds at different time periods and intervals would be beneficial. During precipitation events the amount of contaminants in the ponds will change and other abiotic elements change the bioavailability of contaminants. During this study there was no tunnel wash events performed with detergents, hence it would be of interest to investigate the direct effect of tunnel wash water on tadpoles, especially to further investigate the mortality observed in Vassum following a tunnel wash event. If there are large effects, adding an extra filter step in already existing ponds could be a solution. To include investigation of several ponds that receive road runoff, and ponds that receive tunnel wash water and runoff would give a clearer picture of whether ponds receiving tunnel wash water are affecting organisms to a greater extent. It would also give a better picture of the general effect the contaminants have on organisms living in ponds receiving road-related contaminants.

In regard to biomarkers it would be interesting to examine the difference in response in tadpoles compared to adult frogs from the same pond. Other organisms are also present in the ponds and to investigate the effects on other organisms would be beneficial. This could be included to get information about effects on the different trophic levels in the ponds and to get a clearer picture of the effect on the ecosystem. Further investigation of the effect of other contaminants normally found in sedimentation ponds, including metals and alkylated PAHs, would further give a better picture of the mixture of contaminants, and sources, that cause negative effects and further enhance the regulatory perspective.

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Appendix A

Table A1: Softwares used in the study.

Software	Version
R Studio	1.0.136
Comet Assay IV	4.2
QGIS	2.18.14
HOBOware	3.7.11

Table A2: The equipment used for the study.

List of equipment	Producer/Supplier
20 L plastic container	Biltema
5 L steel buckets	Biltema
Aquariums 20 L fully molded	VWR
Precellys homogeniser	Bertin Technologies
Cryolys	Bertin Technologies
Haerus Multifuge 3 S-R	Thermofisher
Sorvall MTX150 Micro-Ultracentrifuge	Thermofisher
Fluorescense microscope	Carl Zeiss Axio Scope A1
Real time camera	Allied Vision Technologies
Sonifier 450 (Ultrasound)	Branson
Micro-haematocrit Capillary Tubes	Brand™

Table A3: The chemicals used for the study and in what analysis they were used.

List of Chemicals	Cat/Ca sno	Producer/Supplier	Used in
Alkaline copper tartrate solution (Reagent A)	Kit. No. 5000-0111	BioRad	e
Ascorbic acid (5 %)	A9202/50-81-7	Sigma	c
Bovine serum albumin	A7030	Sigma-Aldrich	g
Bovine gamma globulin	P5369	Sigma-Aldrich	e
Carbonate-bicarbonate buffer (coating buffer)	C3041	Sigma-Aldrich	g
Dimethyl sulfoxide	D5879/67-68-5	Sigma-Aldrich	b, f
Dithiothreitol (DTT)	3483-12-3	Sigma	d
EDTA	20302.260 500g/6381-92-6	VWR	d
Ethanol	200-578-6	Kemetyl	a, b
Folin reagent (Reagent B)	Kit. No. 5000-0111	BioRad	e
Glycerol	G5516 1L/56-81-5	Sigma	d
H_2SO_4	1.00731.1000	Merck	g

HCl (37 %)	20252.290 1L/7647-01-0	VWR	b
K_2HPO_4 trihydrate	221317	Sigma	e
KCl	7447-40-7	Merck	d
KH_2PO_4	P5655/7778-77-0	Sigma	b
KH_2PO_4	P-5379	Sigma	e
LMP agarose	A9414/39346-81-1	Sigma	b
Methanol	32213N 2.5L/67-56-1	Sigma	c
MS-222	E70521/888-86-2	Sigma-Aldrich	a
Na_2EDTA	6381-92-6	VWR	b
$Na_2EDTA{\cdot}2H_2O$	E 5134/6381-92-6	Sigma	b
Na_2HPO_4	795410 100g/7558-79-4	Sigma	b
Na_2HPO_4 (dibasic)	S5136 500g/7558-79-4	Sigma	d
NaCl	27810.295 1kg/7647-14-5	VWR	b, f, g
$NaH_2O_4xH_2O$ (monobasic)	75688250g/144-62-7	Sigma	d
NaOH	28244.295/1310-73-2	VWR	b
Rabbit anti-fish CYP1A antibody	CP2266	Biosense Laboratories	g
Resorufin ethyl ether (7-ethoxyresorufin)	E-3763	Sigma-Aldrich	f
Resorufin sodium salt	R3257	Sigma-Aldrich	f
Goat anti-rabbit IgG conjugated with HRP	A0545	Sigma	g
SYBR gold	S11494	Thermo Fisher scientific	b
TMB Plus	4395L	Kem-En-Tech	g
Triphenylamine	T81604	Sigma-Aldrich	c
Tris-EDTA	T9285	Sigma	b
Triton X-100	93443/9002-93-1	Sigma	b
Trizma base	T1503-500g/77-86-1	Sigma	b,g
Trizma-HCl	1185-53-1	Sigma-Aldrich	b,g
Tween-20	P1379	Sigma-Aldrich	g
β-glucuronidase/aryl sulfatase from Helix pomata	9001-45-0	VWR	c
β-NADP reduced tetrasodium salt	N-1630	Sigma-Aldrich	f

a: Dissection

b: Comet assay

c: PAH metabolites

d: Isolation of S100

e: Quantification of protein

f: EROD

g: ELISA

Appendix B

The following graphs and tables represent the temperature in the water column and precipitation events during the field study. The drop observed in early May in all temperature samples was a period were there was frost on some of the ponds, this also resulted in death of several clusters of eggs. The temperature was measured using HOBO Pendant data loggers from onset. Results from the temperature loggers were analyzed using HOBOware version 3.7.11 (2002-2017 Onset Computer Corporation). The precipitation is the mean per 24 hours, temperature mean per 24 hours and average daily temperature is the "døgntemperatur".

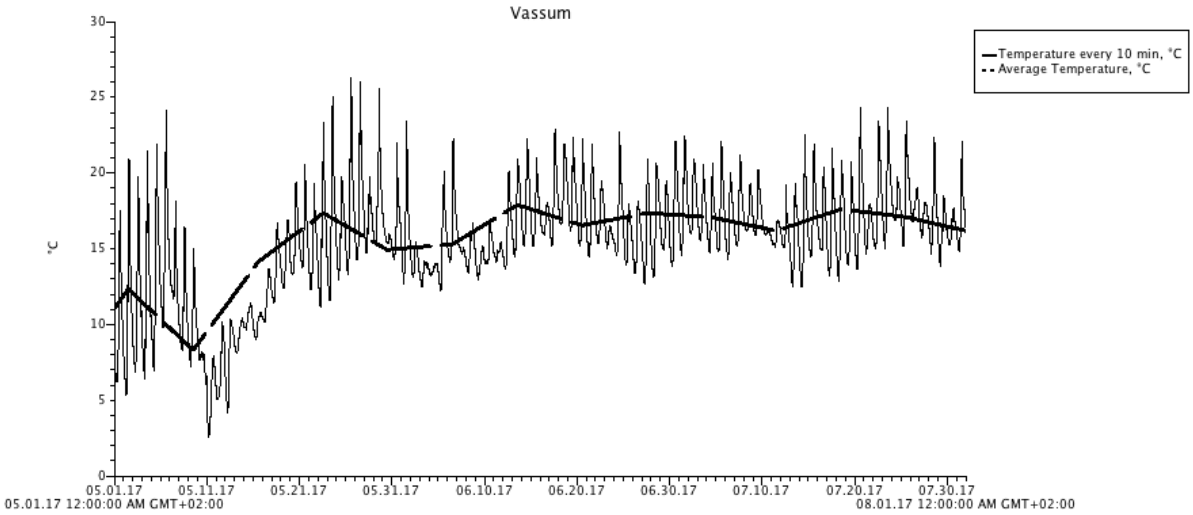

Figure A1: Water temperature in the Vassum pond 01.05-10.07.2017. The line represents the average temperature during 24 hours.

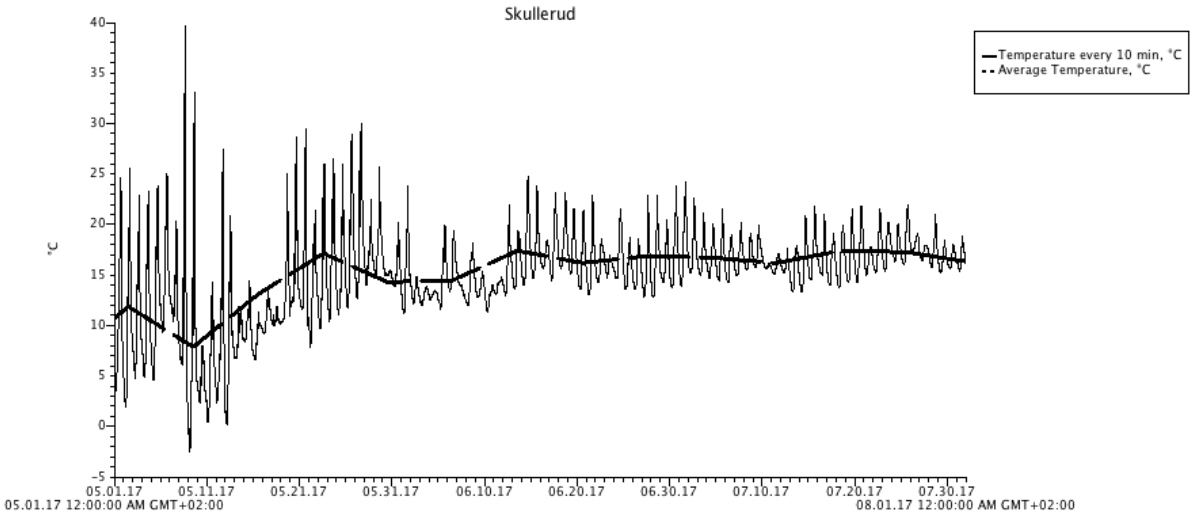

Figure A2: Water temperature in the Skullerud pond 01.05-10.07.2017. The line represents the average temperature during 24 hours.

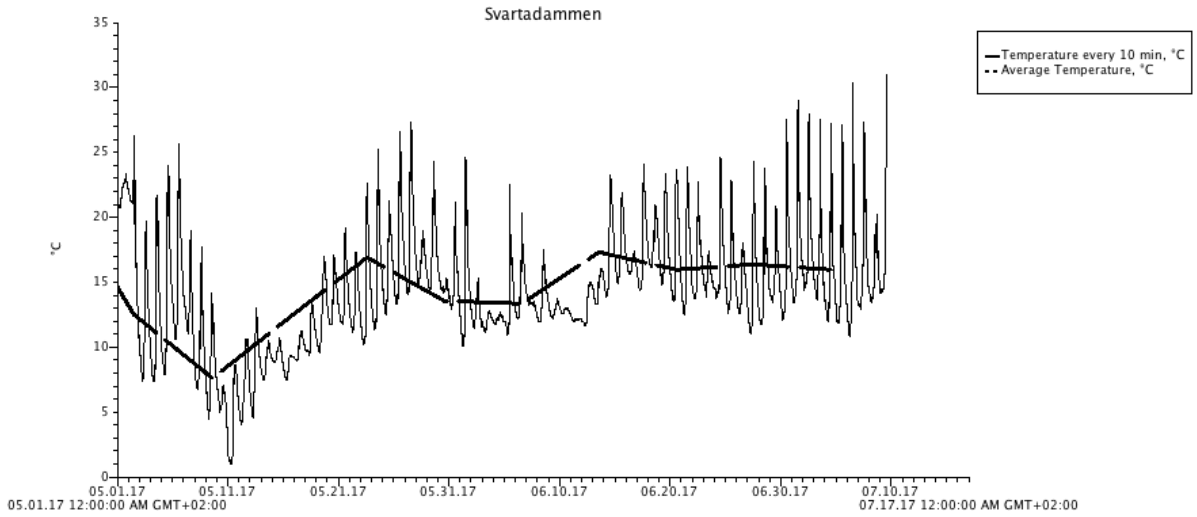

Figure A3: Water temperature in the pond Svartadammen 01.05-10.07.2017. The line represents the average temperature during 24 hours.

Table A4: The precipitation events at Ås (Vassum) and Lambertseter (Skullerud and Svartadammen), Mean water temperature at all ponds and average day temperature in regard to exposure time for tadpoles. The highlighted rows are when the sampling times took place.

Date	**Ås** Precipitation (mm/24hours)	**Lambertseter** Precipitation (mm/24hours)	**Svarta** Mean Temp.°C	**Skullerud** Mean Temp °C	**Vassum** Mean Temp°C	**Svarta** Avg temp*days	**Skullerud** Avg temp*days	**Vassum** Avg temp*days
01.05	0.0	0.0	18.963	10.478	10.483	18.963	10.478	10.483
02.05	**0.0**	**0.0**	**11.854**	**10.883**	**10.833**	**23.708**	**21.766**	**21.666**
03.05	0.0	0.0	12.412	11.232	11.291	37.236	33.696	33.873
04.05	0.0	0.0	13.901	12.338	12.717	55.604	49.352	50.868
05.05	0.0	0.0	15.686	14.831	14.579	78.43	74.155	72.895
06.05	1.9	4.1	12.875	12.767	12.109	77.25	76.602	72.654
07.05	1.9	0.1	10.029	12.003	10.035	80.232	96.024	80.28
08.05	0.2	0.0	8.287	7.351	9.364	74.583	66.159	84.276
09.05	7.4	5.7	5.809	4.463	7.054	58.09	44.63	70.54
10.05	10.9	10.3	4.505	5.693	4.989	49.555	62.623	54.879
11.05	**0.1**	**8.2**	**6.997**	**8.002**	**6.641**	**83.964**	**96.024**	**79.692**
12.05	0.2	0.0	8.313	7.757	7.845	108.069	100.841	101.985
13.05	3.2	1.1	8.89	8.953	9.02	124.46	125.342	126.28
14.05	3.0	3.6	9.465	9.862	10.206	141.975	147.93	153.09
15.05	0.0	1.0	8.561	8.722	9.67	136.976	139.552	154.72
16.05	15.9	19.7	10.027	10.828	11.876	170.459	184.076	201.892
17.05	16.5	19.0	11.041	10.545	13.872	198.738	189.81	249.696
18.05	2.4	2.4	12.651	13.378	14.246	240.369	254.182	270.674
19.05	0.0	0.0	13.628	15.658	15.244	272.56	313.16	304.88
20.05	1.4	0.7	14.06	15.847	15.543	295.26	332.787	326.403

21.05	0.5	0.1	13.458	13.521	14.592	296.076	297.462	321.024
22.05	**0.0**	**0.8**	**14.537**	**15.395**	**15.481**	**334.351**	**354.085**	**356.063**
23.05	0.0	0.0	15.803	16.045	16.298	379.272	385.08	391.152
24.05	0.0	0.0	15.819	15.528	15.948	395.475	388.2	398.7
25.05	0.0	0.0	17.674	17.646	17.57	459.524	458.796	456.82
26.05	0.0	0.0	18.541	18.744	17.91	500.607	506.088	483.57
27.05	0.0	0.0	16.563	16.76	16.679	463.764	469.28	467.012
28.05	0.0	0.0	17.66	18.034	18.389	512.14	522.986	533.281
29.05	0.6	1.1	14.769	15.317	15.771	443.07	459.51	473.13
30.05	0.6	1.8	14.855	15.454	16.403	460.505	479.074	508.493
31.05	2.6	0.8	14.807	15.157	16.099	473.824	485.024	515.168
01.06	0.0	0.0	12.732	13.409	14.127	420.156	442.497	466.191
02.06	1.6	1.7	11.98	12.945	13.587	407.32	440.13	461.958
03.06	4.0	0.5	12.15	12.974	13.284	425.25	454.09	464.94
04.06	6.1	5.0	14.131	14.949	15.48	508.716	538.164	557.28
05.06	0.4	4.9	14.622	15.654	16.063	541.014	579.198	594.331
06.06	1.2	1.3	13.296	13.815	14.809	505.248	524.97	562.742
07.06	12.5	13.8	13.857	14.608	14.441	540.423	569.712	563.199
08.06	14.7	5.1	12.91	13.804	13.688	516.4	552.16	547.52
09.06	31.1	32.6	12.785	12.782	14.803	524.185	524.062	606.923
10.06	0.2	0.7	12.17	13.897	14.404	511.14	583.674	604.968
11.06	1.6	1.7	13.264	15.819	15.575	570.352	680.217	669.725
12.06	**0.0**	**0.9**	**14.648**	**16.097**	**17.084**	**644.512**	**708.268**	**751.696**
13.06	0.9	0.7	17.501	17.907	17.328	787.545	805.815	779.76
14.06	0.0	0.5	17.528	17.618	16.769	806.288	810.428	771.374
15.06	1.4	0.0	16.385	16.839	16.496	770.095	791.433	775.312
16.06	9.3	0.5	17.845	17.773	17.945	856.56	853.104	861.36
17.06	0.0	2.4	18.074	18.243	18.332	885.626	893.907	898.268
18.06	0.0	0.0	17.76	17.458	18.236	88.00	872.9	911.8
19.06	1.2	6.7	17.182	16.581	17.31	876.282	845.631	882.81
20.06	0.0	0.0	16.536	16.38	16.71	859.872	851.76	868.92
21.06	0.0	0.0	16.45	16.222	16.82	871.85	859.766	891.46
22.06	0.0	0.0	15.325	15.879	15.537	827.55	857.466	838.998
23.06	3.4	4.3	17.154	17.055	16.797	943.47	938.025	923.835
24.06	0.0	0.0	15.62	15.714	15.12	874.72	879.984	846.72
25.06	0.2	0.2	14.567	15.677	15.342	830.319	893.589	874.494
26.06	**0.9**	**1.6**	**14.804**	**15.904**	**15.338**	**858.632**	**922.432**	**889.604**

Appendix C

Table A5: The measurements of tadpoles from sampling 3-5 in referene (Svartadammen), Skullerud and Vassum.

Location	Sampling time	Stage	Weight (g)	Length (cm)	Liver (g)	CI	LSI	Intestine (g)
Reference	3	24	0.1245	2.1	0.0045	1.34	3.61	0.02
Reference	3	24	0.1215	2	0.0084	1.52	6.91	0.00
Reference	3	24	0.1027	2.1	0.0067	1.11	6.52	0.02
Reference	3	24	0.0692	1.8	0.001	1.18	1.44	0.01
Reference	3	24	0.108	2.3	0.0049	0.89	4.53	0.01
Reference	3	24	0.0772	1.9	0.0094	1.13	1.17	0.02
Reference	3	23	0.0533	1.6	0.0084	1.30	15.75	0.01
Reference	3	23	0.0686	1.8	0.0084	1.17	12.20	0.01
Reference	3	24	0.242	1.9	0.0051	3.52	2.10	0.01
Reference	3	24	0.137	2.4	0.009	0.99	6.56	0.01
Skullerud	3	25	0.1748	2.5	0.003	1.11	1.71	0.01
Skullerud	3	25	0.2524	2.9	0.0072	1.03	2.85	0.06
Skullerud	3	25	0.3739	2.7	0.0081	1.89	2.16	0.06
Skullerud	3	26	0.3349	3.4	0.0049	0.85	1.46	0.07
Skullerud	3	26	0.334	3.3	0.0196	0.92	5.86	0.07
Skullerud	3	25	0.3295	3.2	0.0015	1.00	0.45	0.06
Skullerud	3	26	0.3205	3.3	0.0256	0.89	7.98	0.07
Skullerud	3	25	0.3827	3	0.0178	1.41	4.65	0.08
Skullerud	3	24	0.1984	1.9	0.0034	2.89	1.71	0.03
Skullerud	3	25	0.1966	2.4	0.0056	1.42	2.84	0.04
Skullerud	3	26	0.3508	3.4	0.0126	0.89	3.59	0.06
Vassum	3	25	0.3548	3.5	0.0092	0.82	2.59	0.03
Vassum	3	25	0.1089	1.8	0.0049	1.86	4.49	0.01
Vassum	3	25	0.3218	3.9	0.0017	0.54	0.52	0.01
Vassum	3	26	0.451	2.7	0.0106	2.29	2.35	0.04
Vassum	3	25	0.2988	3.1	0.0075	1.00	2.51	0.02
Vassum	3	25	0.2572	3.1	0.0083	0.86	3.22	0.04
Vassum	3	26	0.3083	3.3	0.0122	0.85	3.95	0.04
Vassum	3	25	0.2466	2.9	0.0057	1.01	2.31	0.02
Reference	4	29	0.2712	2.5	0.0131	1.73	4.83	0.06
Reference	4	32	0.3252	3.1	0.0133	1.09	4.08	0.07
Reference	4	31	0.2667	2.9	0.015	1.09	5.62	0.05
Reference	4	33	0.2826	3.4	0.0085	0.71	3.00	0.07
Reference	4	32	0.2586	2.8	0.0032	1.17	1.23	0.05

Reference	4	30	0.2005	2.5	0.0114	1.28	5.68	0.03
Reference	4	31	0.2611	2.6	0.0068	1.48	2.60	0.41
Reference	4	32	0.316	2.8	0.0013	1.43	0.39	0.06
Reference	4	35	0.5554	3.8	0.0117	1.01	2.10	0.09
Reference	4	28	0.1764	2.3	0.015	1.44	8.50	0.03
Skullerud	4	37	0.8191	4.4	0.0085	0.96	1.03	0.17
Skullerud	4	35	0.4214	3.5	0.0032	0.98	0.75	0.09
Skullerud	4	36	0.6332	3.9	0.0024	1.06	0.37	0.13
Skullerud	4	34	0.357	2.9	0.0017	1.46	0.47	0.06
Skullerud	4	36	0.5114	3.9	0.0034	0.86	0.66	0.1
Skullerud	4	34	0.4914	2.1	0.0016	5.30	0.32	0.09
Skullerud	4	36	0.6765	3.6	0.0106	1.44	1.56	0.1
Skullerud	4	36	0.6192	4.1	0.0075	0.89	1.21	0.12
Skullerud	4	36	0.6846	4	0.0041	1.06	0.59	0.15
Skullerud	4	36	0.4671	3.9	0.0082	0.78	1.75	0.10
Vassum	4	37	0.5972	36	0.0118	1.28	1.97	0.07
Vassum	4	38	0.6169	4.5	0.0142	0.67	2.30	0.09
Vassum	4	36	0.5601	3.9	0.0029	0.94	0.51	0.08
Vassum	4	38	0.699	4.6	0.0112	0.71	1.60	0.09
Vassum	4	31	0.2594	3	0.0047	0.96	1.81	0.03
Vassum	4	38	0.793	5	0.013	0.63	1.63	0.09
Vassum	4	36	0.6983	4.5	0.0079	0.76	1.13	0.08
Vassum	4	37	0.5161	4.1	0.0102	0.74	1.97	0.06
Vassum	4	36	0.6136	4.2	0.0082	0.82	1.33	0.09
Vassum	4	36	0.5087	4	0.0042	0.79	0.82	0.05
Reference	5	36	0.7295	4	0.0042	1.13	0.57	0.16
Reference	5	34	0.4961	3.4	0.0091	1.26	1.83	0.09
Reference	5	36	0.6201	4	0.008	0.96	1.29	0.12
Reference	5	34	0.4934	3.3	0.0043	1.37	0.87	0.07
Reference	5	33	0.2507	2.9	0.0021	1.02	0.83	0.04
Reference	5	34	0.2753	2.7	0.0037	1.39	1.34	0.04
Reference	5	34	0.1206	2.6	0.0047	0.68	3.89	0.04
Reference	5	34	0.3534	3	0.0064	1.30	1.81	0.05
Reference	5	34	0.2263	2.7	0.0025	1.14	1.10	0.03
Reference	5	34	0.3526	3.1	0.0035	1.18	0.99	0.07
Reference	5	37	0.733	4.4	0.0108	0.86	1.47	0.13
Skullerud	5	39	0.6846	4.1	0.0024	0.99	0.35	0.09
Skullerud	5	39	0.6976	3.7	0.0126	1.37	1.80	0.09
Skullerud	5	37	0.497	3.1	0.0059	1.66	1.18	0.08
Skullerud	5	39	0.5193	3.8	0.012	0.94	2.31	0.09

Skullerud	5	38	0.4306	3.5	0.001	1.00	0.23	0.06
Skullerud	5	38	0.6667	3.8	0.022	1.21	3.29	0.11
Skullerud	5	38	0.5747	4.2	0.0236	0.77	4.10	0.09
Skullerud	5	37	0.3856	3.1	0.0101	1.29	2.61	0.07
Skullerud	5	38	0.6629	4	0.0229	1.03	3.45	0.12
Skullerud	5	38	0.6932	4.2	0.0073	0.93	1.05	0.12
Vassum	5	40	1.0601	4.7	0.0316	1.02	2.98	0.18
Vassum	5	40	0.8862	4.8	0.0099	0.80	1.11	0.16
Vassum	5	40	1.1767	5.2	0.036	0.83	3.05	0.17
Vassum	5	39	0.9527	4.2	0.0178	1.28	1.86	0.15
Vassum	5	39	0.6452	3.5	0.0075	1.50	1.16	0.11
Vassum	5	40	0.8571	4	0.0189	1.33	2.20	0.25
Vassum	5	39	0.6166	4.3	0.0109	0.77	1.76	0.07
Vassum	5	39	0.9145	3.8	0.017	1.66	1.85	0.08
Vassum	5	40	1.0401	4	0.0323	1.62	3.10	0.13
Vassum	5	40	0.8906	4.6	0.0189	0.91	2.12	0.13