Biological mapping and biomarker responses in northern crested newt (*Triturus cristatus*) and smooth newt (*Lissotriton vulgaris*) inhabiting highway sedimentation ponds

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Master Thesis in Toxicology

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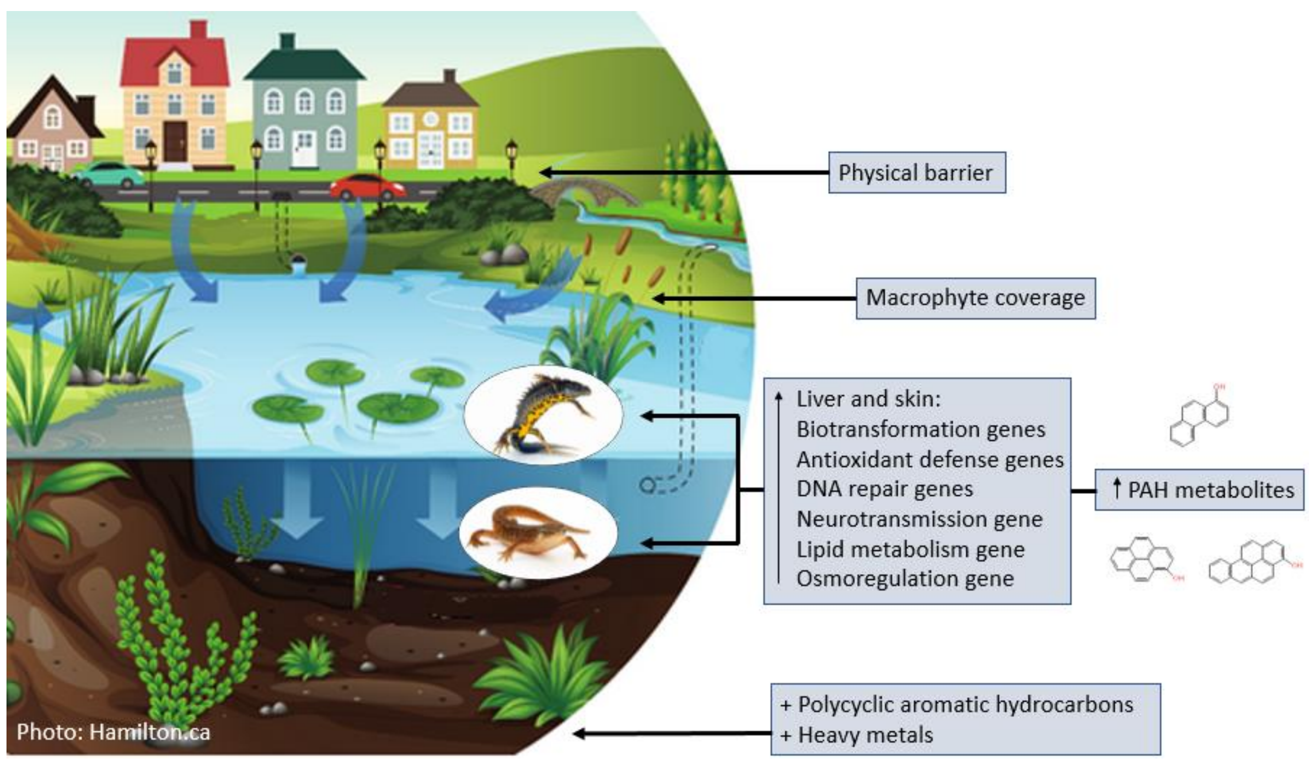
Graphical abstract



Abstract

Road runoff contains a plethora of various contaminants threatening the aquatic environment. Sedimentation ponds have been constructed approximate to heavy traffic roads to treat contaminated road runoff and tunnel wash water as a measure to reduce negative impacts on receiving water bodies. Additionally, these sedimentation ponds are rapidly colonized by aquatic organisms including amphibians. Amphibians are especially vulnerable to pollutants due to their highly permeable skin and multiple life stages in both water and on land. Hence, there may be an apparent conflict between the effective treatment function of the ponds and the species inhabiting them.

This study aimed to determine the presence of northern crested newt (*Triturus cristatus*) and smooth newt (*Lissotriton vulgaris*) in sedimentation ponds and assess potential determinants affecting their abundance. In addition, the study aimed to assess the ecotoxicological effects of the newts inhabiting sedimentation ponds. For future ecotoxicological purposes, this study also aimed to assess whether skin biopsies can replace liver for non-lethal biomonitoring of environmental stress in Norwegian newts.

A biological mapping was conducted to assess the abundance and to see if the abundance could be linked to various chemical, landscape and biological variables. Real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) was conducted using several effect and exposure biomarker genes to assess the transcriptional patterns in skin and liver samples. High performance liquid chromatography (HPLC) was used to measure PAH metabolites in bile samples.

The results obtained from the biological mapping showed that sedimentation ponds to a large extent are inhabited by both northern crested newt and smooth newt. Pollution levels in the sedimentation ponds did not affect newt abundance. However, physical barrier and vegetation coverage were significant determinants of newt abundance. The results obtained from the ecotoxicological analysis showed that induction of oxidative stress, DNA damage, and biotransformation of xenobiotics were common modes of action (MoA) in newts inhabiting sedimentation ponds. Furthermore, PAH metabolites 1-OH-pyrene, 1-OH-phenanthrene, and 3-OH-benzo[*a*]pyrene was measured in the bile of the individuals. Finally, the tissue comparison of gene expression showed that all biomarker responses were expressed

differently in skin and liver. However, some selected genes (e.g., CYP1A1, CYP1B1, and MT) indicated parallel transcriptional patterns in skin and liver.

The biological mapping and biomarker responses in northern crested newt and smooth newt showed that adult newts inhabiting sedimentation ponds experience sublethal effects due to road-related contaminant exposure. However, how these effects influence population dynamics is unclear. For future biomonitoring, non-lethal biopsies (skin) might be an adequate substitution for liver samples to minimize the impact on the threatened species.

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Abbreviations

AA-EQS	Annual average environmental quality standards
AADT	Annual Average Daily Traffic
AChE	Acetylcholinesterase
ACTIN	Beta actin
AMPKB1	5'-AMP-activated protein kinase subunit beta-1
ATP1A1	Sodium/potassium-transporting ATPase subunit alpha-1
CAT	Catalase
cDNA	Complementary DNA
CYP1A1	Cytochrome P450 1A1
CYP1B1	Cytochrome P450 1B1
dsDNA	Double stranded DNA
EEA	European economic area
EF1A1	Elongation factor 1-alpha 1
EQS	Environmental quality standards
GADD45g	Growth arrest and DNA damage-inducible protein GADD45 gamma
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GPX3	Glutathione peroxidase 3
GSTK1	Glutathione S-transferase kappa 1
HMM	High molecular mass species
HPLC	High-performance liquid chromatography

LMM	Low molecular mass species
LOD	Limit of detection
MAC-EQS	Maximum allowable concentration environmental quality standards
MoA	Mode of action
MT	Metallothionein
NIVA	Norwegian Institute for Water Research
NPRA	Norwegian Public Roads Administration
NRT	No reverse transcriptase control
NTC	Non-template control
RAD51	DNA repair protein RAD51
RDA	Redundancy analysis
ROS	Reactive oxygen species
PAH	Polycyclic aromatic hydrocarbon
PCA	Principal component analysis
PNEC	Predicted no effect concentration
PPAR	Peroxisome proliferator-activated receptor alpha isoform X1
RIN	RNA integrity number
RT-qPCR	Real-Time quantitative Polymerase Chain Reaction
ssDNA	Single stranded DNA
TE-buffer	Tris-EDTA buffer
WFD	Water framework directive

XRCC1	DNA repair protein XRCC1/X-ray repair cross-complementing protein1

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1 Introduction

The road network in Norway is continuously expanding to obtain a functioning infrastructure. There are currently over 4 million registered vehicles in Norway and the number of private cars has increased with 9.2 per cent over the past five years (Statistisk sentralbyrå 2019). The growth in the quantity of traffic maintains vital functions of society such as the transport of goods and passengers. However, this development comes with an environmental cost. Traffic is an extensive source of pollution to the environment. For example, comprehensive research has been conducted on the effects of traffic-related air pollution (Xianglu and Naeher 2005). Less conspicuous, but equally important, is the effects on the aquatic environment. A cocktail of traffic-related contaminants such as polycyclic aromatic hydrocarbons (PAHs) and metals enters the aquatic environment through road runoff (Meland 2010). Road runoff pollution has received growing awareness in recent years and non-point source pollution such as road runoff is now emphasized by the European Water Framework Directive (WFD). WFD was implemented in Norwegian law in 2007 through the Water Management Regulation. The directive obliges all member states and the European economic area (EEA) countries that all the water bodies should have good ecological- and chemical status (Lovdata 2010). As a measure to comply with the criteria of the law, the Norwegian Public Roads Administration (NPRA) construct sedimentation ponds approximate to heavy traffic roads (Meland et al., 2016). Sedimentation ponds treat contaminated road runoff and tunnel wash water mainly by sedimentation of particle bound pollutants which will be retained in the sediment (Åstebøl and Hvitved-Jacobsen 2014). This ensures mitigated spread of various pollutants from road runoff to recipient water bodies.

In addition to the intended function of the sedimentations ponds, these ponds are rapidly colonized by various aquatic organisms such as amphibians (Le Viol et al., 2012; Sun et al., 2019). Amphibians are especially vulnerable to pollutants due to their highly permeable skin and multiple life stages in both water and on land (Sparling 2010). Amphibians inhabiting the sedimentation ponds are affected both by contaminated sediment and runoff water, especially during acute discharge of tunnel wash water (Johansen 2013). Hence, sedimentation ponds may act as ecological traps compromising the survival of sensitive species (Snodgrass et al., 2007). Numerous toxicological studies on aquatic organisms from sedimentation ponds have

reported both lethal and sublethal effects such as increased mortality, decreased growth rate, affected metabolism and increased DNA damage (Snodgrass et al., 2007; Brand et al., 2010; Meland et al., 2010b; Liane 2018). However, these studies were mostly restricted to fish or frog species. Additionally, most of the studies conducted on frogs solely investigated larval or tadpole exposure. Few studies have focused on adult amphibian responses, even less on adult newts.

Many amphibian populations, including newt populations, are experiencing declines and extinctions worldwide (Stuart et al., 2004) including the Norwegian northern crested newt (*Triturus cristatus*) which has experienced a 15-30% decline the last decade (Artsdatabanken 2019). This negative trend has several causes, one of which is the effects of environmental contaminants (Dodd 2010). There is extensive literature on the effects of environmental contaminants on amphibians as reviewed in Sparling (2010), yet little is known about how contaminants affect amphibian population dynamics (Alford and Richards 1999). The present study addressed questions from a molecular level to population responses to gain knowledge about the sublethal effects and population status of Norwegian newts in sedimentation ponds. To the authors knowledge, no other study has investigated the presence, sublethal effects and population determinants of adult newts in sedimentation ponds.

1.1 Objectives and hypotheses

By combining ecological and ecotoxicological analysis this study aimed to investigate the presence of northern crested newt (*Triturus cristatus*) and smooth newt (*Lissotriton vulgaris*) in sedimentation ponds and factors potentially influencing the abundance of the species. Moreover, the study aimed to assess biomarker responses and sublethal effects on the two amphibian species. Due to the endangered status of northern crested newt, the final aim was to assess whether non-destructive biopsy samples (skin) can replace destructive biopsy samples (liver) for biomonitoring of environmental stress in Norwegian newts. To achieve this, the following three objectives were assessed.

Objective 1: Determine whether northern crested newt and smooth newt inhabit sedimentation ponds and which factors affect their presence in the ponds.

H_1: Northern crested newt and smooth newt inhabit sedimentation ponds.
H_2: Chemical, landscape and biological variables determine the abundance of northern crested newt and smooth newt.
a) Environmental landscape variables such as vegetation, physical barriers, and distance to other ponds and road determine the abundance of the species.
b) Pollution levels, represented by polycyclic aromatic hydrocarbons and trace metals, in sediment determine the abundance of the species.

Objective 2: Assess the ecotoxicological effects of newts inhabiting sedimentation ponds receiving road runoff.

H_3: PAH-metabolite concentrations in bile are elevated in individuals inhabiting sedimentation ponds compared individuals inhabiting the reference pond.
H_4: Genes related to biotransformation, oxidative stress and DNA repair will be differentially expressed in individuals from sedimentation ponds compare to reference pond due to elevated contaminant exposure.

Objective 3: Determine whether skin samples can replace liver for non-lethal biomonitoring of environmental stress in newts.

H_5: The biomarker gene responses in the skin have similar sensitivity as that in the liver and in general express the same ecotoxicological impact.

2 Background

2.1 Environmental contaminants in highway runoff

Polluted road surfaces are washed by rain or snowfall including snow melting resulting in road runoff. Road runoff contains a wide range of both inorganic and organic compounds. Inorganic compounds such as copper, cadmium, nickel, lead, and zinc originate from break and tire wear (Meland 2010). Organic compounds in road runoff include, among others, phenols and polycyclic aromatic hydrocarbons (PAHs) (Sternbeck et al., 2002; Brown and Peake 2005). These compounds originate from diverse sources such as tire wear, combustion, and oil spills (Meland 2010). In addition to the precipitation-based runoff, highway runoff also includes tunnel wash water. Tunnels are accumulating toxic pollutants over time which are frequently washed out. The washing frequency depends on traffic density and can vary from less than once per year to 12 times per year, and tunnel wash water likely contains higher concentrations of pollutants compared to normal road runoff (Meland 2010).

In Norway, sedimentation ponds are one common measure for the protection of receiving waters from polluted road runoff. The ponds are constructed to receive road runoff and tunnel wash water from heavily trafficked roads. The construction of the ponds normally includes two basins, one slam basin (forebay) and one main basin as illustrated in figure 1. The first basin removes large particles of particulate material through sedimentation following by an accumulation in the bottom sediment of the pond. Smaller particles sediment in the main basin (Åstebøl and Hvitved-Jacobsen 2014). This ensures enhanced protection of recipient waters.

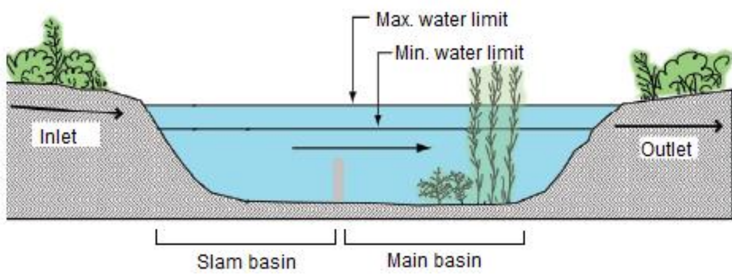

Figure 1. Illustration of a cross-section of a sedimentation pond. Modified from Åstebøl et al., (2010).

2.1.1 Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are a group of ubiquitous hydrocarbons containing one or more benzene rings fused together. PAHs derive from both anthropogenic and natural sources. Anthropogenic sources include the utilization of petroleum products and incomplete combustion of fossil fuels. Natural sources include forest fires or volcano eruptions (Stogiannidis and Laane 2015). PAHs are divided into three major classes depending on their source of origin: pyrogenic, petrogenic or diagenetic (Saber et al., 2006).

The mobility of PAHs largely depends on differences in various physical and chemical properties such as vapour pressure, solubility, octanol-water partitioning coefficient (log K_{ow}) and resistance to oxidation and reduction. High molecular weight PAHs with five or more rings (e.g., perylene and benzo[*a*]pyrene) have low solubility and vapour pressure and are therefore often associated with particles. PAHs with two or three rings (e.g., naphthalene and phenanthrene) are more volatile (Baek et al., 1991). Organisms exposed to PAHs often detoxifies the compounds through biotransformation. During biotransformation, PAHs are metabolized into a wide range of metabolites. Some metabolites become detoxified products and are easily excreted from the organism, while others, become carcinogenic and mutagenic (Dreij 2005). The carcinogenicity of PAHs increases with increased number of rings. Four or five-ringed PAHs such as benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[k]fluoranthene and B[*a*]P, indeno[1,2,3-cd]pyrene, and dibenzo[*a,h*]anthracene are known for their carcinogenic properties (Ravindra et al., 2008).

Acute exposure to high concentrations of PAHs may cause mortality, whereas chronic exposure to sublethal concentrations of PAHs may lead to decreased growth rate, impaired reproduction and tumor growth as reviewed in Logan (2007). Studies of PAH related effects on aquatic organisms exposed to road runoff and tunnel wash water have revealed increased DNA damage in amphibians (Liane 2018) and fish (Grung et al., 2016). Moreover, elevated levels of cytochrome P450 1A1 (CYP1A1) enzyme and induced EROD activity were found in fish hepatocytes inhabiting sedimentation ponds (Petersen et al., 2016). Other sublethal effects such as increased antioxidant defense system, reduced growth rate (Meland et al., 2010b), increased levels of glucose and pCO_2 in blood and affected metabolism (Meland et al., 2009) have been reported for fish exposed to highway runoff.

2.1.2 Metals

Trace metals are an additional main group of contaminants in road runoff. Metals are naturally occurring and are released into the environment by human activities such as mining, smelting, and combustion of fossil fuel. These activities result in widespread contamination of air, water, soil and sediments (Sparling 2010). Metals cannot be destroyed nor created by biological or chemical processes. Hence, detoxification of metals in organisms occurs through binding to proteins such as metallothionein or storage in intracellular granules (Walker et al., 2012).

Trace metals are mostly toxic to aquatic organisms however some (e.g., copper, selenium, and zinc) are nutritionally essential at low levels (Fairbrother et al., 2007). Metals and metalloids appear in different physio-chemical forms (i.e. metal species). Metal species vary in characteristics such as nominal molecular size, oxidation state, charge properties, density, and structure (Salbu 2009). The mobility and bioavailability of metal species are strongly related to the molecular mass. Low molecular mass species (LMM) are more mobile and bioavailable compared to high molecular mass species (HMM) such as colloids, polymers, pseudo colloids, and particles (Salbu 2009). In aquatic environments, several water quality parameters such as pH, ionic strength, redox potential, temperature, suspended soils, and organic matter influence the partitioning of metal species. Thus, these parameters determine the toxicokinetics of metals in aquatic environments. Consequently, the toxicokinetics of metals largely depends on speciation and the ability of the organism to store and regulate the metal (Fairbrother et al., 2007).

Previous studies have documented both lethal and sublethal effects in organisms due to trace metal exposure. Sparling (2010) reported sublethal effects in reptiles such as neurological toxicity, immunological, genetic and endocrine disruption. Documented effects on amphibians after trace metal exposure vary widely due to species sensitivity. For example, Snodgrass el al. (2007), exposed both sensitive and insensitive embryonic and larvae amphibians to polluted sediment from sedimentation ponds. They recorded 100% mortality in sensitive species while insensitive species only displayed sublethal effects (i.e., smaller size at metamorphosis). Moreover, trace element accumulation have been reported in frog embryos exposed to tunnel wash water (Johansen 2013).

2.1.3 Environmental quality standards (EQS)

Environmental quality standards (EQS) is a limit for environmental disturbances due to ambient concentrations of pollutant. EQS is used to assess the ecological and chemical status of water bodies. EQS consists of five ecological classes (class I -V) and two chemical classes (figure 2). The limit values are given as two values; annual average (AA-EQS) and maximum allowable concentration (MAC-EQS). AA-EQS represent the limit for chronic effects of long-time exposure (upper limit of class II) whereas MAC-EQS represent the limit for acute effects from short-term exposure (upper limit of class III). The predicted no-effect concentration (PNEC) is represented at the upper limit of class II (Committee of directorates for the water framework directive 2018).

Chemical condition	Ecological condition	Effects	Deviation from natural state
Good	Background I	No toxic effects	Pristine
	Good II	No toxic effects	Small
Bad	Moderate III	Possible chronic effects	Moderate
	Poor IV	Possible acute effects	Significant
	Very poor V	Considerable toxic effects	Very large

Figure 2. EQS classes for water and sediment. The chemical classes are represented by two classes (blue and red). The ecological classes are represented by five classes (I-V). Source: Modified from Committee of directorates for the water framework directive (2018).

2.2 Amphibians; study species northern crested newt (*Triturus cristatus*) and smooth newt (*Lissotriton vulagris*)

Northern crested newt (*Triturus cristatus*) and smooth newt (*Lissotriton vulgaris*) are two amphibian species distributed in Norway at latitudes stretching to Trøndelag county and altitudes reaching 600 meters above sea level (figure 3). Newts play a key role in the ecosystem since they are intermediate predators with a combined aquatic life cycle. They have a composed diet of diverse larvae, crustaceans, and tadpoles in aquatic habitats and insects, spiders and earthworms in terrestrial habitats (Dolmen 2008).

Newts have a complex life cycle including egg, larvae and adult stages. During the reproduction period in the spring, the adult newts migrate to small freshwater ponds for mating and egg laying. Once the eggs have been laid, they spend the rest of the summer months on land before hibernation during autumn and winter. The newly hatched larvae remain in the pond until the metamorphosis is complete and the lungs are fully developed. Following metamorphosis, the juvenile newts crawl on land and remain there until sexual maturity (1-3 years) before returning to the pond (Dolmen 2008). This complex life cycle leads to a diverse exposure range for environmental contaminants. The uptake of waterborne chemicals begins during the egg stage as water moves into the egg capsule. It enhances at the larvae stage as the chemicals penetrate their permeable skin. The skin continues to play a major role as an exposure route in adult newts. In addition, lungs and diet serve as secondary exposure pathways on land. Hence, adult newts are exposed to water bound-, volatile- and solid phase chemicals from water, air and sediment (Sparling 2010).

Amphibian populations are experiencing decline and extinctions worldwide (Stuart et al., 2004). Biotope modification/destruction, global climate change, introduced predators/foreign species, environmental contaminants and emerging infectious diseases are identified causes to this negative trend (Dodd 2010). Amphibian populations have often been studied in road context regarding their vulnerability to collisions (Ashley and Robinson 1996) and biotope destruction due to urbanization. Amphibious underpasses and artificial hibernation sites have been built approximate to road construction as a mitigation measure for newt populations in Norway (Norwegian Institute for Nature Research 2019).

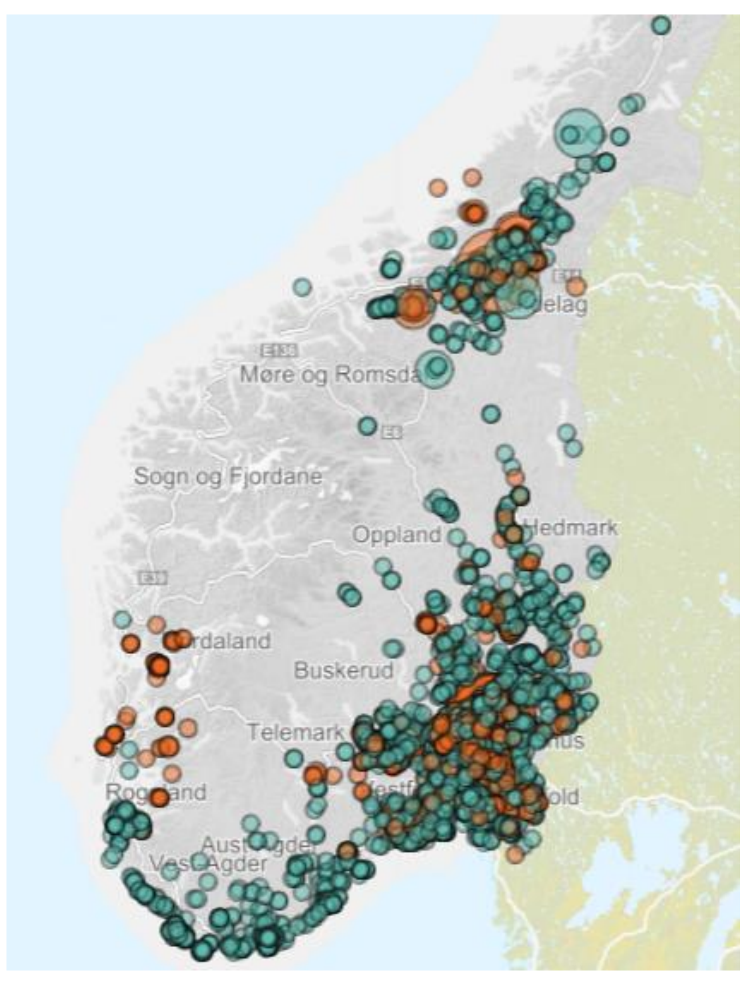

Figure 3. Map showing the geographic range of smooth newt (indicated in green) and northern crested newt (indicated in red) in Norway. The map is retrieved from artsdatabanken.no.

2.3 Biomarkers

Several definitions have been given to the term “biomarker” which generally refers to a molecular, physiologic or organismal response caused by contaminant exposure (Di Giulio et al., 2015. One definition of a biomarker is; “Any biological response to an environmental chemical at the individual level or below” (Walker et al., 2012). This definition will be employed in this thesis. The most widely used biomarkers in ecotoxicology are molecular markers such as genes and proteins (enzymes). Molecular markers are highly sensitive to xenobiotic exposure and can be used to understand the effects the chemicals with different modes of actions (MoAs) (Ankley et al., 2006). In addition, molecular markers are also widely used to indicate specific chemical exposure in environmental monitoring (Amin et al., 2002).

In the present study, a selection of antioxidant defense, DNA repair, xenobiotic biotransformation, neurotransmission, osmoregulation, and lipid metabolism genes were examined based on their cellular expression level in liver and skin samples. In addition, quantification of PAH metabolites served as a biomarker for PAH exposure. Figure 4 illustrates the selected biomarkers and mechanisms involved in the cellular response to PAH and metal exposure.

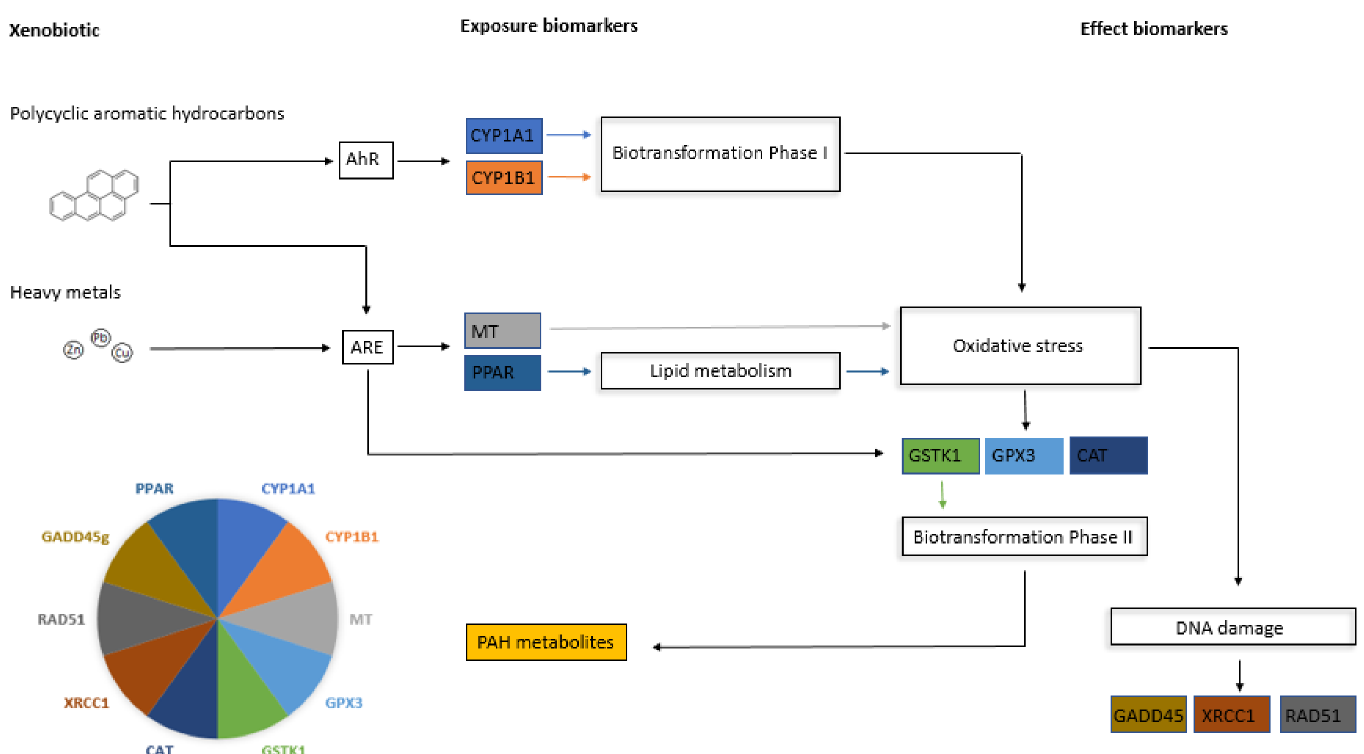

Figure 4. Simplified overview of selected biomarkers and mechanisms involved in cellular response to PAH and metal exposure. **AhR**, Aryl hydrocarbon receptor; **ARE**, Antioxidant response element; **CYP1A1**, Cytochrome P450 1A; **CYP1B1**, Cytochrome P450 1B; **MT**, Metallothionein; **PPAR**, Peroxisome proliferator-activated receptor; **GSTK1**, Glutathione S-transferase kappa 1; **GPX3**, Glutathione peroxidase 3; **CAT**, Catalase; **GADD45g**, Growth arrest and DNA damage-inducible protein gamma; **XRCC1**, DNA repair protein XRCC1; **RAD51**, DNA repair protein RAD51.

2.4.1 Biological responses to xenobiotic exposure

Exposure to lipophilic xenobiotics or endogenous compounds induces a metabolic conversion of these compounds into more water-soluble forms, a process known as biotransformation. Biotransformation occurs in two phases, phase I and phase II. The first phase of biotransformation introduces or expose a functional group of the xenobiotic by hydrolysis,

reduction, and oxidation. The second phase conjugates the xenobiotic parent compound with an endogenous polar ligand such as sugar (glucuronide), sulfate or glutathione. Phase I biotransformation reactions only result in a small increase of hydrophilicity of the compound whereas phase II results in a larger increase. Hence, phase II reactions greatly promote excretion of the xenobiotic compound (Parkinson et al., 2015).

Biotransformation of PAHs is activated when the compounds interact with the aryl hydrocarbon (Ah) receptor (figure 4). This interaction triggers induction of the cytochrome P450 system which initiates phase I biotransformation (Hankinson 1995). Cytochromes P450 (cyt P450) comprise a large family of heme proteins predominantly located in the endoplasmic reticulum of the liver (Van der Oost et al., 2003). Two subfamilies of cyt P450 include CYP1A and CYP1B comprised by genes coding for Cytochrome P450 1A1 (CYP1A1) and Cytochrome P450 1B1 (CYP1B1) enzymes. These enzymes are the major cyt P450 isoforms involved in the biotransformation of PAHs (Chi et al., 2009). Induction of these enzymes is therefore widely used as a biomarker of PAH exposure (Van der Oost et al., 2003). Following phase I biotransformation, the PAHs are subsequently facilitated by phase II enzymes such as UDP-glucuronosyltransferases or sulfotransferase (Parkinson et al., 2015). Finally, the PAH metabolites are excreted through bile or urine. Detection of PAH metabolites in the bile represents the constant flux of PAHs through the organism. The flux is a relevant estimation of biotic stress due to resent PAH exposure (Hylland et al., 2006).

Biotransformation usually results in detoxification of xenobiotics, however, toxification of compounds also occur. Phase I enzymes activate non-toxic compounds into several intermediate reactive molecules such as hydroxylated derivatives and epoxides. Several reactive oxygen species (e.g., superoxide, hydrogen peroxide and ozone) are formed during this process which can lead to the production of toxic free radicals (Pollak 1998). Oxygen radicals are counterbalanced by antioxidants to evade oxygen stress in organisms. Phase II biotransformation enzymatic antioxidant defense includes enzymes such as glutathione S-transferase (GST), glutathione peroxidase (GPX) and catalase (CAT). These enzymes are critical to detoxify radicals to non-reactive molecules (Van der Oost et al., 2003).

Oxygen radicals left unbalanced by antioxidants can lead to oxidative DNA lesions, carcinogenesis, oxidative stress, and impaired cell growth regulation. DNA lesions can be introduced in cells not only by internal factors (e.g., reactive oxygen species) but also various external sources such as ionizing radiation, ultraviolet light, chemicals and endogenous agents

(Houtgraaf et al., 2006). Following exposure to a hazardous agent, lesions can occur as single and double-strand breaks, DNA adducts or cross-links between DNA basis and proteins. Various repair pathways, specific for each type of lesion, remediate the damage. In order to repair double-strand breaks, two major pathways are involved: homologous recombination and nonhomologous end-joining (Parkinson et al., 2015. Homologous recombination is initiated by DNA repair protein RAD51 which binds to ssDNA and recruit recombination factories to complete the process. Single strand break repair processes involve base excision repair, nucleotide excision repair and mismatch repair. In the base excision repair pathway, damaged bases are removed and the gaps are filled in with new nucleotides by DNA polymerase-β subsequently ligated by the Ligase3/XRCC1 complex (Houtgraaf et al., 2006). RAD51 and XRCC1 are important proteins involved in various DNA repair pathways. Induction of these genes therefore serves as a biomarker for DNA damage. In addition, the growth arrest DNA damage gamma (GADDg) gene transcript levels are induced following DNA damage and extrinsic stressors. The protein encoded by this gene responds to environmental stresses by mediating the p53 pathway (Takekawa and Saito 1998). The p53 pathway responds to various intrinsic and extrinsic stressor signals by controlling DNA replication and cell cycle progression (Harris and Levine 2005). Hence, GADD45g is widely used as a biomarker for p53 activation (Takekawa and Saito 1998).

Stress proteins protect and repair the cell against harmful conditions. The most abundant stress proteins are heat shock proteins. A special group of stress proteins is metallothioneins (MTs) (Van der Oost et al., 2003). These proteins are a group of cysteine-rich metal binding proteins. MTs has a wide range of functions such as detoxification of toxic metals, metal ion homeostasis, and protection against reactive oxygen species (ROS). Transcription of MTs is strongly controlled by exposure to metal ions (Sutherland and Stillman 2011). Hence, MTs are used as a biomarker for toxic metals (Van der Oost et al., 2003).

Neural functions of organisms are predominantly associated with cholinesterases. Acetylcholinesterase (AChE) is a cholinesterase with high affinity for the neurotransmitter acetylcholine. AChE catalyze the degradation of acetylcholine at nerve endings to prevent continuous nerve signaling. This process is vital for the normal function of sensory and neuromuscular systems. With respect to neural functions, AChE is one of the most important biomarkers to assess organophosphate and carbamate pesticide exposure as these compounds are known to inhibit AChE (Mathieu et al., 1996).

Exposure to various chemical compounds triggers certain conditions involving drastic changes in cellular morphology and enzymatic activity, a cellular response called peroxisome proliferation. Various xenobiotics such as phthalates and herbicides are known to induce peroxisome proliferation by activating peroxisome proliferator-activated receptors (PPARs) (Schoonjans et al., 1996). These receptors play a central role in lipid metabolism as ligand-activated transcriptional factors controlling gene expression of various genes. PPARs are categorized into three groups: PPAR-α, PPAR-δ/β, and PPAR-γ, which all are encoded by separate genes. PPAR-α will be employed in this thesis. PPAR-α has various functions such as regulation of the expression of genes involved in fatty acid β-oxidation and energy homeostasis (Kota et al., 2004).

The AMP-activated protein kinase (AMPK) regulates cellular energy homeostasis by maintaining the balance between ATP production and consumption. During physiological and pathological stress, the cellular energy charge decreases (increased AMP/ATP ratio). AMPK protects the cells by coordinating ATP production and inhibit anabolic pathways. All AMPK kinases consist of heterotrimeric complexes including catalytic (α subunits) and regulatory (β and γ subunits) (Hardie 2007). The β-subunit is an important signal transducer for metabolic stress signals to initiate the metabolic stress sensing by AMPK (Oakhill et al., 2010). AMPK is activated by numerous of drugs and xenobiotics many of which inhibit mitochondrial functions. In addition, high ROS concentrations activate AMPK as a secondary response to inhibition of mitochondrial ATP production (Hardie et al., 2012). AMPK is a crucial energy sensor and thus used as a biomarker for energetic stress in aquatic organisms (Goodchild et al., 2015)

The sodium/potassium-transporting ATPase (Na^++K^+ -ATPase) pump is a membrane-bound electrogenic ion pump which maintains the sodium/potassium balance in cells. The pump maintains vital physiological processes such as osmoregulation, membrane excitability and transport of amino acids and sugars (Lebovitz et al., 1989). Exposure to xenobiotics can inhibit the Na^++K^+ -ATPase pump in fish and invertebrates (Davis et al., 1972; Yadwad et al., 1990; Issartel et al., 2010).

2.4 Biopsy sampling methods

Tissue sampling methods are broadly characterized into three different groups; destructive, non-destructive and non-invasive sampling methods. Destructive methods involve killing the animal whereas non-destructive methods obtain tissue samples invasively. Non-invasive sampling is restricted to when the source of DNA can be collected without handling the animal. This includes the collection of feces, feathers or skin left behind by the animal. These tissue samples have a limited number of subsequent genetic applications due to low DNA quantity or quality (Taberlet et al., 1999). Hence, tissue samples obtained from non-destructive or destructive methods are commonly used for genetic application.

It is important to consider using the least destructive sampling method when sampling endangered species such as the northern crested newt. The liver is often a popular target in environmental toxicology due to its primary function as a detoxification organ. Liver samples cannot be obtained without a destructive method. However, tail tips can be harvested non-lethally and regenerate a fully functioning limb after amputation (Iten and Bryant 1973). Tissue (skin) samples such as tail tips may provide an adequate substitute for liver samples for genetic analysis in amphibians. Comparative reviews for tissues have been conducted on newts for stable isotopes analysis (Milanovich and Maerz 2012) and mercury concentration analysis (Townsend and Driscoll 2013) showing that tail-clips can provide reliable measurements. While non-lethal biopsy samples have been shown to yield a sufficient amount of DNA for DNA analysis, it is unclear whether these samples are useful for gene expression analyses. Gene expression analyses provide challenges due to tissue-specific gene expression (Czypionka et al., 2015). However, similar gene expression patterns have been shown in different tissues in the same species (Bernatchez et al., 2010; Veldhoen et al., 2013). Thus, it is likely that transcriptome analysis of non-lethal biopsies such as skin tissue can represent the whole organism (Czypionka et al., 2015).

3 Materials and methods

3.1 Site selection and fieldwork

27 sedimentation ponds along the highways E6 and E18 in Oslo, Akershus, Østfold and Vestfold county and three reference locations were chosen for this study (figure 5). The sedimentation ponds were chosen based on technical condition reports conducted by the Norwegian Public Roads Administration (NRPA) (Rise Gregersen et al., 2015). Conditions such as water level, proximity to heavy traffic roads and accessibility underlie the selection. All sedimentation ponds receive road runoff from road or tunnel, or both. All sedimentation ponds included in the study were approved by the NPRA.

The fieldwork was conducted in May and June 2018. It was divided into two parts: biological mapping and ecotoxicological sampling. The biological mapping consisted of registration of northern crested newt and smooth newt and measurements of various chemical, biological and landscape variables (data shown in Appendix A and B). Following the biological mapping, a sampling for toxicological analysis was conducted, further referred to as ecotoxicological sampling (data shown in Appendix A). Three reference locations were chosen based on advice from the environmental protection department of County Governor in Oslo according to the permit of the Norwegian Environment Agency. The reference locations were unconnected to heavy traffic roads and other known sources of emissions for the pollutants of interest. One of these locations were used in the ecotoxicological sampling (see section 3.1.3). All reference locations were situated in the municipality of Drøbak in Akershus county. Both the biological mapping and the ecotoxicological sampling were approved by the Norwegian Environment Agency.

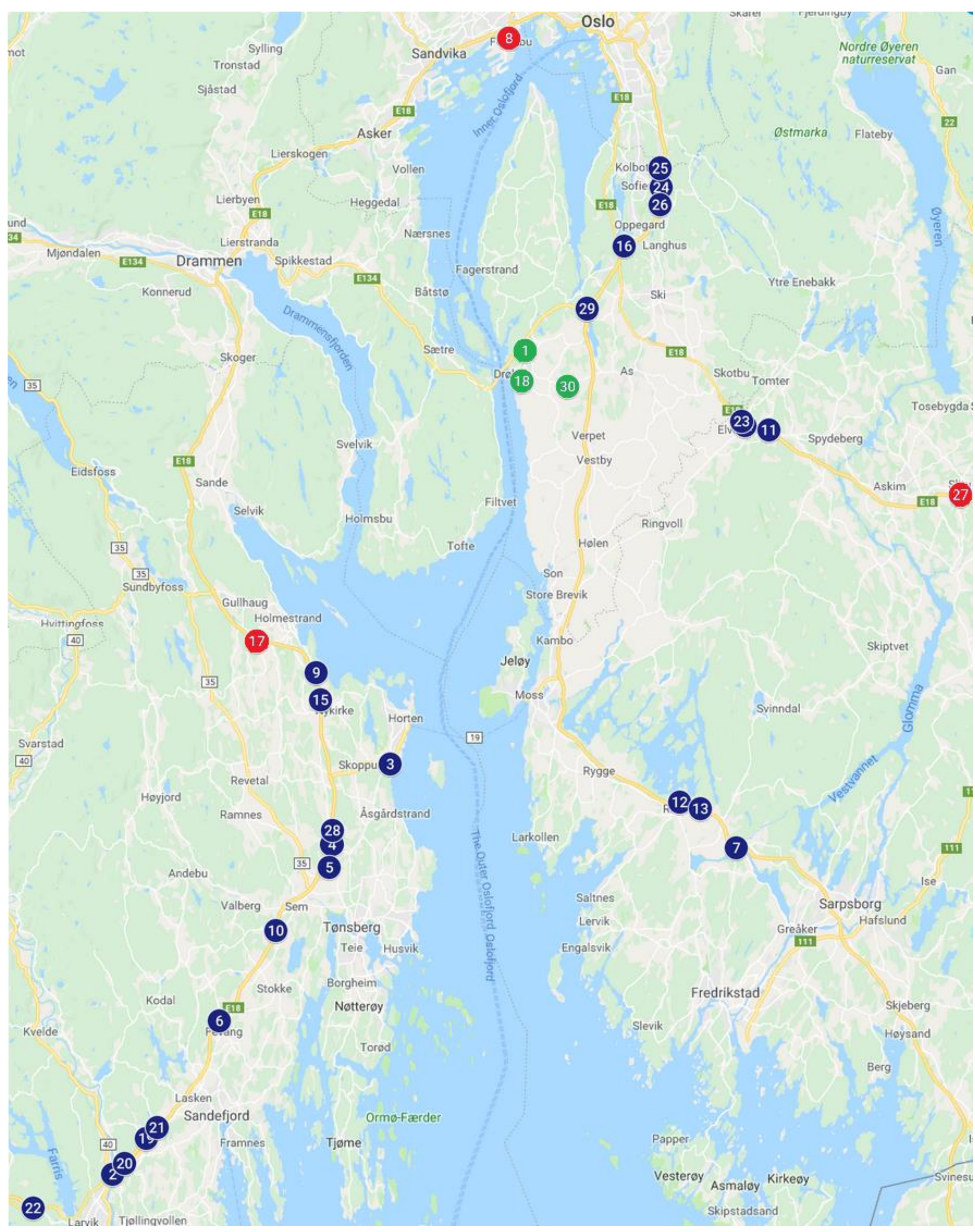

Figure 5. Map showing the locations for all investigated sedimentation ponds including the reference sites. The reference sites are indicated in green. The sites of ecotoxicological sampling are indicated in red. The numbers correspond with the location names presented in table 1. The map was retrieved from Google Maps.

Table 1. General information about the study sites from figure 5 presented in alphabetical order.

Location name	Coordinates (Latitude, Longitude)	Area (m^2)[a]
1. Berle (Reference)	59.680248, 10.649857	7563
2. Bommestad East	59.088436, 10.069917	170
3. Borre	59.384533, 10.461393	410
4. Eikeberg	59.327088, 10.379692	1440
5. Emmerud	59.309804, 10.374559	1370
6. Fevang	59.199533, 10.220526	2640
7. Finstad	59.324181, 10.949016	180
8. Fornebu	59.901158, 10.625914	820
9. Helland	59.449740, 10.356981	3400
10. Holmane	59.264799, 10.299267	1120
11. Holt	59.622986, 10.993750	1860
12. Idrettsveien	59.356753, 10.869128	780
13. Lundeby	59.352789, 10.898544	105
14. Neset	59.626536, 10.958999	1670
15. Nøklegard	59.430760, 10.362965	2780
16. Nøstvedt	59.754345, 10.789175	340
17. Nygård	59.472279, 10.273086	1550
18. Rånås (Reference)	59.659005, 10.644901	1273
19. Ringdal	59.114514, 10.118924	960
20. Seierstad	59.096307, 10.087428	2060
21. Skinmo	59.122158, 10.133539	2060
22. Sky	59.064802, 9.957088	2500
23. Støttum	59.629363, 10.955182	2100
24. Talaldrud Junction	59.796628, 10.840751	1400
25. Taraldrud North	59.809678, 10.839625	780
26. Talaldrud South	59.784055, 10.840021	474
27. Tenor	59.57755, 11.262071	655
28. Tveiten	59.337030, 10.380328	970
29. Vassum	59.709889, 10.736694	431
30. Vennersrød (Reference)	59.654629, 10.709542	4168

[a] Pond area m^2 obtained from NPRA reports or measured from Google Maps

3.1.1 Biological mapping

In order to record the presence of the two species, Ortmann`s funnel traps were used (figure 6). The traps are constructed of a plastic bucket enclosed with a lid with several funnels leading in. The bucket was ensured floating by a floating device attached to each bucket. Additionally, inside the trap, there was a small floating device made of cell polyethylene. These two measures enabled the newts to get air. The traps were lowered below the water

surface and collected no longer than 24 hours after being deployed. The number of traps and time deployed was used to calculate catch per unit (CPU) by the formulae: number of caught animals / hours deployed (data shown in Appendix A). The animals were examined, and weight, length, sex, and species were noted before releasing them back into the pond (data shown in Appendix A).

Figure 6. Left: Ortsmann`s funnel trap with floating device attached. Middle: a male northern crested newt (big) and a smooth newt (small). Right: a female smooth newt being weighed. All photos: ©Sofie Lindman.

Several landscape and local variables of each sedimentation pond were measured (table 2). A multiparameter water quality sonde (YSI 6600V2) was used to measure general water quality variables described in table 2. These measurements were taken approximately 1m from the shoreline. Recorded observations of the two amphibian species from Artsdatabanken was used to assess nearest occupied pond. Google Maps was used to assess the number of ponds within 1km radius and distance to the closest pond. 1 km radius was chosen based on the dispersion capabilities of newts (Dolmen 2008). The percentage of macrophytes coverage was assessed by eye measurement. Reports from the NPRA provided average annual daily traffic (AADT) numbers from 2018.

Table 2. Overview of measured landscape, chemical and biological variables in all sedimentation ponds.

Landscape variable	Unit
Sunexposure	Low, medium, high
Physical barrier[a]	Yes / no
Numer of ponds 1km[b]	
AADT[c]	Vehicles / day
Distance to road	m
Distance known newt location[d]	m
Chemical variable	
pH	M/L
Conductivity	S/m
Temperature	°C
ORP[e]	mV
Dissolved oxygen	mg/L
Oxygen concentration	%
Biological variable	
Macrophyte coverage	%
Area[f]	m^2
Depth	m

[a] Physical barriers (road) between pond and neighboring ponds in addition to highway

[b] Number of neighboring ponds within 1km radius

[c] Average annual daily traffic on connected stretch of highway retrieved from NPRA reports 2018

[d] Distance to closest pond inhabited by northern crested newt or smooth newt obtained from Artsdatabanken

[e] Oxidation reduction potential

[f] Pond surface area

Sediment samples were collected using a Van Veen grab. 3-5 subsamples of sediment were collected from different sites in the pond and mixed. The mixed sample was stored in incinerated glass jars with aluminum foil between the lid and the sample. These samples were analysed for PAHs and trace metals. Analyse of PAHs was conducted by gas chromatography coupled to mass spectrometry (GC/MC) at the Norwegian Institute for Water Research (NIVA). Analyses for metals was conducted at Eurofins. Water samples were collected in plastic bottles to analyze total phosphorus and nitrogen content.

All used equipment was sterilized and dried between every pond using disinfectant solution Virkon S according to the permit of Norwegian Environment Agency. This prevents any potential spreading of the fungi *Batrachochytrium dendrobatidis* (bd) which is newly discovered in Norway.

3.1.2 Ecotoxicological sampling sites and methods

Fornebu, Tenor and Nygård sedimentation pond were chosen for ecotoxicological analysis. All three ponds showed high numbers of both species during the first examination (data shown in Appendix A). These sites represent a good selection of variety regarding AADT and runoff from different sources (data shown in Appendix A). In addition, one reference pond (Rånås) was used for ecotoxicological analysis.

Fornebu sedimentation pond (figure 7) is located in the municipality of Bærum in Oslo county in an area previously used as an airport for Oslo. The pond was constructed in 2004 and receives road runoff from state highway 166. The pond is located in an intersection of roads, buildings and park areas. AADT was 23193 cars in 2018 according to NRPA.

Figure 7. Left: Fornebu sedimentation pond. Right: The surrounding area of Fornebu sedimentation pond seen from above. Photo: ©Sofie Lindman, map: Google Maps.

Tenor (Slitu) sedimentation pond (figure 8) is located in the municipality of Eidsberg in Østfold county. The sedimentation pond was constructed in 2007 and receives road runoff from highway E18. The pond is located adjacent to woodland and the highway and has planted trees surrounding it. According to the NPRA the AADT was 13132 vehicles/day in 2018.

Figure 8. Left: Tenor sedimentation pond. Right: The surrounding area of Tenor sedimentation pond seen from above Photo: ©Sofie Lindman, map: Google Maps.

Nygård sedimentation pond (figure 9) is located in the municipality of Holmestrand in Vestfold county. The sedimentation pond was constructed during 2001-2002. The sedimentation pond receives road runoff from highway E18 and the Botne tunnel. According to the NPRA the AADT was 23680 vehicles/day in 2018.

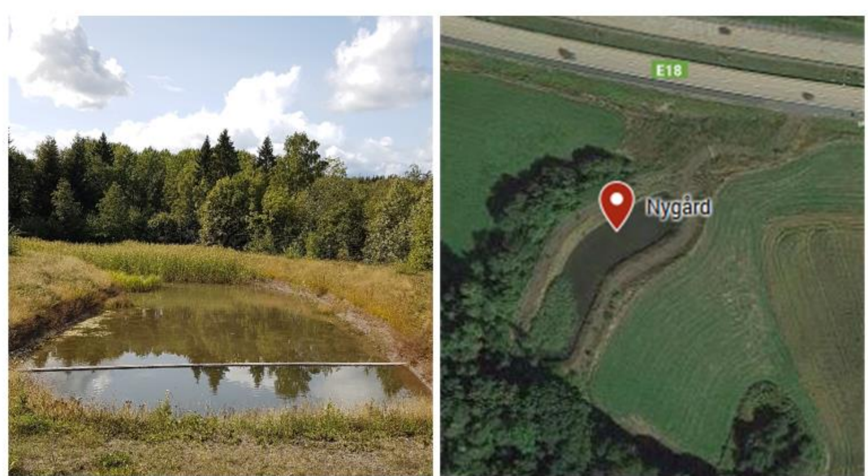

Figure 9. Left: Nygård sedimentation pond. Right: The surrounding area of Nygård sedimentation pond seen from above. Photo: ©Sofie Lindman, map: Google Maps.

Rånås pond (figure 10) is the reference site. Rånås is a naturally occurring pond located in a residential area in the municipality of Drøbak in Akershus county.

Figure 10. Left: Rånås pond. Right: The surrounding area of Rånås pond seen from above Photo: ©Sofie Lindman, map: Google Maps.

Six individuals of each species were collected from Fornebu sedimentation pond, Tenor sedimentation pond and Rånås reference pond. Seven individuals of northern crested newt were collected from Nygård sedimentation pond due to the absence of captured smooth newts. Apparent pregnant females were excluded from sampling according to the permit from the Norwegian Environmental Agency.

The newts were killed by a blow to the head according to the guidelines provided by the Norwegian Food Safety Authority. The tail was cut off and blood was collected from the ventral tail vein using a heparinized microhematocrit tube. The blood was transferred to a pre-filled Eppendorf-tube containing 200µL PBS-EDTA. A piece of the outermost part of the tail was collected and transferred to a pre-filled Precellys-tube containing 700µL RNAlater® solution (RNA stabilization reagent). After identifying the liver and gall bladder, the gall bladder was collected whole and a piece of liver was collected and stored in RNAlater in 1,5mL Eppendorf tubes. The dissection equipment and workspace were washed with ethanol 90% and RNase AWAY® between each individual to avoid cross-contamination. All samples were kept on ice until further processed or stored.

Skin and liver samples were stored in the fridge overnight and transferred to -80°C the following day according to the protocol for RNAlater Sigma. The gallbladder samples were kept dark on ice maximum 4 hours before transferring to a 4°C fridge. The samples were kept in the fridge until all samples were collected after four days. After, the samples were kept in -20°C until further processed. The blood samples were processed the same day as sampled, for comet assay preparation (Appendix D). Due to error in the comet assay protocol, the analysis

was unsuccessful. Five more individuals (juvenile newts) were captured post-sampling in Rånås pond to modify the protocol. In addition, micronucleus assay was performed on these individuals. The comet assay and micronucleus assay methods with modifications are described in appendix D. However, the results from these analysis are not shown in this thesis.

3.2 Gene expression analysis

Gene expression analysis was conducted using real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Liver and skin samples from both northern crested newt and smooth newt were analysed. RT-qPCR is a method to detect and quantify nucleic acids. In RT-qPCR, mRNA is first reversed transcribed to complementary DNA (cDNA) by reverse transcriptase. The amount of cDNA formed is comparable with the original amount of mRNA in the sample. Following cDNA synthesis, the cDNA is amplified using polymerase chain reaction. This reaction contains three repeatable steps; denaturation, annealing, and elongation. The high temperature (95°C) in the first denaturation step causes the double stranded DNA (dsDNA) to separate into single stranded DNA (ssDNA). In the annealing step, the annealing temperature is set to optimize the binding of the primers to the ssDNA. Finally, the temperature is raised (60-78°C) in the elongation step to enable the DNA polymerase to synthesize new cDNA (Freeman et al., 1999). These three repeatable steps enable the amount of cDNA to double after each cycle. To allow quantification of the amplified DNA molecules a fluorescent dye called SYBRgreen is used. SYBRgreen binds to dsDNA and during each cycle and the amount of SYBRgreen is measured. The fluorescent signal will increase proportionally to the amount of synthesized cDNA and hence the DNA is quantified in real time (Morrison et al., 1998). Table 3 shows selected genes used in the gene expression analysis.

Table 3. General information of selected genes.

Function	Biomarker name	Abbreviation
PAH biotransformation	Cytochrome P450 1A1	CYP1A1
PAH biotransformation	Cytochrome P450 1B1	CYP1B1
Metal biotransformation	Metallothionein	MT
Neurotransmission/endocrine	Acetylcholinesterase	AChE
Antioxidant	Glutathione peroxidase 3	GPX3
Antioxidant	Glutathione S-transferase kappa 1	GSTK1
Antioxidant	Catalase	CAT
DNA repair	DNA repair protein XRCC1	XRCC1
DNA repair	DNA repair protein RAD51	RAD51
DNA repair	Growth arrest and DNA damage-inducible protein gamma	GADD45g
Lipid metabolism	Peroxisome proliferator-activated receptor alpha isoform X1	PPAR
Energy sensor	5'-AMP-activated protein kinase subunit beta-1	AMPKB1
Osmoregulation	Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1

3.2.1 Tissue homogenization and RNA isolation

RNA isolation was conducted using a RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany) as described in Song et al., (2014). Briefly, 20-30mg liver tissue and 25-30mg muscle tissue were lysed in 600µL RLT buffer™ containing β-mercaptoethanol (1% v/v) (Sigma-Aldrich Chemie, Steinheim, Germany). The lysate was homogenized (2 x 10 sec, 6000rpm) in Precyllys CK14 beads using a Precelly orbital shaker bead mill (Bertin, Montigny-leBretonneux, France). Following homogenization, the tissue homogenate was centrifuged (30.000rpm) for 3 min. The supernatant was transferred to a gDNA EliminatorTM spin column (Qiagen). The column was centrifuged (10.000 rpm) for 30 sec to remove genomic DNA. 400µL ethanol (50%) was added to the eluate and gently mixed by pipetting and transferred to a RNeasy spin column (Qiagen). The column was centrifuged (10.000rpm) for 15 sec. To wash the spin column membrane, 700µL Buffer RW1 was added to the column followed by 15 sec centrifuging (10.000rpm). The column was then washed with 700µL RW1 buffer and centrifuged for 15sec (10.000rpm). Finally, the column was washed twice with 500µL buffer RPE and centrifuged (10.000rpm) for 15sec to ensure that no ethanol was carried over. 50µL nuclease free-water was added to the column and centrifuged (10.000rpm) for 1 min to ensure pure RNA elute.

3.2.2 RNA quantity and quality control

To determine the quantity and purity of the RNA samples, a NanoDrop® Spectrophotometer (ND 1000, Nanodrop Technologies, Wilminton, Delaware USA) was used. The RNA concentration was measured by reading the absorbance at 260nm. The purity of the RNA samples was determined using the ratio between RNA at 260nm and protein at 280nm (260/280 ratio). Only samples with sufficient quantity (>50ng/µL) and purity (260/280 ratio between 1,8-2,0) were used for further transcriptome sequencing analysis.

The Agilent 2100 Bioanalyzer and RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, California USA) were used to determine the RNA integrity number (RIN). The RIN number is inferred based on the ratio between ribosomal subunit 18S and 28S and range from 1 (totally degraded) to 10 (intact) (Schroeder et al., 2006). A RIN above 8 was used.

Briefly, Nano Gel Matrix was added to a spin filter and centrifuged at 1500g for 10 minutes. 65µL Gel Matrix was transferred to a 1.5mL Eppendorf tube. Nano Dye was vortexed and 1µL was pipetted and added to the Gel Matrix. The gel-dye mix was vortexed and centrifuged at 13000g for 10 minutes. 9µL of the gel-dye mix was added to one well at an RNA Nano chip. The chip was placed in a priming station and a plunger was used for 30 seconds to distribute to gel-dye mix throughout the chip. Further, 9µL of the gel-dye mix were added to two adjacent wells. 5µL Nano Marker was added to all the remaining wells. Meanwhile, the RNA samples were placed in a heating block (70°C) for 2 minutes to remove secondary structures (denaturation). One µL of RNA sample and 1 µL RNA ladder was added to the chip.

The chip was vortexed (MS3 (IKA) vortexer) at 2400rpm for 1 min prior to analysis in the Agilent 2100 Bioanalyzer. The samples with a RIN value above 8 were considered intact. The RNA sample was stored at -80°C until further analysis.

Liver and skin RNA samples with sufficient quantity (>5µg), purity (280/260 >1.8, 260/280>2) and quality (RIN>8) were chosen for transcriptome sequencing and qPCR.

3.2.3 RNA sequencing and de novo transcriptome assembly

Since the genome information of the two newt species was not available, transcriptome sequencing (RNA-seq) was performed to obtain the mRNA (contig) sequences for qPCR

primer design. RNA-seq and *de novo* transcriptome assembly (without reference genome) were performed as an external analytical serve by Beijing Genome Institute (BGI, Hong Kong, China) using the Illumina NovaSeq 6000 platform (Illumina Inc., San Diego, USA).

3.2.4 Quantitative real time RT-qPCR

16μg of RNA sample was reversely transcribed to complementary DNA (cDNA) using High Capacity cDNA Archive kit (Applied Biosystems, Foster City, California, USA). RNA sample and 4μL cDNA SuperMix (containing buffer, dNTPs, $MgCl_2$, primers, RNase inhibitor protein, qScript reverse transcriptase, and stabilizers) was mixed and incubated in Mastercycler gradient (Eppendorf) running primer annealing (25°C, 5min), elongation (42°C, 60min) and finally termination (85°C, 5 min). Following cDNA synthesis, the cDNA template was amplified in technical duplicates using the PerfeCTa® SYBR® Green Fastmix® (Quanta, BioSciencesTM, Gaihers-burg, MD, USA) and 100μM forward/reverse primers in a 20μL reaction. The primers were designed based on the contig sequences obtained from transcriptome sequencing using the online primer design tool Primer3 v0.4.0 (frodo.wi.mit.edu/primer3). The primers were purchased from Invitrogen™ (Carlsbad, USA) and optimized for annealing temperatures (table 4 and 5). To calculate the amplification efficiencies (90-105%), standard curves were generated using pooled cDNA. In addition, non-template controls (NTC) and no-reverse-transcriptase controls (NRC) were included in the qPCR reaction to ensure adequate quality.

The relative gene expression of each gene was calculated based on the quantification cycle (Cq) values using the Pfaffl method (Pfaffl 2001) and normalized to the geometric mean expression of the housekeeping genes using the ΔΔCq method (Vandesompele et al., 2002) . To calculate the relative fold change, the relative gene expression of the sampling sites was further normalized to the reference site.

Table 4. Primer sequences for smooth newt (*Lissotriton vulgaris*). ACTIN, GADPH and EF1A1 are housekeeping genes.

Gene name	Gene symbol	Primer sequence (5'-3')	Species	E[a]	Annealing temp[b]
Beta actin	ACTIN	F:CCTGCTTGCTGATCCACATC R:AAGATCATTGCCCCACCTGA	Cynops pyrrhogaster	1	60
Glyceraldehyde 3-phosphate dehydrogenase	GADPH	F:CGCGTTGTGGATCTGATGAG R:AGGGCATAAGTGGTGGAGAC	Ranodon sibiricus	1.03	62.5
Elongation factor 1-alpha	EF1A1	F:CTTCCGTTCAACCTTCCAGC R:GTTACAACCCAGCCACAGTG	Cricetulus griseus	0.923	56.6
Cytochrome P450 1A1	CYP1A1	F:ACCCAGCTCCGAAAAGATCA R:ACTCTCTGATCGACCACTGC	Andrias davidianus	0.95	62.5
Catalase	CAT	F:GAGGATAACGTCACCCAGGT R:TCTGGATGAAAGGCTGAGCA	Calidris pugnax	0.9	62.5
DNA repair protein RAD51	RAD51	F:GCACAAGTAGATGGAGCTGC R:TTTCGCCTCTTCCCTTCCTT	Cynops pyrrhogaster	1	62.5
Cytochrome P450 1B1	CYP1B1	F:TCACGGACCACTGATTGACA R:TGCCTGTTACCATTCCCCAT	Latimeria chalumnae	0.999	56.6
Metallothionein	MT	F:CTTGTTCATGTGCTGGGTCG R:TCTCCGTCGATCCTCCTTTG	Cynops orientalis	0.92	56.6
Glutathione peroxidase 3	GPX3	F:GGGCAAGAAAGAGAAGGCAC R:TCCTGGCTGCAGTAAATGGA	Nanorana parkeri	0.901	56.6
Peroxisome proliferator-activated receptor alpha isoform X1	PPAR	F:AGTAGAGGATGCAAGGAGGC R:TGACCTCCGACAACTTGTGA	Picoides pubescens	0.965	60
X-ray repair cross-complementing protein 1	XRCC1	F:GAAGGTCCCAGGCAGTACTT R:TGCCTACAAATCACCCCAGT	Nanorana parkeri	1	60
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	F:TCCGGAACAGATTGACGACA R:ACAGCCCTCCACAATGATCA	Crocodylus porosus	1	60
Glutathione S-transferase kappa 1	GSTK1	F:TAGAGAGGTGGGAAATGGGC R:TGCATACCGTTGAAGCGATG	Chelonia mydas	0.991	51.6
Growth arrest and DNA damage-inducible protein gamma	GADD45G	F:ATGCATTTAAAGACCCCGCC R:GCACCCAGTCGTTGAAGTTT	Nanorana parkeri	0.9	51.6
Acetylcholinesterase	AChE	F:TCTTCCGTGTAGTTGAGCGT R:CAAGTGGATGGGTGTGCTTC	Rhincodon typus	0.94	51.6
5'-AMP-activated protein kinase subunit beta-1	AMPKB1	F:GCTTCAAATCGCCACCCATA R:GAAATGCCGGTGTCCTTGTT	Monodelphis domestica	0.927	51.6

[a] Efficiency

[b] Annealing temperature (°C)

Table 5. Primer sequences for northern crested newt (*Triturus cristatus*). ACTIN, GADPH and EF1A1 are housekeeping genes.

Gene name	Gene symbol	Primer sequence (5'-3')	Species	E[a]	Annealing temp[b]
Beta actin	ACTIN	F:CCCATTGAACACGGCATCAT R:CGTTGTAGAAGGTGTGGTGC	Dasyatis akaje	0.911	53.8
Glyceraldehyde 3-phosphate dehydrogenase	GADPH	F:AGAAGGTGGTAAAGGCAGCT R:CGAAGTGGTTGTTGAGTGCA	Ranodon sibiricus	0.938	64.2
Elongation factor 1-alpha	EF1A1	F:CAAAGTCACCAAGTCCGCAA R:TGCTCAGTAACCAGTCCCTG	Ovis aries	0.9	62.5
Cytochrome P450 1A1	CYP1A1	F:CACTTGCCACTGGTTCACAA R:CCACACAGCACGACAAGAAA	Andrias davidianus	0.914	62.5
Catalase	CAT	F:GGATAAAGGACGGGAACAGC R:ACACCGATGATGGGATCTGG	Nanorana parkeri	0.901	56.6
DNA repair protein RAD51	RAD51	F:GCACAAGTAGATGGAGCTGC R:TTTCGCCTCTTCCCTTCCTT	Cynops pyrrhogaster	0.975	50.3
Cytochrome P450 1B1	CYP1B1	F:ACGTGTGGAGCTTTCCCTAA R:CTGGAAGTGGCTGAGAAGGA	Latimeria chalumnae	0.909	50.3
Metallothionein	MT	F:GTTCTCACACTTGCACGACC R:GCTGCAAAACATGGACCCTA	Triturus carnifex	0.993	53.8
Glutathione peroxidase 3	GPX3	F:CAGGAGGTGGTGGGTTAAGT R:TCTGACACCTTCCAGCTCAG	Nanorana parkeri	1.05	56.6
Peroxisome proliferator-activated receptor alpha isoform X1	PPAR	F:ATCCCTGGCTTTGTGGACTT R:CGACCAGCATCCCATCTTTG	Picoides pubescens	0.913	56.6
X-ray repair cross-complementing protein 1	XRCC1	F:GGAAGCAATCTGAATCGCGT R:ATCTTCACCCGGTCCCATTT	Anolis carolinensis	0.91	53.8
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	F:CGTCACCATCCTCTGCATTG R:ATCTGTCTTGGGGTTCCTGG	Rhinella marina	0.904	60
Glutathione S-transferase kappa 1	GSTK1	F:TAGGCAAGAACTGAGAGGGC R:TAGAGTGGGGCAATGACAGG	Chelonia mydas	0.907	53.8
Growth arrest and DNA damage-inducible protein gamma	GADD45G	F:GCCACACATGCAGAGACTTT R:TTCAGCTTCCCCATGTCAGT	Nanorana parkeri	0.904	56.6
Acetylcholinesterase	AChE	F:TCCCTCCCATTCCAACTCAC R:TTGACTGGTTGTGTGCAGTG	Austrofundulus limnaeus	0.952	60
5'-AMP-activated protein kinase subunit beta-1	AMPK	F:TGTTTTCCTCTGGACACCGA R:CAAGGGCAGACTTCGACAAC	Monodelphis domestica	0.908	64.2

[a] Efficiency

[b] Annealing temperature (°C)

3.3 PAH metabolites

High-performance liquid chromatography (HPLC) was used to measure the concentration of PAH metabolites in bile samples of northern crested newt and smooth newt. Preparation of the bile samples was done by the author. HPLC analysis was performed at the laboratory at NIVA.

The gallbladders were thawed on ice. The samples contained the whole gallbladder and were punctured using soda lime glass capillaries (Hilgenberg) to collect the bile. The length of the capillary filled with bile was measured using a caliper to calculate the total volume of each sample. The bile was transferred to Eppendorf tubes containing 10µL of internal standard (triphenylamine). 20µL MillQ® water and10µL β-glucoronidase/arylsulfatase was added to each sample and mixed by pipetting. The samples were incubated at a heating block at 37°C for 60 minutes. Following incubation, 50µL methanol was added. The samples were then centrifuged (13.000rpm) for 10 min. The supernatant was transferred to 1mL clear glass vials (Waters) and kept in the freezer -20°C prior to HLPC analysis.

A Waters 2695 Separations Module attached with a 2475 fluorescence detector was used to perform the HPLC analyses. A Waters PAH C18 column with 5µm particles was used. The separation was done by two mobile phases (acetonitrile and ammonium acetate). Fluorescence was measured at the optimum for each analyte. 70µL extract was injected for each analysis. The results were calculated by subtracting the highest blank for each metabolite. The limit of detection was set to the highest blank making the limit of detection two times the blank value.

3.4 Statistical analysis

Raw data was processed using Microsoft® Excel® for Office 365 MSO. Statistical analyses were conducted using Rstudio version 1.1.456. Following additional packages in Rstudio was used: tidyverse, vegan, car, ggpubr, plsdepot, ggbiplot, gplots, RcolorBrewer and reshape2.

3.4.1 Univariate statistics

Residuals of the data were evaluated for normal distribution and homologous variance using the Shapiro-Wilk test and Levene's test respectively. If these assumptions were met, parametric tests were performed. The data was log transformed when necessary. When

parametric tests were not applicable, non-parametric tests were used. The level of significance was set to 0.05. Values below the limit of detection (PAH data) were substituted by a random value between 0 and the limit of detection using the extrapolated distribution (Helsel 1990).

PAH metabolites data were analyzed using Welch sample t-test to evaluate differences between sedimentation and reference pond. Nested one-way analysis of variance (ANOVA) followed by post hoc Tukey's test was used to compare differences between locations.

3.4.2 Multivariate statistics

The use of multivariate statistics is a powerful tool to handle complex multivariate data sets which reveal patterns and inter-relationships between variables. The use of multivariate statistics also reduces the probability of performing type I error by reduced multiple statistical testing. Hence, the use of multivariate statistics in this study was appropriate to summarize the overall picture of biological effects on the newts inhabiting contaminated sedimentation ponds.

Principal component analysis (PCA) is an unconstrained ordination method which creates a linear relationship between variables and the latent variables in a data set. PCA reduces the dimensionality of the data by obtaining the variables which explains as much as possible of the variance in the original data. This variance is summed on a new set of uncorrelated variables called principal components. The principal component that maximizes the variation is called axis 1, the following principal components explain successively smaller quantities of the total variation. Each component has associated eigenvalues and explanation percentage (Bro and Smilde 2014). The results of PCA is often plotted as a biplot with the two first axis as perpendicular planes. Samples are plotted as points and variables are plotted as vectors (arrows). Two samples close to each other express similar response whereas units far from each other express different responses. The angle between the vectors represents the correlation between them. The length of the vector indicates the variation in the vector. Thus, longer vectors indicate more variability within the variable (Sparks et al., 1999).

Redundancy analysis (RDA) is a constrained parallel ordination method to PCA. Constrained ordination optimizes the fit of the response variable to a set of explanatory variables (constrained variables). The variation expressed on the redundancy axes is therefore restricted to the variation also explained by the explanatory variables (Sparks et al., 1999). The

variation explained by the constrained variables is presented as constrained axes while unexplained variation is presented as unconstrained axes.

Principal component analysis (PCA) was performed on explanatory variables for abundance data and gene expression data. This was done to identify potential interrelationships between the variables and to investigate patterns between locations and species. Following PCA on the explanatory variables for the abundance of newts, a redundancy analysis (RDA) was performed to investigate associations between the response variable (i.e. abundance) and explanatory variables. The level of significance was set to 0.05 and 1499 Monte Carlo permutations were used. By using Monte Carlo permutation tests the distribution of the original data is simulated to derive a pseudo F ratio. Pseudo F ratio is the counterpart of F ratio used in the parametric significant test in regression models. In parametric regression models, normality is required. However, in permutation based models, assumptions of normality are abandoned.

The number of explanatory variables for the abundance data was reduced before the RDA analysis to reduce the risk of overfitting the RDA model. This was done by using sample scores extracted from axis 1 of the PCA analysis on metal and PAH data to use this as a proxy for metal and PAH pollution in the sediment of the ponds. The pollution concentrations in the sediment represent a time-integrated proxy for the overall pollution levels in the ponds. In contrast, variables related to water quality only represents a snapshot of the water quality and has therefore not been assessed as potential explanatory variables for newt abundance. In addition, Partial RDA was performed on the gene expression data to remove the effects of variation from other co-variables (tissue, species and location).

4 Results and discussion

4.1 Water quality

Water quality parameters measured in all reference and sedimentation ponds are presented in table 6. The reference ponds displayed similar wet characteristics (e.g., conductivity, pH, temperature and oxygen reduction potential) within each parameter. The sedimentation ponds varied within each parameter among all ponds. For example, the reference ponds had 17-55µg/L total phosphorus whereas 6-460µg/L was measured in sedimentation ponds. Furthermore, the conductivity varied between 141-3338µS/cm in sedimentation ponds while 124-274µS/cm was measured in the reference ponds. Conductivity is the concentration of anions and cations in the water, representing the waters ability to carry an electrical current (Dodd et al., 2010). Hence, conductivity is often used as a proxy for de-icing salt in sedimentation ponds. The pH concentration varied in sedimentation ponds between 6.8-9.1. 15 sedimentation ponds had alkaline waters which may present harm to aquatic organisms (Dodd et al., 2010). However, none of the sedimentation ponds had acidic waters.

Table 6. Water quality parameters measured in all reference and sedimentation ponds. The number prior to the name represents the location in the location map (figure 5).

Reference pond	pH	Conductivity (µS/cm)	Temp (°C)	Oxygen (%)	Oxygen (mg/L)	ORP[d]	TOT P[e]	TOT N[f]
1. Berle	7.7	274	18.2	45	4.3	533	52	1000
18. Rånås	7.2	124	17.8	83	11.1	413	17	590
30. Vennersrød	7.5	216	19.6	70	6.4	425	55	800
Sedimentation pond								
2. Bommestad East	8.2	141	17.9	113	10.7	436	12	990
3. Borre	7.4	870	20.3	53	4.8	528	29	480
4. Eikeberg	8.4	1309	24.4	118	9.9	731	37	680
5. Emmerud	7.3	945	20.7	45	4.1	729	150	1300
6. Fevang	7.6	1794	24.8	106	8.8	727	24	820
7. Finstad	7.7	1492	17.4	121	11.4	636	410	1800
8. Fornebu	6.8	3338	17.5	23	2.1	499	95	1100
9. Helland	7.1	2044	24.6	99	7.7	699	15	350
10. Holmane	8.4	885	23.8	223	19.1	722	100	1200
11. Holt	9.1	1017	23.2	123	10.5	504	6	270
12. Idrettsveien	6.8	594	14.4	42	4.2	753	120	1700
13. Lundeby	7.0	864	11.7	85	9.0	637	10	660
14. Neset	8.3	897	23.8	102	8.6	568	15	270
15. Nøklegard	8.2	1783	22.7	83	6.4	796	460	1600
16. Nøstvedt	7.2	1321	17.4	38	3.5	542	19	450
17. Nygård	7.2	473	14.0	47	4.4	725	180	1100
19. Ringdal	7.1	191	17.4	25	7.9	452	440	1100
20. Seierstad	9.1	622	18.1	117	2.3	375	9	710
21. Skinmo	8.3	1156	19.5	119	10.8	378	13	550
22. Sky	8.0	1546	19.8	102	9.2	548	38	580
23. Støttum	8.0	1496	23.3	96	8.1	689	7	330
24. Talaldrud Junction	7.4	1253	16.6	69	9.8	749	29	670
25. Taraldrud North	7.6	1227	13.9	96	9.9	807	51	430
26. Talaldrud South	7.0	1310	19.7	80	7.3	757	15	480
27. Tenor	7.4	1890	15.4	120	12.0	618	98	850
28. Tveiten	7.7	399	21.5	80	7.0	756	38	500
29. Vassum	7.6	1518	18.7	53	4.7	561	120	930

d Oxygen reduction potential

e Total phosphorus concentration measured in µg/L

f Total nitrogen concentration measured in µg/L

4.2 Pollution level in sediment

4.2.1 Metal concentration

The concentration of trace metals in sediment samples is shown in Appendix B. Figure 11 shows a representation of each metal normalized to their associated AA-EQS, chronic toxicity see figure 2, values in order to compare all metals to their associated PNEC values (metal concentration / AA-EØS).

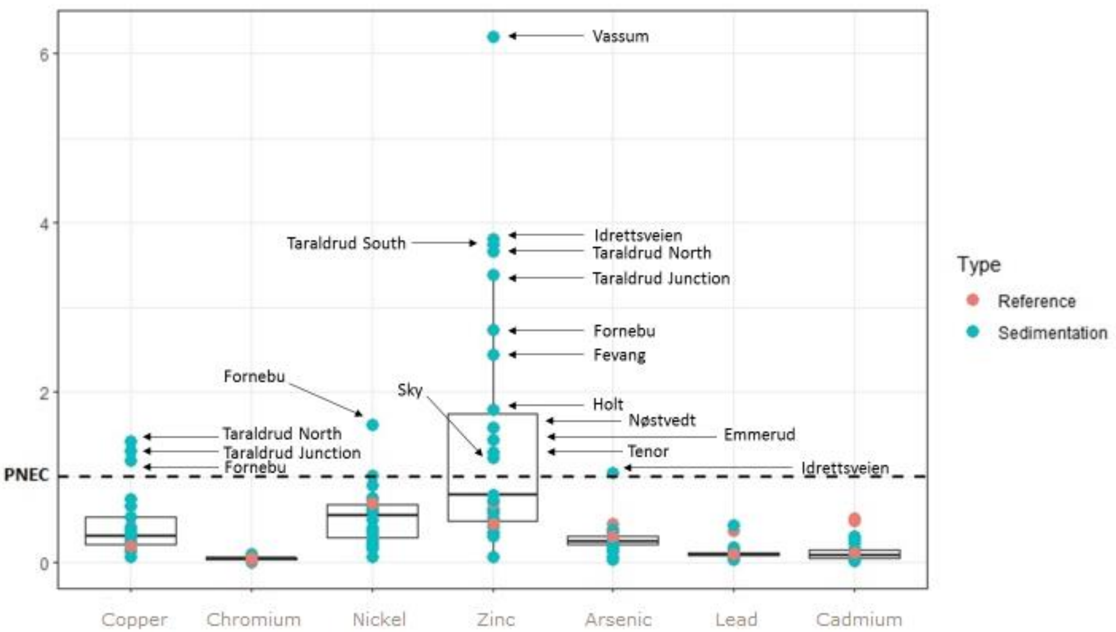

Figure 11. Boxplot showing metal concentration in sediment from reference (n=3) and sedimentation ponds (n=27). The box represents the interquartile range. The upper borderline represents the third quartile whereas the lower borderline represents the first quartile. The middle line represents the median value. The reference sites are indicated in red whereas sedimentation ponds are indicated in green. All concentrations are normalized to the AA-EQS for each metal. The PNEC value (1) is indicated with a dashed line. All locations exceeding the PNEC value are named. The y-axis is unitless. The x-axis shows all metals.

Copper (Cu) concentrations were below PNEC in all locations apart from Taraldrud North/Junction and Fornebu sedimentation pond. Chromium (Cr) concentration did not exceed the PNEC value in any location. Nickel (Ni) concentrations solely exceeded PNEC in Fornebu sedimentation pond. Zinc (Zn) concentrations were high in several sedimentation ponds. 12 sedimentation ponds displayed exceeded PNEC values for zinc. Arsenic (As)

concentrations solely exceeded PNEC values in Idrettsveien. Lead (Pb) and cadmium (Cd) concentrations did not exceed the PNEC value in any location.

Metals of concern were primarily zinc and copper which had several locations exceeding the PNEC value. This result was consistent with other studies examining the toxicity in sediment from sedimentation ponds. For example, Karlsson et al., (2010) reported that zinc and copper concentrations exceeded EQS class IV in sedimentation ponds in Sweden. Zn and Cu mainly originate from tires and breaks whereas Cd, Ni, Pb and Cr mainly originate from combustion, road surface and vehicle bodies (Meland 2010).

4.2.2 PAH concentration

The PAH16 concentration in sediment from all locations is shown in figure 12 (data shown in Appendix B). For comparison, both PAH16 and PAH16 and their associated alkylates concentration are shown. Fevang sedimentation pond displayed over 150 000 µg/kg DW of PAH16+alkylates concentration. One plausible explanation for this high concentration was an oil spill reported by the NPRA four years ago. Fevang sedimentation pond was therefore excluded in this presentation.

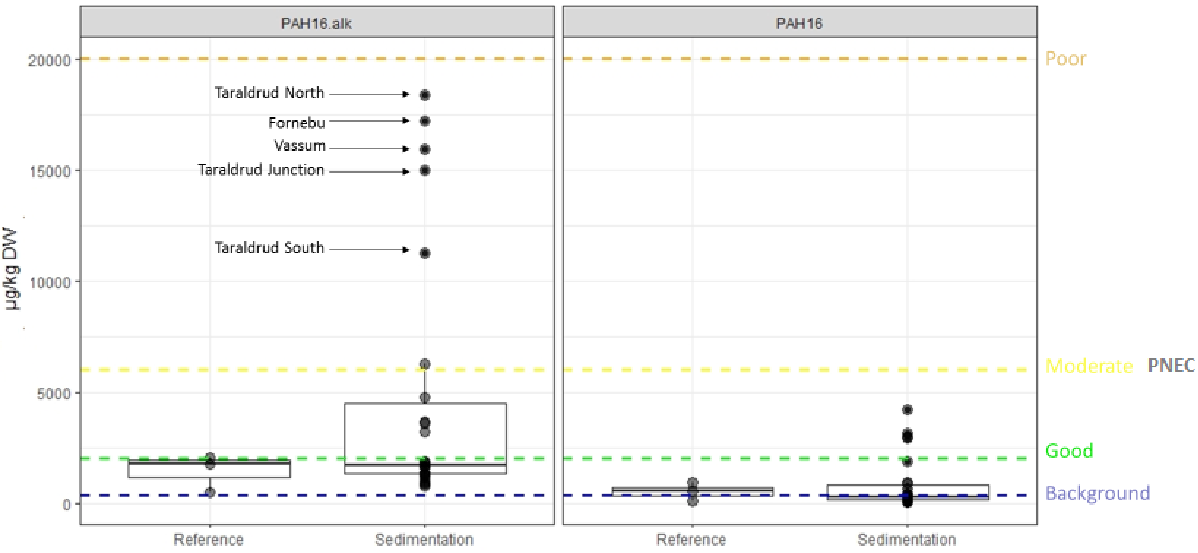

Figure 12. Boxplot showing PAH16 and their associated alkylates concentration (left) and PAH16 concentration (right) in reference and sedimentation ponds. EQS classes and PNEC are indicated in dashed lines. The y-axis represents concentration (µg/kg DW).

PAH16 concentration did not exceed PNEC in any location. PAH16 and associated alkylates concentration exceeded PNEC in five locations namely Taraldrud South/North/Junction, Vassum and Fornebu. These sedimentation ponds were classified as the EQS level moderate (figure 2) indicating possible chronic effects of pollution on the organisms. Alkylated PAHs have in many cases been identified as equally toxic as parent PAHs (Andersson and Achten 2015). It is therefore reason to believe that they also effect organisms. However, to conduct a final risk assessment it is essential to include EQS for biota and water in addition.

There was no difference in parent PAH16 concentration between sedimentation ponds and reference ponds ($t = -0.23251$, $p = 0.8341$). Neither was there evident differences for PAH16 and associated alkylates concentration between sedimentation and reference ponds ($W = 29$, $p = 0.4665$). One plausible explanation for the observed result is few reference ponds and large variation in PAH concentration between sedimentation ponds.

4.3 Abundance of newts in sedimentation ponds

The survey showed that 14 (52%) of the 27 investigated sedimentation ponds were inhabited by newts (figure 13, Appendix A). Smooth newt was present in all these ponds, four of which had northern crested newt in addition. The survey showed variation ranging between 1-51 individuals of smooth newt and 1-17 individuals of northern crested newt per location. The number of caught animals in this study were relatively low compared to naturally occurring reproduction locations where 1-549 smooth newts and 1-97 northern crested newts was caught during a survey in Oslo (Elgtvedt and Dervo 2018).

The trapping success varied between traps at the same location. The abundance of newts in the investigated sedimentation ponds is indicated in figure 13. The biological mapping showed that both northern crested newt and smooth newt inhabit sedimentation ponds. This result is in line with hypothesis I, and confirms the species presence in sedimentation ponds.

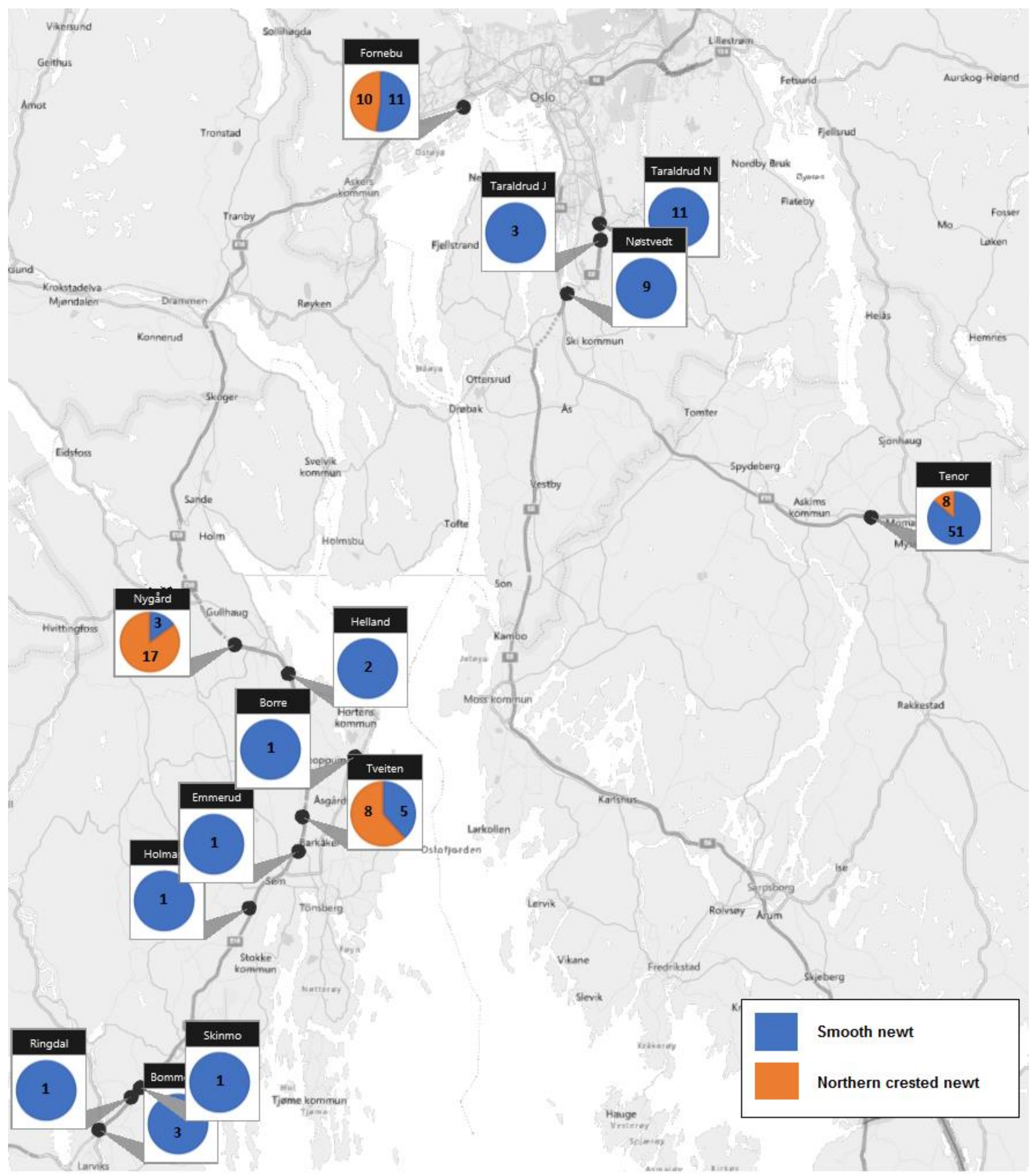

Figure 13. Map showing all locations inhabited by newts. Pie chart colors indicate the percentage of species found in each pond. Orange represent northern crested newt. Blue represents smooth newt. The numbers represent the actual number of individuals caught.

4.3.1 Abundance determinants

Table 7 shows selected explanatory variables for newt abundance. Figure 14 shows a principal component analysis (PCA) for selected explanatory variables.

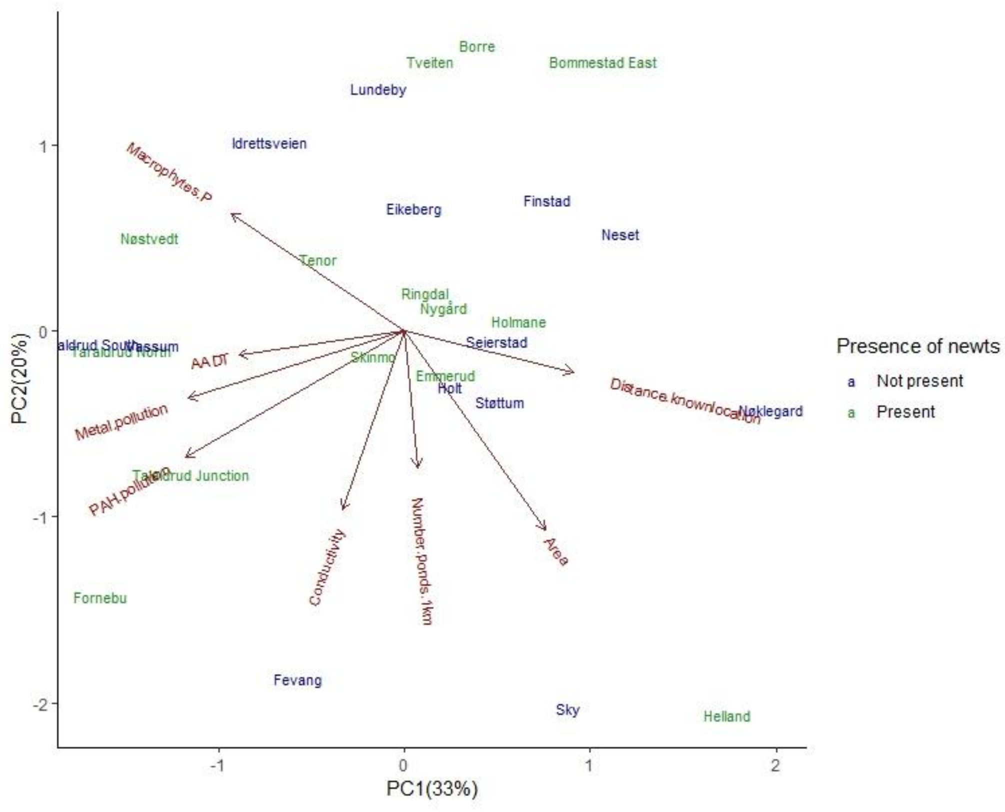

Figure 14. Principal component analysis (PCA) on selected explanatory variables listed in table 7. Locations indicated in green have newts present in the ponds. Locations indicated in blue lack newt presence.

The first two PCA axes captured 33 % and 20 % of the total variation, respectively. The PCA showed a positive correlation between AADT, PAH pollution, and metal pollution. This was confirmed by a linear regression analysis (data are shown in Appendix C) showing a significant correlation between AADT and PAH16 ($r=0.74$, $p=0.00001$). Area and macrophyte coverage showed a strong negative correlation indicating decreased macrophyte coverage with increased pond size.

A redundancy analysis (RDA) was conducted on the abundance of newts modelled by the explanatory variables. The abundance data were log10(x+1) transformed to reduce skewness and achieve normality of the data. Due to the relatively small number of northern crested newt, it was decided to group the two species to just “newts”. The results from the RDA

analysis is displayed in table 7. The RDA showed that the explanatory variable physical barrier (p=0.010) and macrophytes coverage (p=0.011) contribute to explain the abundance of newts in sedimentation ponds. Physical barrier explained 24% of the variation whereas macrophyte coverage explained 6% of the variation observed in the abundance data. The remaining seven explanatory variables were not found to have a significant association with the response variable.

Table 7. Redundancy analysis (RDA) results for all explanatory variables using abundance of newts as response variable. Permutations=1999. Abundance of newts are log10(x+1) transformed. The grey shaded areas show significant results of simple effects.

Explanatory variable	Explain%	pseudo-F	P-value
Physical barrier[a]	24.1	7.9	0.010
Area (m^2)[b]	6.5	1.7	0.202
Macrophyte coverage (%)	6.1	7.2	0.011
Distance known newt location[c]	5.6	1.5	0.246
Metal pollution	3.1	0.8	0.333
Annual average daily traffic	2.0	0.01	0.946
PAH pollution	1.7	0.4	0.511
Number of ponds 1km[e]	1.2	1.1	0.273
Conductivity	0.8	0.8	0.364

[a] Physical barrier (road) between pond and neighboring ponds in addition to highway
[b] Pond surface area in m^2
[c] Distance to closest pond inhabited by northern crested newt or smooth newt in meters provided by Artdatabanken
[d] Average annual daily traffic retrieved from NPRA
[e] Number of neighboring ponds within 1 km radius

Physical barrier was the most important statistically significant explanatory variable. The results suggest that sedimentation ponds bounded by a road have a lower abundance of newts. This result was expected since roads increase the mortality rate of newts (Ashley and Robinson 1996) resulting in decreased dispersion capabilities to sedimentation ponds bounded by roads. Following physical barrier, macrophyte coverage was the second most important variable to explain newt abundance. This was in accordance with results obtained from previous studies showing the importance of macrophyte coverage when predicting newt occurrence (Hartel et al., 2010) and abundance (Joly et al., 2008). Aquatic vegetation provides shelter and is used as oviposition sites for eggs (Egan and Paton 2004). Thus, macrophyte coverage is of vital importance to reproduction and survival of newts. Taken together, these findings confirmed that biological and landscape variables contribute to determining newt abundance in sedimentation ponds (H2a).

The explanatory variables conductivity, number of ponds within 1km, and distance to the closest pond were not found to have significant associations with newt abundance in this study (table 7). Contradictory to our findings, these variables have previously been shown to affect newt abundance. Karraker et al., (2008) reported reduced embryonic and larvae survival in spotted salamander (*Ambystoma maculatum*) exposed to moderate and high conductivity concentrations. However, this study was conducted on early life stages of salamander which may be more sensitive to pollutants compared to adults (Carey and Byrant 1995). The number of ponds within 1km radius was an important factor explaining the dispersion and prediction of newt presence in a study conducted by Joly et al. (2008). Furthermore, Hartal et al. (2010) reported that the distance to nearest occupied pond was an important predictor of newt occurrence.

Contradictory to expectations, there were no evident results that pollution level (PAHs and metals) had a negative impact on the abundance of the species (H2b). Previous ecotoxicological research has revealed both sublethal and lethal effects in early life stages in frogs and salamanders due to PAH and trace metal exposure. Mortality has been registered in early life stages of the wood frog (*Rana sylvatica*) (Snodgrass et al., 2007), common frog (*Rana temporaria*) (Johansen 2013) and the grey tree frog (*Hyla versicolor*) (Brand et al., 2010). Moreover, reports of sublethal effects showed reduced size at metamorphosis for tadpoles of the wood frog (Snodgrass et al., 2007) exposed to highway runoff. Nebeker et al., (1995) reported altered growth in northwestern salamander (*Ambystoma gracile*) larvae due to cadmium exposure. However, these studies were conducted on frogs or salamander in early life stages such as larvae or tadpoles. Early life stages of amphibians can be more susceptible and affected by xenobiotics compared to adults (Carey and Byrant 1995). In addition, adult individuals are only present in the ponds during limited periods of time during the year (Canova and Fasola 1992), probably decreasing their exposure to xenobiotics compared to the larvae or tadpoles.

Compared to the Norwegian EQS the concentrations of toxic metals e.g., chromium, nickel, arsenic, lead, copper, and cadmium (figure 11) were below PNEC and thus low in most investigated sedimentation ponds. Furthermore, Kerby et al., (2009) argue that amphibians as a group exhibit relatively low sensitivity to heavy metal pollution. Thus, the low metal concentrations in sediment coupled with potentially low species sensitivity to metals might explain our findings that metal pollution did not impact the abundance of adult newts.

4.4 PAH metabolites

To assess any uptake of PAH in the newts, PAH metabolites were analysed in bile samples. The HPLC analysis showed presence of 1-OH-pyrene, 1-OH-phenanthrene and 3-OH-benzo[*a*]pyrene in bile samples from all locations. There were no statistical differences between females and males or between species, therefore newt has been used as a group to assess PAH metabolites.

The results presented in figure 15 showed a difference in biliary concentration of 1-OH-pyrene between individuals from sedimentation ponds and reference pond ($t = -2.67$, $p = 0.01145$). No difference was observed between individuals from sedimentation ponds and reference pond for 1-OH-phenanthrene ($p=0.418$) and 3-OH-benzo[*a*]pyrene ($p = 0.2073$) concentrations. One male northern crested newt from Fornebu had a substantially higher concentration of 1-OH-pyrene and 3-OH-benzo[*a*]pyrene compared to other individuals in the same location. This individual represents the two outliers for these metabolites.

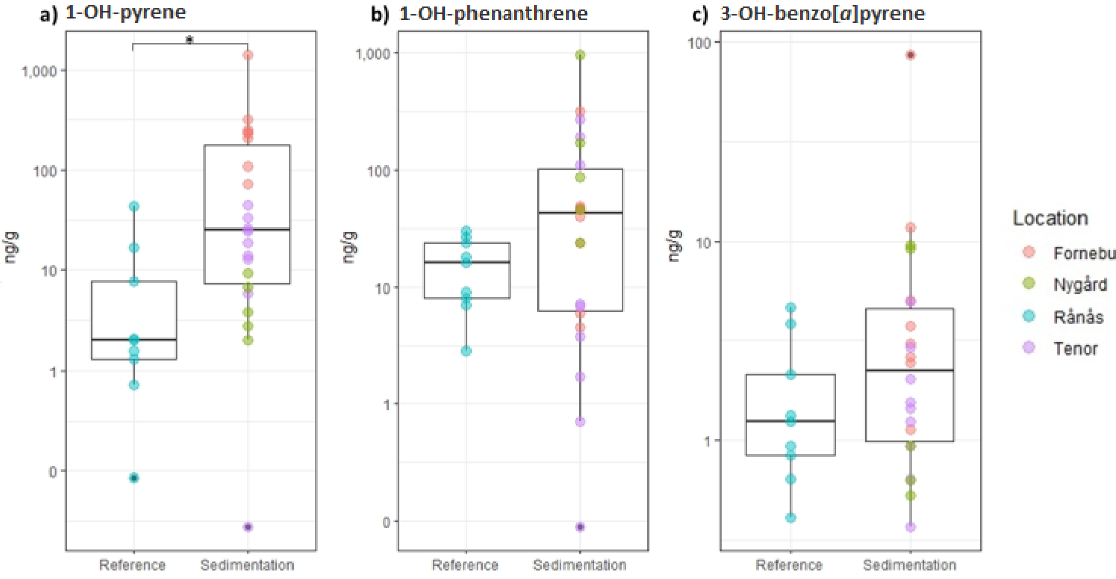

Figure 15. Boxplot showing the concentration (ng/g) of PAH metabolites in bile in individuals from sedimentation (Fornebu, Nygård, Tenor) and reference pond (Rånås). The y axis is on a log10 scale. a) 1-OH-pyrene concentration b) 1-OH-phenanthrene concentration c) 3-OH-benzo[*a*]pyrene concentration. Significant results are indicated with an Asterix.

The biliary concentrations of 1-OH-pyrene, 1-OH-phenanthrene and 3-OH-benzo[*a*]pyrene in individuals from different locations are presented in figure 16-18. An analysis of variance (ANOVA) on concentration of 1-OH-pyrene yielded variation among locations ($F = 12.12$, $p = 0.000033$). A post hoc Tukey test showed that biliary concentrations in newts from Fornebu sedimentation pond were different from the newts from the reference pond (Rånås), Nygård sedimentation pond, and Tenor sedimentation pond. There was no difference in biliary concentrations of 1-OH-phenanthrene ($p = 0.061$) or 3-OH-benzo[*a*]pyrene ($p = 0.0582$) between locations.

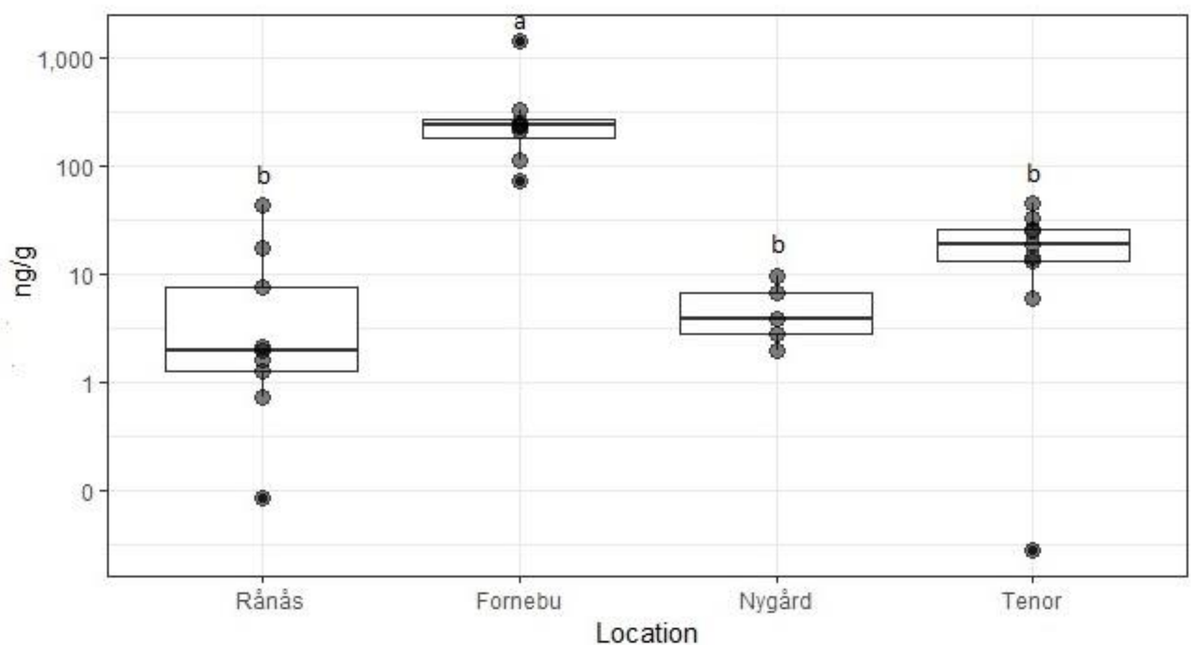

Figure 16. Boxplot showing the biliary concentration of 1-OH-pyrene from all sampling locations. The y-axis shows the concentration (ng/g) on a log10 scale. Rånås is the reference site. Fornebu, Nygård and Tenor are sedimentation ponds. Letters indicate significant results.

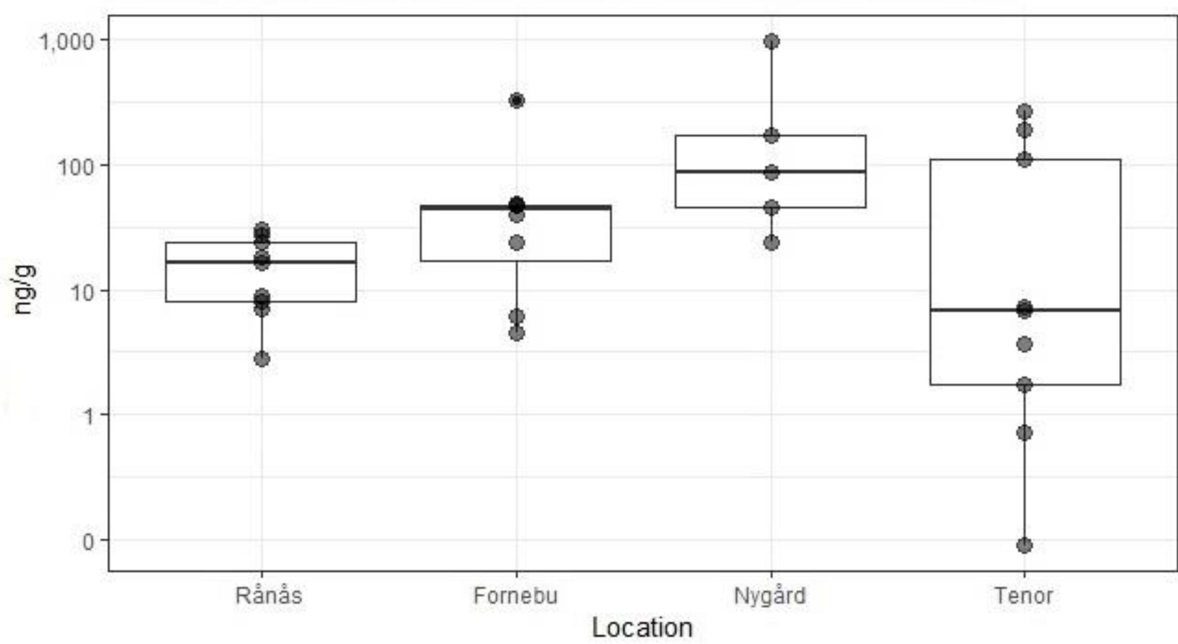

Figure 17. Boxplot showing the biliary concentration of 1-OH-phenanthrene from all sampling locations. The y-axis shows the concentration (ng/g) on a log10 scale. Rånås is the reference site. Fornebu, Nygård and Tenor are sedimentation ponds.

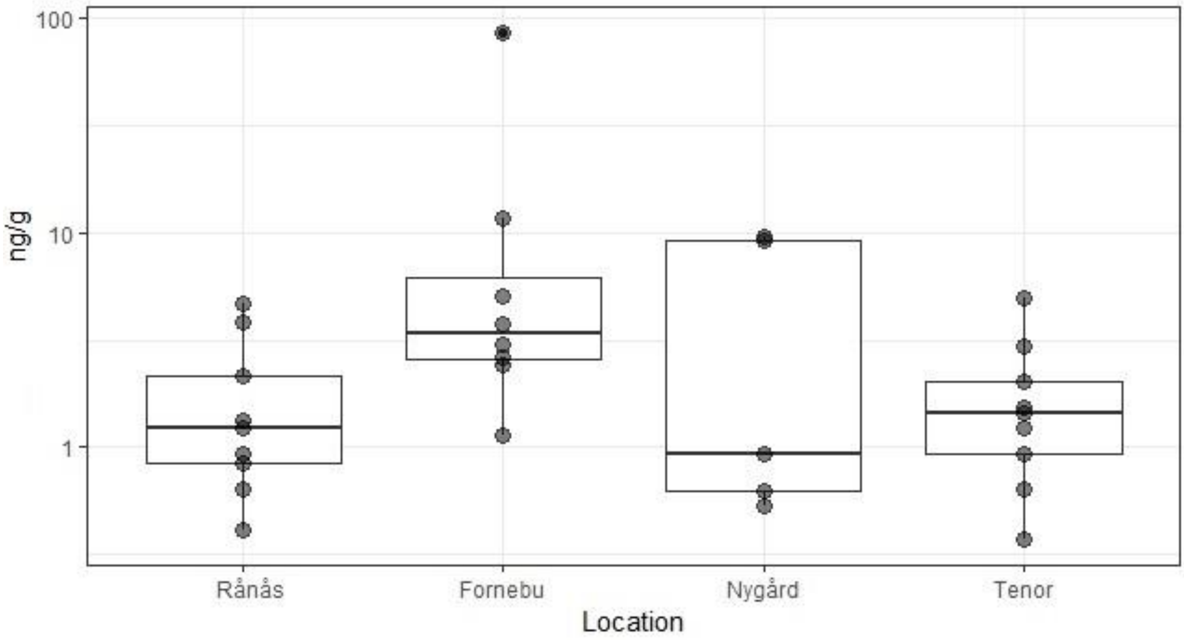

Figure 18. Boxplot showing the biliary concentration of 3-OH-benzo[*a*]pyrene from all sampling locations. The y-axis shows the concentration (ng/g) on a log10 scale. Rånås is the reference site. Fornebu, Nygård and Tenor are sedimentation ponds.

The results show that northern crested newt and smooth newt metabolize phenanthrene, pyrene and benzo[*a*]pyrene to hydroxy metabolites indicating that these individuals are exposed to and readily take up various PAHs. Two bile samples were below the limit of detection (LOD) for 1-OH-phenanthrene. These two samples aside, all three metabolites were detected in all samples.

The biliary concentration of 1-OH-pyrene was elevated in individuals from sedimentation ponds compared to the reference pond. In contrast, this result was not observed for 1-OH-phenanthrene or 3-OH-benzo[*a*]pyrene. Nor did these two metabolites show significant differences in concentration between the locations separately. It is widely known that the numbers of rings on individual PAHs influence the metabolic rate of PAHs. Uptake and depuration rates in newts vary with properties such as the number of rings, molecular weight, and K_{OW}. Comparatively uptake rates for phenanthrene (three ringed), pyrene (four ringed) and benzo[*a*]pyrene (five ringed) in Iberian ribbed newt (*pleurodeles waltl*) larvae have been shown to be 17%, 48%, and 96% respectively and steady state levels have been reached after 8 days (Garrigues et al., 2010). The findings in the present study indicated that pyrene (four ringed) were taken up and metabolized to a greater extent compared to benzo[*a*]pyrene (five ringed). However, pyrene concentrations in sediment (data shown in Appendix B) were substantially higher (10-659 µg/kg) compared benzo[*a*]pyrene concentrations (6-149µg/kg) likely explaining the observed difference. Taken together, these results partly confirm that PAH-metabolite concentrations are elevated in individuals from sedimentation ponds (H_3). These findings were consistent with previous studies investigating PAH metabolites in aquatic organisms inhabiting sedimentation ponds. For example, high concentrations of PAH metabolites have previously been reported in fish (Dybwad 2015; Hauge Skarsjø 2015; Grung et al., 2016) and tadpoles of frogs (Liane 2018). In addition, Stephensen et al., (2009) reported presence of hydroxylated PAHs in bile due to leakage of PAHs from tires.

The reference site (Rånås) in this study mainly displayed higher concentrations in the sediment of pyrene, phenanthrene and benzo[*a*]pyrene compared to both Nygård and Tenor sedimentation pond. This likely explained the absence of evident difference between individuals from sedimentation and reference ponds for 1-OH-phenanthrene and 3-OH-benzo[*a*]pyrene.

4.5 Gene expression

4.5.1 Transcriptional patterns

To assess the transcriptional patterns of individual genes in different locations, heatmaps were conducted for species and tissues individually (figure 19-22).

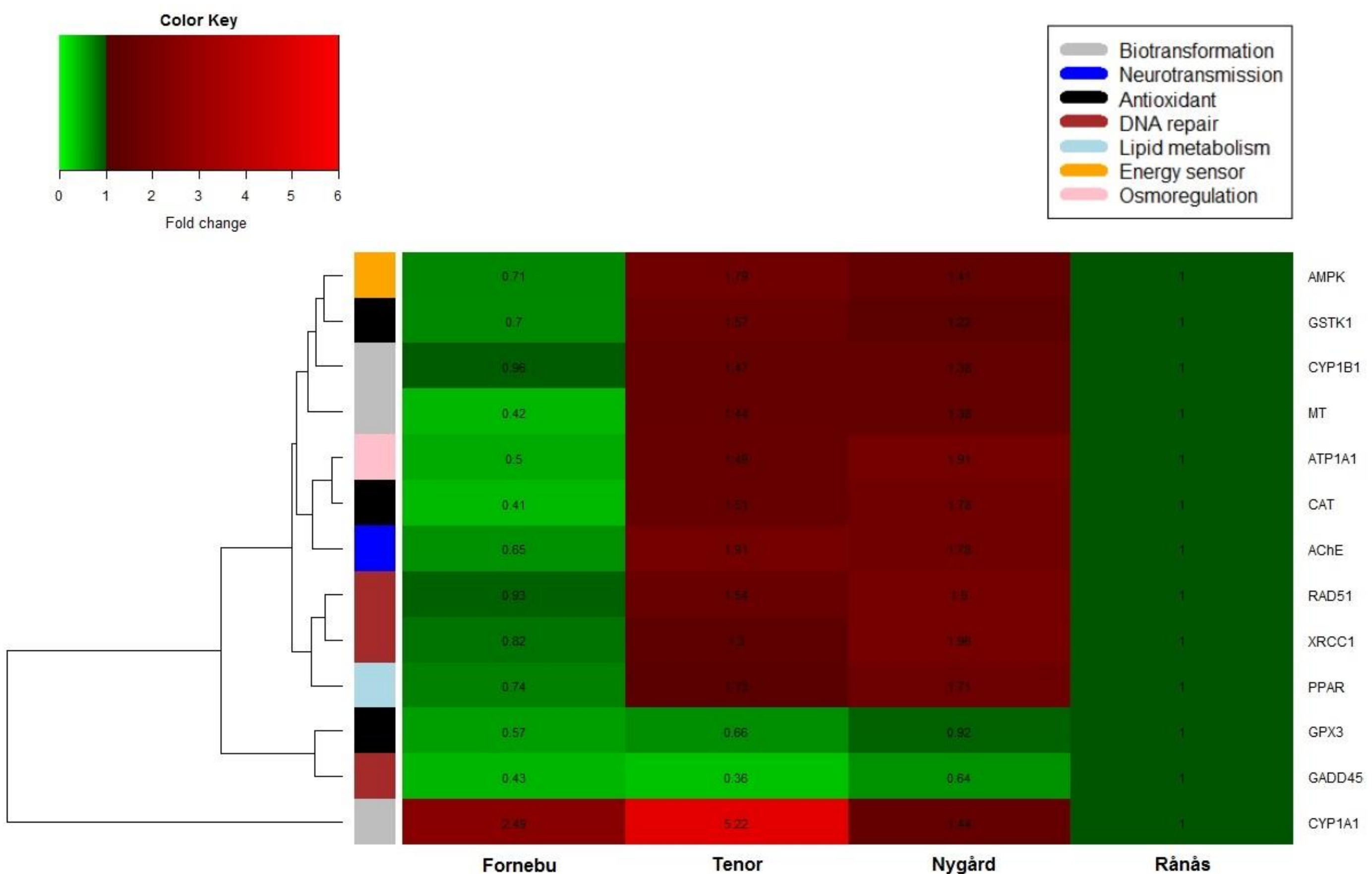

Figure 19. Hierarchical clustering heatmap with dendrogram showing up- (red) and down (green) regulated genes in comparison to the reference (Rånås) in liver from northern crested newt. Genes clustered together in the dendrogram are similar in their expression patterns. The colors in the dendrogram shows the functions of the genes.

Liver samples (figure 19) from northern crested newt expressed upregulation of genes related to biotransformation such as cytochrome P450 1B1 (CYP1B1), cytochrome P450 1A1 (CYP1A1) and metallothionein (MT) in individuals from Nygård and Tenor sedimentation pond. Moreover, genes related to DNA repair such as DNA repair protein RAD 51 and XRCC1 (RAD51, XRCC1) were upregulated in these locations. Antioxidant defense genes glutathione S-transferase kappa 1 (GSTK1) and catalase (CAT) were upregulated. In addition, energy sensor (5’-AMP-activated protein kinase, AMPK), neurotransmission

(Acetylcholinesterase, AChE), lipid metabolism (Peroxisome proliferator-activated receptor, PPAR) and osmoregulation (Sodium/potassium-transporting ATPase, ATP1A1) genes were upregulated in these locations. Two genes associated with antioxidant defense (Glutathione peroxidase 3, GPX3) and DNA repair (Growth arrest and DNA damage-inducible protein gamma, GADD45g) were downregulated in Tenor and Nygård sedimentation pond. Interestingly, liver samples from individuals inhabiting Fornebu sedimentation pond expressed downregulation of all genes apart from CYP1A1.

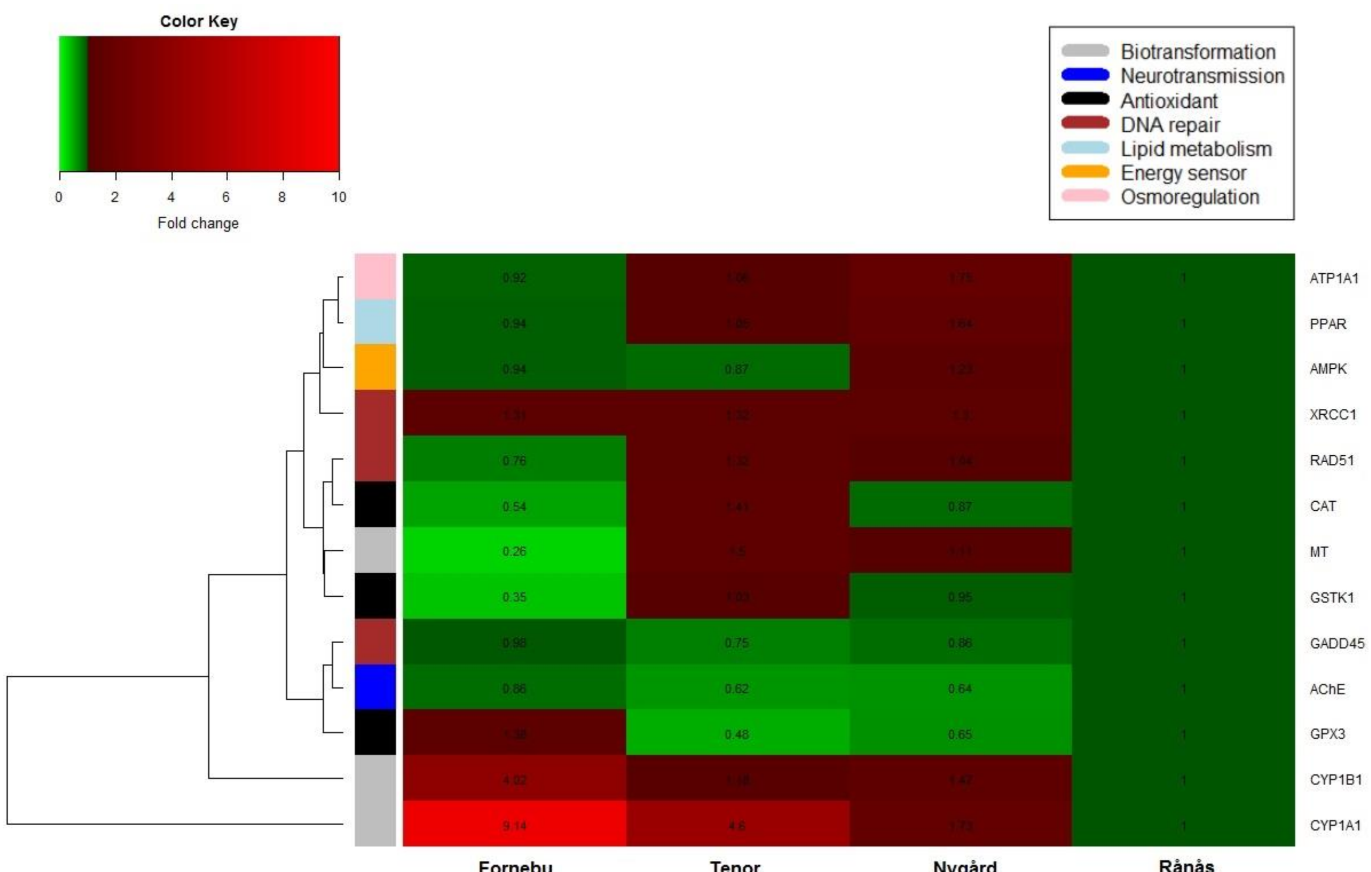

Figure 20. Hierarchical clustering heatmap with dendrogram showing up (red) and down (green) regulated genes in comparison to the reference (Rånås) in skin from northern crested newt. Genes clustered together in the dendrogram are similar in their expression patterns. The colors in the dendrogram show the functions of the genes.

Skin samples (figure 20) from individuals of northern crested newt inhabiting Nygård sedimentation pond expressed up-regulation of biotransformation, DNA repair, energy sensor, lipid metabolism and osmoregulation genes. However, all antioxidant and neurotransmission genes were downregulated in this pond. Individuals from Tenor sedimentation pond expressed upregulation for all genes apart from GPX3, AChE, GADD45g, and AMPK. Individuals

inhabiting Fornebu sedimentation pond displayed upregulation in transcripts of CYP1A1, CYP1B1, GPX3, and XRCC1. Remaining genes were downregulated in this location.

Northern crested newt displayed different gene expression patterns between tissues in Fornebu sedimentation pond where more genes were upregulated in skin samples compared to liver samples. Northern crested newts inhabiting Tenor and Nygård sedimentation pond displayed similar patterns between tissues with exceptions for a few genes.

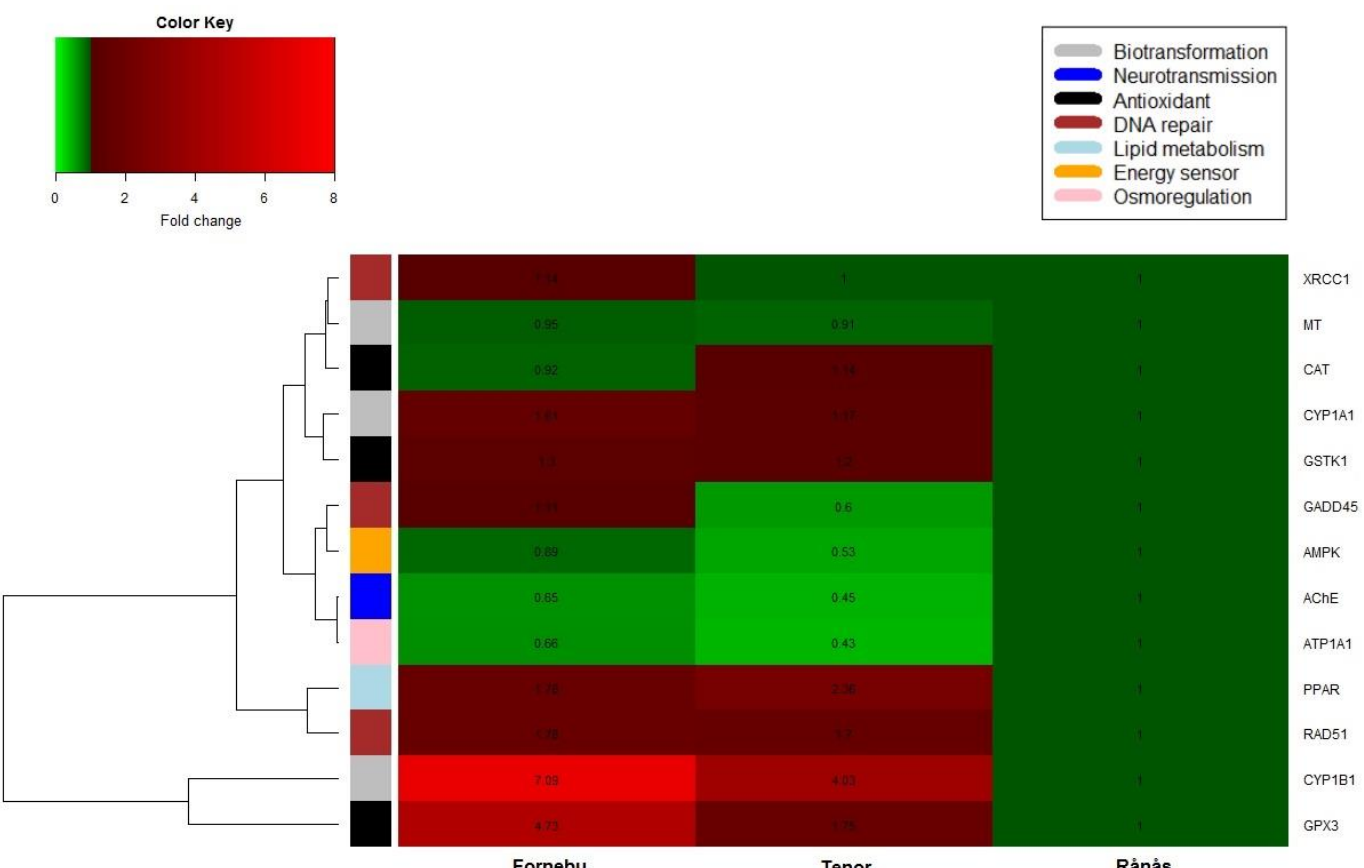

Figure 21. Hierarchical clustering heatmap with dendrogram showing up (red) and down (green) regulated genes in comparison to the reference (Rånås) in liver from smooth newt. Genes clustered together in the dendrogram are similar in their expression patterns. The colors in the dendrogram show the functions of the genes.

Liver samples (figure 21) from individuals of smooth newt inhabiting Fornebu sedimentation pond displayed up-regulation of all DNA repair and lipid metabolism genes. In addition, genes related to biotransformation (CYP1B1, CYP1A1) and antioxidant defense (GSTK1, GPX3) were upregulated in this location. Moreover, osmoregulation, energy sensor neurotransmission and remaining antioxidant and biotransformation genes were

downregulated. Individuals from Tenor sedimentation pond expressed a similar transcriptional profile as individuals from Fornebu sedimentation pond.

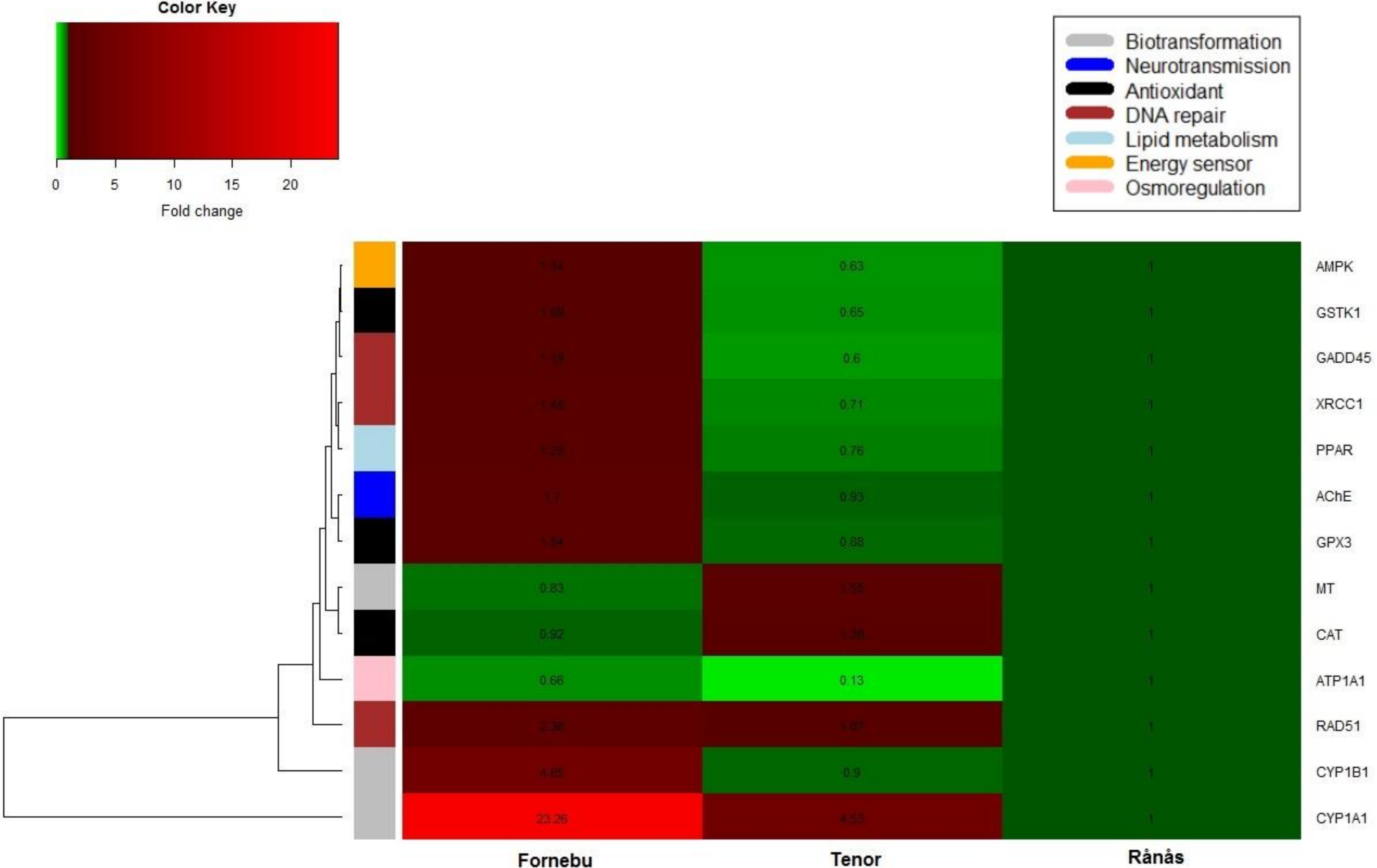

Figure 22. Hierarchical clustering heatmap with dendrogram showing up (red) and down (green) regulated genes in comparison to the reference (Rånås) in skin from smooth newt. Genes clustered together in the dendrogram are similar in their expression patterns. The colors in the dendrogram show the functions of the genes.

Skin samples (figure 22) from smooth newt inhabiting Fornebu sedimentation pond displayed upregulation in 77% of all genes. All DNA repair, neurotransmission, lipid metabolism, and energy sensor genes were upregulated. In addition, biotransformation genes (CYP1A1, CYP1B1) and antioxidant genes (GSTK1, GPX3) were upregulated in this location. CYP1A1 expressed a 23-fold up-regulation compared with the reference pond. Three genes related to biotransformation (MT), antioxidant defense (CAT) and osmoregulation (ATP1A1) were downregulated in Fornebu. Contrary to individuals inhabiting Fornebu, individuals from Tenor sedimentation pond displayed downregulation for most genes apart from biotransformation (CYP1A1, MT), antioxidant (CAT), and DNA repair (RAD51) genes.

Smooth newt displayed similar transcriptional patterns between tissues. Skin samples expressed upregulation of all gene functions except for osmoregulation. Liver samples showed upregulation of all gene functions except for osmoregulation, neurotransmission and energy sensor genes.

The transcriptional pattern in the two species showed induction of oxidative stress, DNA damage, and biotransformation of xenobiotics as the main modes of actions (MoAs). These results were consistent with previous studies reporting transcriptional upregulation of xenobiotic biotransformation and oxidative stress genes in brown trout (*Salmo trutta*) following tunnel wash water exposure (Meland et al., 2011; Dybwad 2015). In addition, Stephensen et al., (2009) reported activation of the CYP1A system and increased oxidative stress in rainbow trout (*Oncorhynchus mykiss*) due to leakage of PAHs and aromatic nitrogen compounds from tires.

Xenobiotic biotransformation and oxidative stress are commonly recognized as responses to benzo[*a*]pyrene exposure (Carlson et al., 2004). The presence of several PAHs in the sediment coupled by the transcriptional upregulation of these genes suggests that the species are exposed to and readily take up PAH compounds such as benzo[*a*]pyrene. In addition, the xenobiotic biotransformation gene metallothionein (MT) was mostly upregulated indicating that detoxification of metals occurred as a response to metal exposure. Cadmium, zinc, and copper have been shown to induce strongest transcription of MT in fish (Rhee et al., 2009). Interestingly, individuals inhabiting the sedimentation pond with the highest zinc and copper concentration (Fornebu sedimentation pond) solely expressed downregulation of the gene. Other compounds, besides PAHs and heavy metals, such as phthalates are present in road runoff (Clara et al., 2010). Di-(2-ethylhexyl)-phthalate (DEHP) have been shown to modulate genes involved in protein metabolism (e.g., PPAR) in zebra fish (Mardonna et al., 2013). Presence of phthalates, although not measured in the present study, may potentially have contributed to the observed transcriptional upregulation of PPAR. Organophosphates (OPs) is another group of contaminants abundant in road runoff (Marklund et al., 2005) which are known to inhibit the neurotransmitter acetylcholinesterase (Mathieu et al., 1996). AChE was mostly downregulated in individuals indicating limited organophosphate exposure.

To summarize, these results showed that genes related to xenobiotic biotransformation, DNA repair and oxidative stress were differentially expressed, mostly upregulated, in individuals from sedimentation ponds compare to individuals from reference ponds (H_4) probably due to elevated PAH and metal exposure.

4.5.2 Tissue differences in gene transcripts

To assess whether species, locations or tissue affected the gene transcripts a PCA was conducted for both species together (figure 23) and individually (figure 24 and 25). RDA was used to assess any differences in the biomarker responses between species (northern crested newt and smooth newt) and tissue (liver and skin).

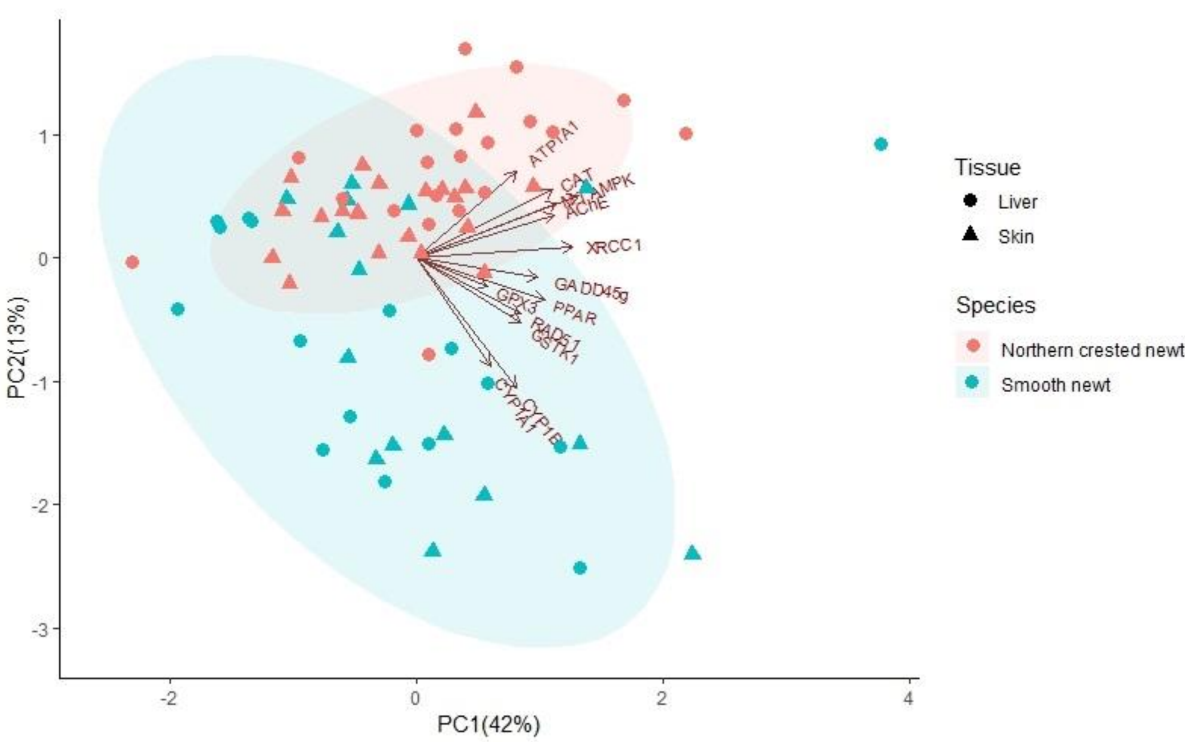

Figure 23. Principal component analysis (PCA) for biomarker responses in northern crested newt and smooth newt in skin and liver tissue. Liver tissue is marked with circle. Skin tissue is marked with a triangle. Blue color represents smooth newt. Red color represents northern crested newt. The gene expression data is log10(x+1) transformed.

The first two dimensions of the principal component analysis for both species (figure 23) explained 42% and 13% respectively of the variation for biomarkers response data. Smooth newt and northern crested newt clustered in two separate groups. The results revealed differences in biomarker responses between the two species (p=0.001) when the effect of variation between tissues and locations were excluded. The biomarker responses are largely covaried but cluster to a certain degree in three groups. The first group contained biotransformation genes (e.g., CYP1A1 and CYP1B1). This group expressed high gene transcription in smooth newt. The second group contained antioxidant genes (GSTK1 and GPX3), all DNA damage genes (XRCC1, RAD51, GADD45g) and lipid metabolism gene (PPAR). The last group contained osmoregulation gene (ATP1A1), oxidative stress (CAT), neurotransmission (AChE), energy sensor (AMPK) and one biotransformation gene (MT). This group expressed high gene transcription in northern crested newt. Differences were also observed between tissues (p=0.001) when the effect of variation between species and locations were excluded. In addition, differences between locations (p=0.002) were observed when the effect of variation between species and tissues were excluded.

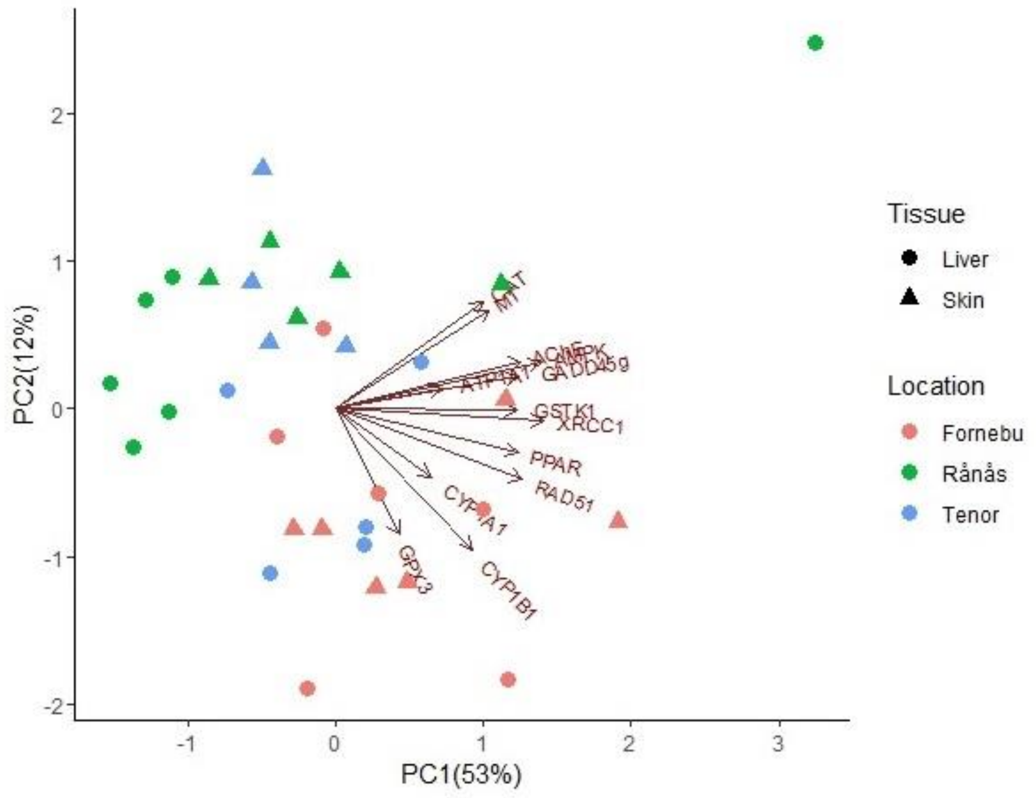

Figure 24. Principal component analysis (PCA) for biomarker responses in smooth newt. The colors represent different locations. Skin is shown as a triangle. Liver is shown as a circle. The data is log10(x+1) transformed. Rånås is the reference site.

The first two dimensions of the PCA for smooth newt (figure 24) explained 53% and 12% respectively of the variation in the biomarker responses. The results revealed differences in biomarker responses between tissues (p=0.001) when the effects of variation between locations were excluded. Differences were also observed between locations (p=0.001) when the effects of variation between tissues were excluded.

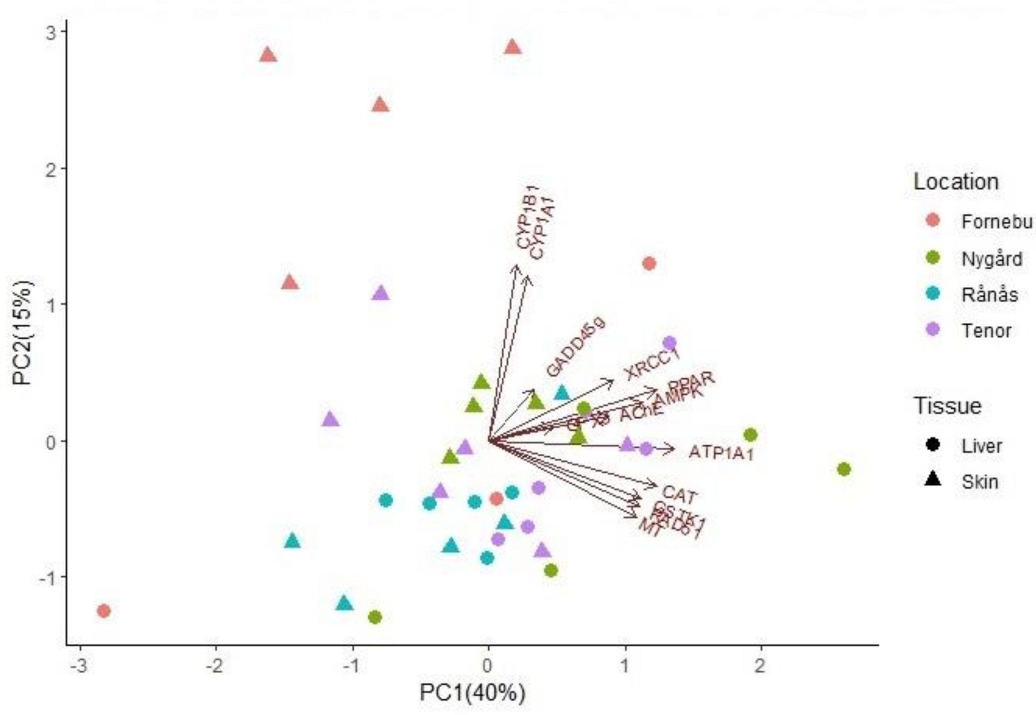

Figure 25. Principal component analysis (PCA) for northern crested newt. The colors represent different locations. Skin is shown as a triangle. Liver is shown as a circle. The data is log10(x+1) transformed. Rånås is the reference site.

The first two dimensions of the PCA for northern crested newt (figure 25) explained 40% and 15% respectively of the variation in biomarker responses. The results revealed a difference in biomarker responses between tissues (p=0.018) when the effect of variation between locations was excluded. Similar results were evident between locations (p=0.001) when the effects of variation between tissues were excluded.

The biomarker gene responses showed differences between tissues in both species as a group and individually. This result indicated that skin and liver samples do not have similar sensitivity in gene expression, hence rejecting hypothesis 5. However, this result was obtained using all biomarkers together. Despite the fact that the liver is the main detoxifying organ, the transcriptional patterns in skin biopsies were not less informative compared to liver samples (figure 19-22). Interestingly, skin samples in smooth newt (figure 22) were induced 22-fold for CYP1A1 compared to liver samples. Furthermore, the transcriptional patterns indicated parallel transcriptional responses in both skin and liver for some genes although not tested for statistical significance. This was observed for xenobiotic biotransformation genes (CYP1A1, CYP1B1, and MT), lipid metabolism gene (PPAR), and osmoregulation gene (ATP1A1).

These genes were upregulated in the same locations in both liver and skin in northern crested newt (figure 19 and 20) indicating similar gene induction. Moreover, when comparing the tissue-specific transcriptional pattern in each species individually the biomarker gene responses generally expressed the same ecotoxicological impact. For example, northern crested newt expressed upregulation of most biomarker functions (figure 19-20) apart from DNA repair gene GADD45g in both tissues. Similar patterns were observed for smooth newt where both tissue types expressed downregulation of the osmoregulation gene (ATP1A1) and upregulation for most other biomarker functions (figure 21-22).

Tail biopsies (skin) have previously been shown to provide equally complex and informative as whole body samples from fire salamander larvae in transcriptome analysis (Czypionka et al., 2015). Furthermore, recent research have shown that biopsies of peripheral tissue in rainbow trout (*Oncorhynchus mykiss*) can represent the whole organism for individual genes such as cytochrome P450 family (Veldhoen et al., 2013). Similar results have been reported in Arctic grayling (*Thymallus arcticus*) exposed to heavy metals, where the metal body burden and caudal fin mRNA abundance showed a strong correlation, demonstrating the potential use for non-lethal biomonitoring in this species (Veldhoen et al., 2014). Thus, it is likely that skin biopsies in newts can be representative of the whole organism. The results obtained in this study indicate that for some selected genes (e.g, CYP1A1, CYP1B1, and MT), the skin biopsies might be an adequate substitution for liver for biomonitoring in Norwegian newts.

4.6 Sedimentation ponds – ecological traps or just compromised habitats?

Rich biodiversity in sedimentation ponds was observed in this study as well as previous studies recording a large variety of macroinvertebrates and amphibians (Sun et al., 2019). Sedimentation ponds are constructed to protect the surrounding environment, yet it affects the organisms inhabiting them. The results obtained in this study showed transcriptional upregulation of xenobiotic biotransformation, antioxidant, and DNA repair genes and PAH metabolites as sublethal endpoints in Norwegian newts inhabiting sedimentation ponds probably due to polluted road runoff. However, since pollution levels did not influence the abundance of the species, adult newts might be relatively insensitive for pollution. The adult newts are only present in the ponds for a short annual period. They may experience sublethal

effects during this aqueous period, however not strong enough to influence population dynamics in the long term.

Fish constitute natural predators to amphibians and often restricts the assemblages of amphibians in natural ponds (Smith et al., 2001). However, fish species rarely inhabit sedimentation ponds due to the limited size of the ponds. The absence of predatory fish in sedimentation ponds may act beneficial for newts. Reduced predation and competition against pollution sensitive species might act beneficial for newts despite experiencing sublethal effects due to pollution.

The results obtained in this study can be used when designing and constructing sedimentation ponds. To facilitate for dispersion species such as newts, the ponds could be constructed on the adjacent side of the highway as natural ponds inhabited by newts to provide the least possible barrier effect. In the event of limited construction area, underpasses may be constructed to allow dispersion. In addition, some vegetation could be planted in and around the pond to facilitate breeding activities. To ensure minimal contaminant exposure for the species, the ponds could be constructed with a closed forebay or with a sedimentation tank. It is also important to consider not only the lipophilic compounds but the hydrophilic compounds. Filters could be installed as a remedial agent to remove dissolved compounds. These measures could potentially transform sedimentation ponds from ecological traps to nature-based solutions safeguarding both the surrounding environment and the species inhabiting them.

5 Conclusions

A large extent of the investigated sedimentation ponds were inhabited by smooth newt and northern crested newt. The newt abundance was not affected by pollution levels. However, sedimentation ponds bounded by additional roads had a negative impact on newt abundance whereas increased macrophyte coverage had positive impacts on newt abundance.

The newts inhabiting sedimentation ponds showed molecular responses through induction of genes related to antioxidant defense, xenobiotic biotransformation, DNA damage repair, elevated osmoregulation, lipid metabolism, and neurotransmission. In addition, the newts experienced sublethal effects through elevated levels of PAH metabolites such as 1-OH-pyrene, 1-OH-phenanthrene, and 3-OH-benzo[*a*]pyrene. All the biomarker responses coupled with measured PAHs and trace metals in the sediment indicated that the newts inhabiting sedimentation ponds were exposed to and readily take up xenobiotics such as PAHs and trace metals.

The tissue comparison of gene expression showed that all biomarker responses were expressed differently in skin and liver. However, some selected genes (e.g., CYP1A1, CYP1B1, and MT) showed parallel transcriptional patterns in skin and liver. This indicates that skin biopsies might be an adequate substitution for liver samples for future biomonitoring using these genes.

The biological mapping and biomarker responses in northern crested newt and smooth newt showed that adult newts inhabiting sedimentation ponds experience sublethal effects due to road-related contaminant exposure. However, how these influence population dynamics is unclear. The results obtained in this study may be used for design and construction of sedimentation ponds to facilitate for dispersion species such as newts. This may transform sedimentation ponds from potential ecological traps to nature-based solutions safeguarding both the surrounding environment and the species inhabiting them.

6 Future perspectives

This study showed that a large extent of sedimentation ponds are inhabited by smooth newt and the red-listed species northern crested newt. It is important to take this into account during the construction and maintenance of sedimentation ponds.

Limited research on the effects of road runoff on adult newts stresses the need for further investigations on traffic-related contaminants. It is important to consider investigating more biomarkers and synergistic effects of pollutants. Additionally, more research is needed on the potential effects that might influence population dynamics. This includes if newt populations are more susceptible to xenobiotics when their immune system is weakened by the newly discovered fungi *Batrachochytrium dendrobatidis* (bd).

Current research of traffic-related contaminants on newt larvae is restricted to the effects of de-icing salt. *In vivo* studies on newt larvae using other pollutants in road runoff such as PAH, phenols and phthalates would be complementary to this thesis. This would complement the findings on adult newts from this study with knowledge on early life stages of newts to assess their sensitivity.

Considering the near threatened status of northern crested newt and other amphibians, it is vital to further assess the use of non-lethal biopsies in environmental monitoring. A tissue comparison study related to gene expression for xenobiotic biotransformation and oxidative stress genes restricted to one species and one exposure site would simplify and complement the results in this study.

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Appendices

Appendix A – Sedimentation ponds

A1 – General information sedimentation ponds

Location	County	Municipality	Road	Runoff	AADT	Area	Depth	Sunexposure
Berle (R)	Akershus	Frogn	NA	NA	NA	8700	0.125	Medium
Bommestad East	Vestfold	Larvik	E18	Road	3944	170	0.571	High
Borre	Vestfold	Horten	Rv 19	Road	9000	410	0.342	High
Eikeberg	Vestfold	Tønsberg	E18	Both	29235	1440	0.404	High
Emmerud	Vestfold	Tønsberg	Rv 308	Road	29235	1370	0.372	Medium
Fevang	Vestfold	Sandefjord	E18	Road	31504	2640	0.378	High
Finstad	Østfold	Råde	E6	Road	3318	180	0.456	High
Fornebu	Oslo	Bærum	Rv 166	Road	23193	820	0.496	Medium
Helland	Vestfold	Horten	E18	Both	24875	3400	0.495	High
Holmane	Vestfold	Tønsberg	E18	Road	32303	1120	0.351	High
Holt	Østfold	Hobøl	E18	Road	13132	1860	0.807	High
Idrettsveien	Østfold	Råde	E6	Road	41252	780	0.42	High
Lundeby	Østfold	Råde	E6	Road	41252	105	0.234	High
Neset	Østfold	Hobøl	E18	Road	13132	1670	0.586	High
Nøklegard	Vestfold	Horten	E18	Road	25630	2780	0.411	High
Nøstvedt	Akershus	Ski	E6	Road	50344	340	0.447	High
Nygård	Vestfold	Holmestrand	E18	Both	23680	1550	0.292	High
Rånås (R)	Akershus	Frogn	NA	NA	NA	1400	0.172	High
Ringdal	Vestfold	Larvik	E18	Road	41252	960	0.327	High
Seierstad	Vestfold	Larvik	E18	Road	41252	2060	0.7	High
Skinmo	Vestfold	Sandefjord	E18	Road	41252	2060	0.451	High
Sky	Vestfold	Larvik	E18	Tunnel	17740	2500	0.417	High
Støttum	Østfold	Hobøl	E18	Road	13132	2100	0.623	High
Talaldrud Junction	Akershus	Ski	E6	Road	50344	1400	0.97	High
Taraldrud North	Akershus	Ski	E6	Road	50344	780	0.9	High
Talaldrud South	Akershus	Ski	E6	Road	50344	474	0.7	High
Tenor	Østfold	Eidsberg	E18	Road	13132	655	0.337	Medium
Tveiten	Vestfold	Tønsberg	E18	Both	29235	970	0.478	High
Vassum	Akershus	Frogn	E6	Both	32712	431	0.428	Medium
Vennersrød (R)	Akershus	Ås	NA	NA	NA	3300	0.133	High

A2 – Bommestad East, Borre and Eikeberg sedimentation pond

A3 – Emmerud, Fevang and Finstad sedimentation pond

A3 – Helland, Holmane and Holt sedimentation pond

A4 – Idrettsveien, Lundeby and Neset sedimentation pond

A5 – Nøklegård, Nøstvedt and Ringdal sedimentation pond

A6 – Seierstad, Skinmo and Sky sedimentation pond

A7 – Støttum, Taraldrud North and Taraldrud Junction sedimentation pond

A8 – Taraldrud South, Tveiten and Vassum sedimentation pond

Appendix B - Raw data

B1 – Abundance and CPU northern crested newt and smooth newt.

Location	Smooth newt	Northern crested newt	Total	CPU northern	CPU smooth
Bommestad East	3	0	3	0.000	0.128
Borre	1	0	1	0.000	0.049
Eikeberg	0	0	0	0.000	0.000
Emmerud	1	0	1	0.000	0.048
Fevang	0	0	0	0.000	0.000
Finstad	0	0	0	0.000	0.000
Fornebu	11	10	21	0.426	0.468
Helland	2	0	2	0.000	0.105
Holmane	1	0	1	0.000	0.043
Holt	0	0	0	0.000	0.000
Idrettsveien	0	0	0	0.000	0.000
Lundeby	0	0	0	0.000	0.000
Neset	0	0	0	0.000	0.000
Nøklegard	0	0	0	0.000	0.000
Nøstvedt	9	0	9	0.000	0.429
Nygård	3	17	20	0.919	0.162
Ringdal	1	0	1	0.000	0.041
Seierstad	0	0	0	0.000	0.000
Skinmo	1	0	1	0.000	0.043
Sky	0	0	0	0.000	0.000
Støttum	0	0	0	0.000	0.000
Talaldrud Junction	3	0	3	0.000	0.128
Taraldrud North	11	0	11	0.000	0.463
Talaldrud South	0	0	0	0.000	0.000
Tenor	51	8	59	0.364	2.318
Tveiten	5	8	13	0.471	0.294
Vassum	0	0	0	0.000	0.000

B2 – Ecotoxicological sampling information

Individual	Location	Length[a]	Length 2[b]	Weight[c]	Sex	Species[d]
1	Fornebu	10.5	7	7.8	Female	Northern
2	Fornebu	12	7	7.4	Female	Northern
3	Fornebu	14	7.6	8.9	Female	Northern
4	Fornebu	13	7.5	10.6	Female	Northern
5	Fornebu	12.5	6.5	10.8	Male	Northern
6	Fornebu	10.5	5.5	5.6	Female	Northern
7	Fornebu	7.5	4	1.5	Male	Smooth
8	Fornebu	7	4	2.1	Male	Smooth
9	Fornebu	7	4	1.7	Female	Smooth

10	Fornebu	7	4	1.7	Male	Smooth
11	Fornebu	7.5	4	2.2	Female	Smooth
12	Fornebu	8.5	4.5	2.2	Male	Smooth
13	Tenor	14	7.5	11.2	Male	Northern
14	Tenor	11	5	5.5	Female	Northern
15	Tenor	12.5	6.5	10.5	Male	Northern
16	Tenor	12.5	5.5	10.2	Male	Northern
17	Tenor	12	5	6.4	Male	Northern
18	Tenor	11	5.5	6.6	Female	Northern
19	Tenor	8.5	4	2.9	Male	Smooth
20	Tenor	8	4	1.9	Female	Smooth
21	Tenor	8	3.5	2.1	Male	Smooth
22	Tenor	7	3.5	1.8	Female	Smooth
23	Tenor	9	4	2.6	Male	Smooth
24	Tenor	8	4.5	1.8	Female	Smooth
25	Nygård	13	6	12.6	Male	Northern
26	Nygård	12.5	6	11.8	Male	Northern
27	Nygård	11	5	5.8	Male	Northern
28	Nygård	13	6	10.2	Male	Northern
29	Nygård	11.5	4.5	8	Female	Northern
30	Nygård	9.5	4	4.8	Male	Northern
31	Nygård	15	7	13.3	Female	Northern
32	Rånås (R)	12	7	12	Male	Northern
33	Rånås (R)	11.5	6	8.3	Male	Northern
34	Rånås (R)	12	6.5	7.2	Male	Northern
35	Rånås (R)	11	6	6.9	Male	Northern
36	Rånås (R)	12	6.5	6.6	Female	Northern
37	Rånås (R)	12	6	9.1	Male	Northern
38	Rånås (R)	8	4	1.7	Male	Smooth
39	Rånås (R)	10	5	3	Male	Smooth
40	Rånås (R)	8	3.5	1.5	Male	Smooth
41	Rånås (R)	7.5	3.5	1.5	Male	Smooth
42	Rånås (R)	8	3.5	1.6	Female	Smooth
43	Rånås (R)	7	4.5	1.8	Female	Smooth

[a] Length including tail (cm)
[b] Length tail excluded (cm)
[c] Weight (g)
[d] Northern crested newt or Smooth newt

B3 – Fold change data for smooth newt

ID	Location	Sex	Tissue	GADPH	ACTIN	EF1A1	MT	CYP1A1	CYP1B1	GADD45	XRCC
35	Rånås	Female	Liver	0.68	0.59	1.03	0.45	1.38	0.27	0.71	0.41
37	Rånås	Male	Liver	0.27	0.60	0.67	0.19	0.71	0.37	0.45	0.49
38	Rånås	Male	Liver	1.80	1.28	0.18	4.01	2.40	3.99	3.78	3.74
39	Rånås	Male	Liver	1.82	1.19	2.13	0.48	0.10	0.87	0.17	0.28
40	Rånås	Male	Liver	0.90	1.04	0.92	0.47	0.70	0.29	0.26	0.52
26	Fornebu	Male	Liver	0.53	1.30	1.08	0.40	0.71	0.20	0.62	0.55
27	Fornebu	Male	Liver	1.29	1.24	0.43	1.00	1.99	6.44	1.81	1.35
28	Fornebu	Female	Liver	1.29	1.16	0.66	1.06	1.27	3.42	0.30	1.44
29	Fornebu	Male	Liver	1.01	0.57	0.47	1.00	0.65	1.83	1.70	0.95
30	Fornebu	Female	Liver	1.15	1.22	0.55	1.03	2.70	20.65	1.45	1.44
41	Fornebu	Male	Liver	0.29	0.39	0.49	0.78	0.51	4.53	0.63	0.90
31	Tenor	Female	Liver	1.39	1.31	1.45	0.84	2.53	5.66	0.77	0.75
32	Tenor	Male	Liver	1.86	1.01	0.64	1.40	1.80	5.60	0.46	1.50
33	Tenor	Female	Liver	1.22	1.65	0.98	0.87	0.80	2.39	1.13	1.19
34	Tenor	Female	Liver	0.18	0.19	0.30	0.61	0.67	4.51	0.09	0.89
42	Tenor	Male	Liver	0.74	0.67	0.86	0.80	1.00	2.75	0.57	0.61

ID	Location	Sex	Tissue	RAD51	GPX3	GSTK1	CAT	AChE	ATP1A1	PPAR	AMPK
35	Rånås	Female	Liver	0.30	0.02	0.51	0.70	0.38	0.59	0.47	0.48
37	Rånås	Male	Liver	0.32	2.81	0.25	0.29	0.12	1.37	0.28	0.28
38	Rånås	Male	Liver	4.20	0.09	3.28	3.86	4.69	2.36	4.23	4.39
39	Rånås	Male	Liver	0.50	0.01	0.81	0.22	0.22	0.14	0.30	0.18
40	Rånås	Male	Liver	0.28	0.13	0.68	0.62	0.36	0.36	0.31	0.36
26	Fornebu	Male	Liver	0.39	2.95	0.46	0.32	0.24	1.18	0.42	0.32
27	Fornebu	Male	Liver	2.20	5.63	1.84	1.85	0.85	0.71	2.36	1.23
28	Fornebu	Female	Liver	1.97	3.46	1.25	1.14	0.73	0.97	1.67	0.76
29	Fornebu	Male	Liver	1.30	0.03	0.84	0.60	0.75	0.41	1.28	0.81
30	Fornebu	Female	Liver	2.13	6.26	1.80	0.79	0.87	0.87	3.29	1.69
41	Fornebu	Male	Liver	2.32	12.98	1.09	0.37	0.41	0.49	1.13	0.36
31	Tenor	Female	Liver	0.74	0.01	0.96	0.79	0.32	0.53	0.97	0.48
32	Tenor	Male	Liver	1.66	0.03	1.68	2.53	0.98	0.42	3.11	0.48
33	Tenor	Female	Liver	2.45	3.08	1.44	0.84	0.27	0.25	2.38	0.72
34	Tenor	Female	Liver	2.90	0.02	0.87	0.30	0.46	0.52	1.86	0.24
42	Tenor	Male	Liver	0.48	0.02	0.68	0.64	0.23	0.38	1.31	0.48

ID	Location	Sex	Tissue	GADPH	ACTIN	EF1A1	MT	CYP1A1	CYP1B1	GADD45	XRCC
84	Rånås	Male	Skin	1.89	1.10	1.03	0.92	1.57	4.88	0.76	0.83
85	Rånås	Male	Skin	1.70	1.17	0.87	1.04	1.23	0.67	0.63	0.95
86	Rånås	Female	Skin	1.02	0.76	0.87	0.60	1.25	0.49	0.76	0.94
87	Rånås	Female	Skin	0.93	1.11	1.77	0.70	0.37	1.02	0.62	0.56
93	Rånås	Male	Skin	0.52	1.14	0.69	1.57	1.36	1.70	2.12	1.46
77	Fornebu	Male	Skin	0.82	0.82	0.80	1.09	0.79	1.12	0.87	1.09
78	Fornebu	Female	Skin	1.73	0.67	0.58	0.70	16.86	2.00	2.18	1.04
79	Fornebu	Male	Skin	0.54	0.68	0.59	0.14	28.47	3.65	0.99	1.48
80	Fornebu	Female	Skin	1.47	0.85	0.37	2.08	42.59	8.41	1.79	2.35

81	Fornebu	Male	Skin	0.50	1.05	0.90	1.07	18.45	6.80	0.75	1.60
88	Fornebu	Male	Skin	0.92	0.73	1.07	0.40	18.29	4.07	0.85	1.35
82	Tenor	Male	Skin	1.71	1.03	1.26	0.56	14.87	4.20	0.60	1.07
83	Tenor	Female	Skin	1.86	0.73	0.74	0.92	1.73	0.52	0.45	0.68
89	Tenor	Male	Skin	1.95	0.59	0.99	2.15	1.89	0.81	0.30	0.71
91	Tenor	Male	Skin	1.96	0.46	0.64	1.56	7.62	1.21	0.52	0.61

ID	Location	Sex	Tissue	RAD51	GPX3	GSTK1	CAT	AChE	ATP1A1	PPAR	AMPK
84	Rånås	Male	Skin	1.03	5.63	1.30	1.36	0.31	0.58	3.11	0.73
85	Rånås	Male	Skin	0.86	0.20	0.42	1.19	1.06	0.26	0.73	0.78
86	Rånås	Female	Skin	1.02	0.26	1.65	0.52	0.90	0.74	0.78	0.88
87	Rånås	Female	Skin	0.52	0.10	0.36	0.84	0.60	0.52	0.59	0.64
93	Rånås	Male	Skin	1.83	4.23	1.37	1.83	1.35	2.16	1.61	1.74
77	Fornebu	Male	Skin	0.76	0.21	1.20	0.62	1.10	1.32	1.30	0.96
78	Fornebu	Female	Skin	3.06	2.61	2.43	2.28	1.84	0.67	1.31	1.57
79	Fornebu	Male	Skin	2.45	0.18	0.91	0.38	1.85	0.53	1.26	0.84
80	Fornebu	Female	Skin	3.86	2.83	1.79	1.20	3.10	0.59	2.52	1.93
81	Fornebu	Male	Skin	2.17	1.94	0.56	0.50	1.57	0.67	1.23	1.03
88	Fornebu	Male	Skin	1.72	0.09	0.30	0.53	0.72	1.07	0.91	0.76
82	Tenor	Male	Skin	0.93	1.56	0.53	0.63	1.12	0.41	0.47	0.68
83	Tenor	Female	Skin	0.65	1.40	0.51	1.78	0.81	0.14	1.00	0.57
89	Tenor	Male	Skin	0.92	0.12	0.45	2.18	0.57	0.13	0.60	0.57
91	Tenor	Male	Skin	1.44	0.22	0.65	0.66	0.89	0.08	0.52	0.64

B4 – Fold change data for northern crested newt

ID	Location	Sex	Tissue	GADPH	ACTIN	EF1A1	MT	CYP1A1	CYP1B1	GADD45	XRCC
20	Rånås	Male	Liver	1.04	1.08	0.89	1.08	0.87	1.41	0.86	1.34
21	Rånås	Male	Liver	NA	1.02	NA	0.08	NA	NA	1.04	1.11
22	Rånås	Male	Liver	1.08	0.91	1.19	1.06	0.56	0.39	0.70	0.62
23	Rånås	Male	Liver	1.12	1.07	1.30	1.04	1.44	1.27	1.09	1.38
24	Rånås	Female	Liver	0.76	1.07	0.78	1.48	0.99	1.16	1.01	0.72
25	Rånås	Male	Liver	1.01	0.86	0.84	1.26	1.14	0.77	1.30	0.83
1	Fornebu	Male	Liver	1.21	2.01	3.18	1.40	0.85	0.85	0.99	1.09
2	Fornebu	Female	Liver	1.45	1.27	7.67	1.44	1.12	1.13	1.79	1.14
14	Fornebu	Female	Liver	NA	0.02	NA	NA	NA	NA	0.09	0.05
17	Fornebu	Female	Liver	NA	0.04	0.41	0.06	0.03	0.46	0.24	0.08
18	Fornebu	Female	Liver	0.08	0.04	0.38	0.11	0.10	0.36	0.24	0.09
19	Fornebu	Female	Liver	0.11	0.05	0.83	0.06	0.02	0.33	0.08	0.04
9	Tenor	Male	Liver	1.29	1.44	4.76	1.75	1.27	1.36	0.51	1.94
10	Tenor	Female	Liver	1.27	0.89	2.20	1.14	2.60	1.01	0.65	0.94
11	Tenor	Male	Liver	1.57	1.27	1.68	1.49	1.14	1.16	0.57	1.52
12	Tenor	Male	Liver	3.22	2.26	6.55	1.88	0.95	1.42	0.49	1.72
13	Tenor	Male	Liver	1.29	0.94	4.08	1.26	1.48	0.94	0.84	1.57
16	Tenor	Female	Liver	2.10	1.95	12.08	1.57	1.22	0.89	0.87	1.39
15	Nygård	Male	Liver	0.77	1.18	0.83	1.82	2.37	1.68	0.86	1.67
3	Nygård	Male	Liver	2.34	1.97	2.74	2.45	2.33	2.72	0.69	3.84
4	Nygård	Male	Liver	1.50	1.53	1.36	1.42	1.22	1.60	0.87	2.34

5	Nygård	Male	Liver	0.79	0.51	1.17	0.91	0.65	1.13	1.81	1.48
6	Nygård	Female	Liver	NA	2.08	NA	NA	NA	NA	0.71	1.10
7	Nygård	Male	Liver	1.11	0.59	0.98	0.73	0.60	0.39	0.55	1.00
8	Nygård	Female	Liver	1.94	1.33	1.52	1.63	1.11	0.86	0.93	1.15

ID	**Location**	**Sex**	**Tissue**	**RAD51**	**GPX3**	**GSTK1**	**CAT**	**AChE**	**ATP1A1**	**PPAR**	**AMPK**
20	Rånås	Male	Liver	0.88	1.50	0.73	0.91	1.17	1.16	0.85	1.07
21	Rånås	Male	Liver	1.05	0.72	1.36	0.73	0.72	1.21	1.37	1.12
22	Rånås	Male	Liver	1.01	1.17	0.91	0.74	0.62	0.80	0.58	0.77
23	Rånås	Male	Liver	0.66	0.38	1.19	1.12	1.43	0.98	1.70	1.31
24	Rånås	Female	Liver	0.92	1.49	0.96	1.31	1.72	0.77	0.98	0.91
25	Rånås	Male	Liver	1.49	0.74	0.86	1.19	0.34	1.08	0.52	0.81
1	Fornebu	Male	Liver	0.84	1.32	0.85	0.98	0.61	1.40	1.12	1.25
2	Fornebu	Female	Liver	2.42	1.62	1.21	2.77	2.03	1.81	0.82	1.26
14	Fornebu	Female	Liver	NA	NA	0.05	0.12	0.57	0.17	0.63	0.09
17	Fornebu	Female	Liver	0.12	NA	0.08	0.16	0.52	0.24	0.35	0.14
18	Fornebu	Female	Liver	0.29	0.28	0.25	0.09	0.36	0.32	0.34	0.16
19	Fornebu	Female	Liver	NA	0.50	0.12	0.31	0.80	0.24	0.62	0.14
9	Tenor	Male	Liver	1.04	1.37	0.56	0.94	1.03	1.23	1.24	1.44
10	Tenor	Female	Liver	1.87	0.91	0.33	0.66	0.50	1.56	2.52	1.58
11	Tenor	Male	Liver	1.19	1.12	0.08	0.63	1.45	1.32	0.93	1.17
12	Tenor	Male	Liver	1.27	1.98	0.77	1.13	1.25	1.36	0.20	1.33
13	Tenor	Male	Liver	1.44	1.51	0.22	1.20	2.12	1.84	4.98	1.91
16	Tenor	Female	Liver	2.02	2.32	0.20	2.24	1.48	2.11	1.61	1.50
15	Nygård	Male	Liver	1.70	1.86	0.77	1.07	1.21	0.92	0.22	2.00
3	Nygård	Male	Liver	2.20	3.15	0.75	2.34	1.90	2.13	4.67	3.15
4	Nygård	Male	Liver	1.14	2.11	0.60	3.01	4.14	1.24	5.44	2.19
5	Nygård	Male	Liver	0.38	1.55	0.18	1.45	2.74	1.15	0.33	2.01
6	Nygård	Female	Liver	1.36	1.45	0.76	1.56	2.03	1.07	1.12	1.67
7	Nygård	Male	Liver	0.31	1.35	0.27	0.82	0.67	0.68	0.47	0.55
8	Nygård	Female	Liver	2.58	1.81	1.13	1.67	1.07	1.35	0.23	1.78

ID	**Location**	**Sex**	**Tissue**	**GADPH**	**ACTIN**	**EF1A1**	**MT**	**CYP1A1**	**CYP1B1**	**GADD45**	**XRCC**
69	Rånås	Male	Skin	0.61	0.75	0.43	0.76	0.80	2.20	0.61	1.66
70	Rånås	Male	Skin	0.75	0.29	0.29	0.70	0.76	0.31	0.58	0.50
71	Rånås	Male	Skin	NA	1.05	NA	NA	NA	0.07	3.67	0.73
72	Rånås	Male	Skin	0.51	0.76	0.35	1.02	1.07	0.66	0.43	1.01
54	Rånås	Male	Skin	1.14	1.71	0.91	1.19	1.05	1.48	0.44	1.03
61	Rånås	Female	Skin	2.00	1.43	3.02	1.33	1.32	1.29	0.27	1.07
73	Fornebu	Female	Skin	NA	0.83	NA	NA	NA	NA	5.26	0.46
74	Fornebu	Female	Skin	1.53	1.08	14.58	1.36	0.56	0.49	0.80	1.20
75	Fornebu	Female	Skin	0.51	0.15	5.80	0.51	0.20	0.12	0.94	0.25
76	Fornebu	Male	Skin	0.88	1.02	10.40	0.77	0.17	0.69	0.33	0.66
62	Fornebu	Female	Skin	0.82	0.25	9.93	0.44	0.10	0.29	0.65	0.12
63	Fornebu	Female	Skin	NA	NA	4.99	NA	NA	NA	0.31	0.52
65	Tenor	Female	Skin	0.61	0.31	6.82	0.52	0.33	0.64	0.62	0.98
66	Tenor	Male	Skin	1.08	1.22	4.76	1.27	2.75	2.14	0.63	2.54
67	Tenor	Male	Skin	0.70	1.10	4.20	0.57	0.43	0.47	0.43	0.29
68	Tenor	Male	Skin	0.81	0.53	4.06	0.60	1.66	0.80	0.53	0.54
59	Tenor	Male	Skin	1.12	1.64	4.14	0.99	0.79	1.21	0.40	0.64
60	Tenor	Female	Skin	0.87	1.89	3.61	1.08	0.91	2.90	0.28	3.46
53	Nygård	Female	Skin	1.36	0.79	2.10	1.15	1.48	1.24	0.40	1.27
55	Nygård	Male	Skin	NA	1.48	NA	NA	NA	NA	1.49	0.52

ID	Location	Sex	Tissue	RAD51	GPX3	GSTK1	CAT	AChE	ATP1A1	PPAR	AMPK
56	Nygård	Male	Skin	0.97	1.30	1.95	0.92	1.01	0.70	0.65	1.03
57	Nygård	Male	Skin	1.16	1.76	2.44	0.92	1.07	1.42	0.63	1.32
58	Nygård	Male	Skin	1.07	0.84	0.97	0.77	0.97	0.78	0.62	0.57
64	Nygård	Male	Skin	1.56	1.39	1.20	0.94	1.04	0.90	0.11	0.49

ID	Location	Sex	Tissue	RAD51	GPX3	GSTK1	CAT	AChE	ATP1A1	PPAR	AMPK
69	Rånås	Male	Skin	0.75	1.02	0.91	1.18	1.32	0.63	1.30	0.97
70	Rånås	Male	Skin	1.11	0.74	0.48	0.44	0.63	0.35	0.60	0.42
71	Rånås	Male	Skin	0.94	0.97	0.75	1.40	1.08	1.96	1.00	0.95
72	Rånås	Male	Skin	0.86	0.89	0.30	0.66	0.60	0.31	0.67	0.93
54	Rånås	Male	Skin	0.92	1.04	1.39	1.17	1.06	1.61	1.27	1.19
61	Rånås	Female	Skin	1.42	1.34	2.18	1.14	1.31	1.14	1.17	1.54
73	Fornebu	Female	Skin	3.37	1.38	1.59	1.11	1.44	0.67	0.14	0.70
74	Fornebu	Female	Skin	4.96	0.71	1.33	1.41	1.26	0.21	2.37	1.45
75	Fornebu	Female	Skin	1.55	0.17	0.55	0.88	1.19	0.64	0.32	0.45
76	Fornebu	Male	Skin	3.75	0.51	0.96	1.11	1.62	0.21	0.92	1.00
62	Fornebu	Female	Skin	6.05	0.09	0.16	0.51	1.36	0.25	1.06	0.42
63	Fornebu	Female	Skin	4.46	1.72	1.29	0.63	0.99	0.15	0.35	1.50
65	Tenor	Female	Skin	1.37	0.64	0.97	0.99	1.93	0.52	0.46	0.61
66	Tenor	Male	Skin	1.04	0.73	1.19	1.47	2.33	1.20	1.13	1.94
67	Tenor	Male	Skin	0.95	1.24	0.61	0.77	0.93	0.45	0.25	0.70
68	Tenor	Male	Skin	1.62	1.49	0.54	0.90	0.63	1.61	0.33	0.72
59	Tenor	Male	Skin	1.34	1.98	0.70	1.13	0.92	1.30	0.49	0.91
60	Tenor	Female	Skin	0.79	1.83	0.49	1.05	1.17	1.10	1.05	1.48
53	Nygård	Female	Skin	1.33	0.97	0.67	2.10	1.77	0.89	0.73	2.46
55	Nygård	Male	Skin	0.74	0.74	0.44	1.31	1.21	2.13	0.68	2.01
56	Nygård	Male	Skin	2.29	0.84	1.14	1.77	0.73	0.91	0.53	1.36
57	Nygård	Male	Skin	1.69	1.41	0.94	1.74	1.44	0.68	0.61	1.68
58	Nygård	Male	Skin	1.60	1.34	1.33	1.32	1.65	0.38	0.73	1.47
64	Nygård	Male	Skin	1.20	0.91	0.62	1.59	0.99	0.56	0.58	1.51

B5 – PAH metabolite concentration (ng/g)

Species	Sex	1-OH-pyrene (ng/g)	1-OH-phenanthrene (ng/g)	3-OH-benzo[*a*]pyrene (ng/g)
Northern crested newt	Female	71.99	4.52	1.14
Northern crested newt	Female	239.99	49.12	2.44
Northern crested newt	Female	209.99	47.12	3.74
Northern crested newt	Female	109.99	6.02	2.64
Northern crested newt	Male	1399.99	319.12	85.64
Northern crested newt	Female	229.99	24.12	11.64
Smooth newt	Male	319.99	47.12	3.04
Smooth newt	Female	249.99	40.12	5.04
Northern crested newt	Female	24.99	189.12	1.54
Northern crested newt	Male	25.99	0.09	0.37
Northern crested newt	Male	44.99	269.12	0.64
Northern crested newt	Male	13.99	7.22	1.24
Northern crested newt	Female	32.99	109.12	2.94
Smooth newt	Male	5.89	0.72	4.94
Smooth newt	Female	12.99	6.82	2.04

Smooth newt	Male	18.99	3.72	1.44
Smooth newt	Female	0.028	1.72	0.94
Northern crested newt	Male	9.39	959.12	9.54
Northern crested newt	Male	6.79	169.12	0.94
Northern crested newt	Male	1.99	45.12	9.14
Northern crested newt	Male	3.79	86.12	0.63
Northern crested newt	Female	2.79	24.12	0.53

Location	Berle (R)	Bommestad East	Borre	Eikeberg	Emmerud	Fevang	Finstad	Fornebu	Helland	Holmane
C1-Naphthalene	<20.0	<10.0	<10.0	<10.0	17	113	12	100	13	<15.0
C2-Naphthalene	192	130	141	118	180	3680	215	581	264	<70.0
C3-Naphthalene	692	494	473	408	689	35300	740	2830	973	<230.0
C4-Naphthalene	124	72	73	61	<80.0	19200	106	810	160	<45.0
Acenapththylene	<20.0	<10.0	<10.0	<10.0	<10.0	<50.0	<10.0	26	<10.0	<15.0
Acenaphthene	<8.0	<10.0	2.9	2.7	<10.0	126	<3.0	21	6.7	<5.0
Fluoren	4.6	<3.0	1.6	2.8	<5.0	465	2.6	83	2.1	<8.0
C1-Fluorene	<8.0	<5.0	<13.0	<13.0	<5.0	6210	5.4	269	8.4	<8.0
C2-Fluorene	<20.0	<10.0	<10.0	<13.0	<10.0	15000	<10.0	470	<10.0	<35.0
C3-Fluorene	<50.0	<25.0	<25.0	<45.0	<25.0	30300	<25.0	802	<25.0	<80.0
Dibenzothiophene	<2.0	<2.0	<1.0	<1.0	<2.0	<100.0	<2.0	20	<1.0	<4.0
Phenanthrene	16	5	<5.0	<5.0	12	1460	8.9	270	<5.0	11
Anthracene	3.1	5.2	<1.0	<2.0	9.6	236	1.7	51	<1.0	<8.0
C1-Phenanthrene	<20.0	<11.0	<11.0	<11.0	<11.0	7200	<11.0	366	<11.0	<17.0
C2-Phenanthrene	<40.0	<20.0	<20.0	<20.0	<20.0	23700	<20.0	1370	<20.0	<60.0
C3-Phenanthrene	<20.0	<13.0	<8.0	<8.0	<20.0	10400	<10.0	902	<8.0	<80.0
C4-Phenanthrene	<2.0	<10.0	<3.0	<3.0	<10.0	2710	<10.0	335	<3.0	<90.0
C1-Dibenzothiophene	<12.0	<6.0	<6.0	<6.0	<6.0	462	<6.0	119	<6.0	<10.0
C2-Dibenzothiophene	<25.0	<15.0	<15.0	<15.0	<15.0	671	<15.0	718	<15.0	<20.0
C3-Dibenzothiophene	<25.0	<15.0	<15.0	<15.0	<15.0	1140	<15.0	1340	<15.0	<20.0
Fluoranthene	55	15	<3.0	6.9	37	146	22	392	<3.0	28
Pyrene	29	18	<7.0	7.5	28	946	14	659	<7.0	62
C1-Pyr-Flu	20	13	<10.0	<10.0	30	2270	<10.0	460	<10.0	68
C2-Pyr-Flu	<20.0	30	<7.0	29	34	2150	18	986	<7.0	180
Benzoaantracen	10	6.1	<1.0	2	15	27	4.1	117	<1.0	8.4
Chrysene	34	9.8	<2.0	5.5	29	s102	8.5	215	<2.0	s24
C1-Chrysene	23	28	<3.0	31	40	692	12	858	<3.0	195
C2-Chrysene	16	76	<3.0	82	78	1320	33	1780	<6.0	490
Benzobjfluoranthene	134	30	<1.0	17	90	163	24	351	1.9	57
Benzokfluoranthene	29	7.5	<1.0	3.4	23	45	4.9	95	<1.0	12
Benzoepyrene	56	21	<1.0	16	49	213	14	368	1.3	76
Benzoapyrene	16	8.7	<1.0	4.6	27	58	5.4	149	<1.0	22
Perylene	4.1	<3.0	6	6.9	11	<30.0	2.3	54	10	19
Indeno123cdpyren	59	12	<2.0	7.7	43	58	10	133	<2.0	<17.0
Dibenzacahantracen	11	<3.0	<1.0	<2.0	<10.0	<25.0	<3.0	<50.0	<1.0	<17.0

Benzoghiperylen	37	17	<2.0	8.1	28	158	8	230	<2.0	50

Location	**Holt**	**Idrettsveien**	**Lundeby**	**Neset**	**Nøklegård**	**Nøstvedt**	**Nygård**	**Rånås (R)**	**Ringdal**	**Seierstad**
C1-Naphthalene	17	31	<10.0	20	<10.0	19	<20.0	<40.0	<10.0	<1.0
C2-Naphthalene	186	684	68	98	248	386	413	200	80	87
C3-Naphthalene	715	2920	252	324	1010	1660	2370	<230.0	381	263
C4-Naphthalene	107	488	33	39	191	332	392	84	83	<25.0
Acenapththylene	<10.0	<25.0	<10.0	<20.0	<10.0	<15.0	<20.0	109	<10.0	<15.0
Acenaphthene	<10.0	<20.0	<3.0	<8.0	5.8	<15.0	11	<10.0	<3.0	<4.0
Fluoren	<5.0	8.8	<2.0	<2.0	3.1	18	24	4.8	<3.0	<3.0
C1-Fluorene	<10.0	32	<4.0	<8.0	<13.0	50	<8.0	20	<5.0	<5.0
C2-Fluorene	<20.0	<24.0	<10.0	<20.0	<10.0	90	<20.0	65	<10.0	<15.0
C3-Fluorene	<30.0	<64.0	<25.0	<50.0	<25.0	<190.0	<50.0	<110.0	<40.0	<40.0
Dibenzothiophene	6.1	<3.0	<2.0	<2.0	<1.0	<4.0	<3.0	<6.0	<2.0	<2.0
Phenanthrene	30	<13.0	9.3	<10.0	<5.0	69	<10.0	122	19	<8.0
Anthracene	4.6	3	2.9	<1.0	<1.0	12	<3.0	16	7.8	3.9
C1-Phenanthrene	26	<3.0	<11.0	<20.0	<11.0	67	20	52	22	<17.0
C2-Phenanthrene	137	<50.0	24	<40.0	<20.0	356	<40.0	117	95	<30.0
C3-Phenanthrene	126	<20.0	<10.0	<20.0	<8.0	234	<16.0	46	60	<12.0
C4-Phenanthrene	<70.0	<10.0	<20.0	<7.0	<3.0	<140.0	<5.0	<30.0	<4.0	<12.0
C1-Dibenzothiophene	12	<15.0	<6.0	<12.0	<6.0	16	<12.0	<30.0	5.7	<10.0
C2-Dibenzothiophene	67	<30.0	<15.0	<25.0	<15.0	142	<25.0	<60.0	26	<20.0
C3-Dibenzothiophene	178	<30.0	<15.0	<25.0	<15.0	372	<25.0	<60.0	76	<20.0
Fluoranthene	52	12	36	<4.0	<3.0	107	11	285	40	17
Pyrene	123	<17.0	24	<10.0	<7.0	214	<12.0	213	55	40
C1-Pyr-Flu	83	<25.0	12	<15.0	<10.0	131	<20.0	50	35	31
C2-Pyr-Flu	251	i	28	<20.0	<7.0	372	<15.0	46	90	94
Benzoaantracen	10	5.7	5.8	<2.0	<1.0	21	<2.0	12	13	6.9
Chrysene	s41	6.7	8.1	<3.0	<2.0	s62	6.3	15	s25	s15
C1-Chrysene	342	26	23	20	<3.0	309	<6.0	16	94	106
C2-Chrysene	903	66	48	52	8.5	857	<7.0	17	199	280
Benzobjfluoranthene	78	39	28	10	2.3	88	27	29	74	48
Benzokfluoranthene	13	7	5.9	2.9	<1.0	18	7	8.7	15	9.3

Benzoepyrene	123	44	18	17	2	125	12	16	55	65
Benzoapyrene	25	15	7.5	5.3	<1.0	40	6	7.6	21	19
Perylene	32	5.8	2.9	2.3	5.4	23	<2.0	<4.0	11	15
Indeno123cdpyren	25	33	12	7.3	<2.0	35	<10.0	<20.0	28	18
Dibenzacahantracen	15	7.1	<3.0	<3.0	<1.0	<15.0	<8.0	<4.0	<10.0	<10.0
Benzoghiperylen	63	28	11	9.8	<2.0	117	<20.0	<30.0	33	42

Location	**Skinmo**	**Sky**	**Støttum**	**Talaldrud Junction**	**Taraldrud North**	**Talaldrud South**	**Tenor**	**Tveiten**	**Vassum**	**Vennersrød (R)**
C1-Naphthalene	<10.0	<15.0	<10.0	64	87	51	13	19	45	<10.0
C2-Naphthalene	168	112	97	368	295	284	207	296	287	56
C3-Naphthalene	677	364	336	2520	1340	905	761	1030	1010	197
C4-Naphthalene	97	<40.0	45	1020	428	248	113	155	353	26
Acenapththylene	<10.0	<15.0	<10.0	12	36	22	<10.0	<10.0	<15.0	<10.0
Acenaphthene	3.9	<7.0	<6.0	<15.0	29	<25.0	<6.0	5.9	<40.0	<3.0
Fluoren	3.5	<5.0	<2.0	41	67	32	<7.0	2.7	64	<2.0
C1-Fluorene	<13.0	<13.0	<5.0	231	211	109	<10.0	<13.0	184	<4.0
C2-Fluorene	<10.0	<15.0	<10.0	624	454	268	<15.0	<10.0	478	<10.0
C3-Fluorene	<25.0	<40.0	<25.0	1410	<1100.0	511	<40.0	<25.0	<980.0	<25.0
Dibenzothiophene	<1.0	<2.0	<2.0	17	33	19	<2.0	<1.0	19	<2.0
Phenanthrene	5.8	28	12	213	512	185	13	<5.0	283	<5.0
Anthracene	1.5	7.3	2.5	16	47	40	2.2	<2.0	41	<1.0
C1-Phenanthrene	<11.0	18	13	428	480	219	<11.0	<11.0	425	<11.0
C2-Phenanthrene	<20.0	65	57	1610	1900	213	<40.0	<20.0	1570	<20.0
C3-Phenanthrene	<8.0	76	40	839	1030	674	0	<8.0	1050	<10.0
C4-Phenanthrene	<3.0	<70.0	46	<300.0	<500.0	490	<20.0	<3.0	390	<20.0
C1-Dibenzothiophene	<6.0	8.4	<6.0	88	120	67	<6.0	<6.0	101	<6.0
C2-Dibenzothiophene	<15.0	46	15	501	785	493	<20.0	<15.0	690	<15.0
C3-Dibenzothiophene	<15.0	130	52	996	1590	886	<60.0	<15.0	1550	<15.0
Fluoranthene	9.6	47	23	221	622	386	22	<3.0	415	<3.0
Pyrene	15	146	37	468	1190	641	30	<7.0	825	<7.0
C1-Pyr-Flu	<10.0	105	19	311	638	453	23	<10.0	626	<10.0
C2-Pyr-Flu	28	353	59	700	1210	1070	59	23	1440	<7.0
Benzoaantracen	3.9	13	4.5	39	98	113	6.8	<1.0	67	<1.0

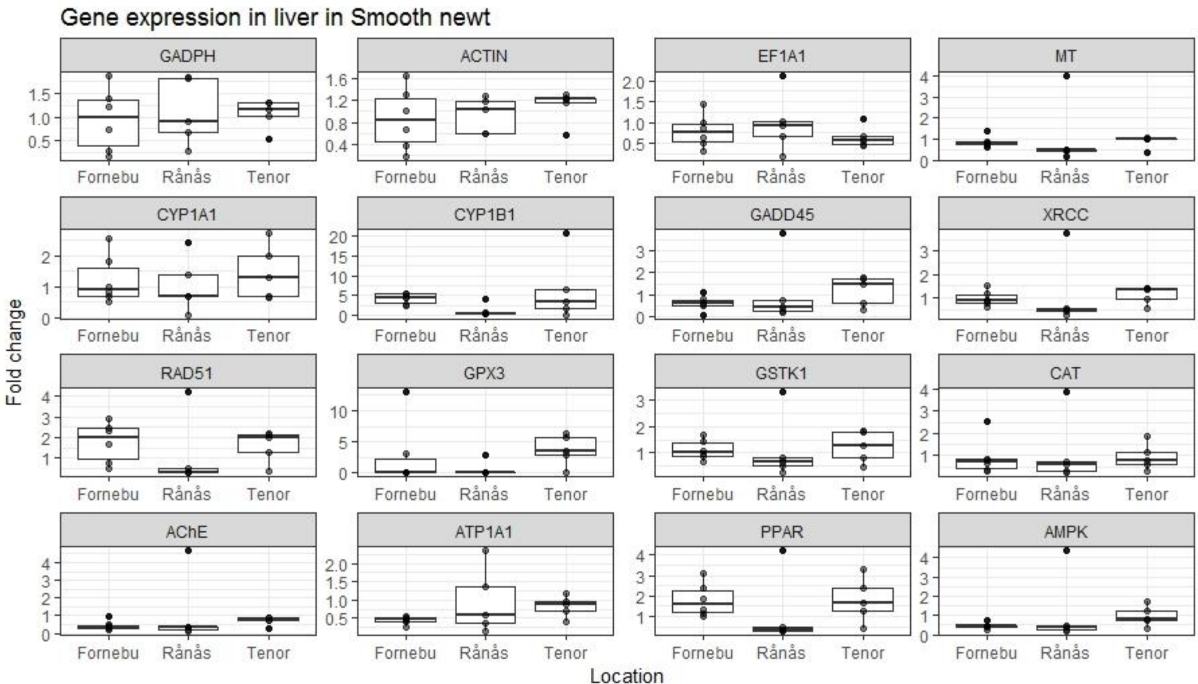
Chrysene	9.3	49	13.8	s73	215	176	17	2.6	118	<2.0
C1-Chrysene	35	309	84	521	1090	749	83	28	993	<3.0
C2-Chrysene	91	958	187	1280	2490	1740	171	78	2610	<8.0
Benzobjfluoranthene	33	111	23	142	281	306	30	9.4	221	3.1
Benzokfluoranthene	7.4	22	4.5	33	67	97	7.7	2.1	54	<1.0
Benzoepyrene	28	171	31	213	406	331	40	12	345	1.8
Benzoapyrene	6.9	52	7.7	73	128	164	13	2.5	108	<1.0
Perylene	10	42	8.6	<40.0	65	80	11	4.6	74	4.8
Indeno123cdpyren	14	43	9.6	54	117	134	13	<3.0	78	<2.0
Dibenzacahantracen	4.6	19	5.1	<30.0	<50.0	44	<10.0	<2.0	<40.0	<1.0
Benzoghiperylen	17	109	17	211	342	262	24	8	235	18

B4 – Metal concentration (µg/kg) in sediment

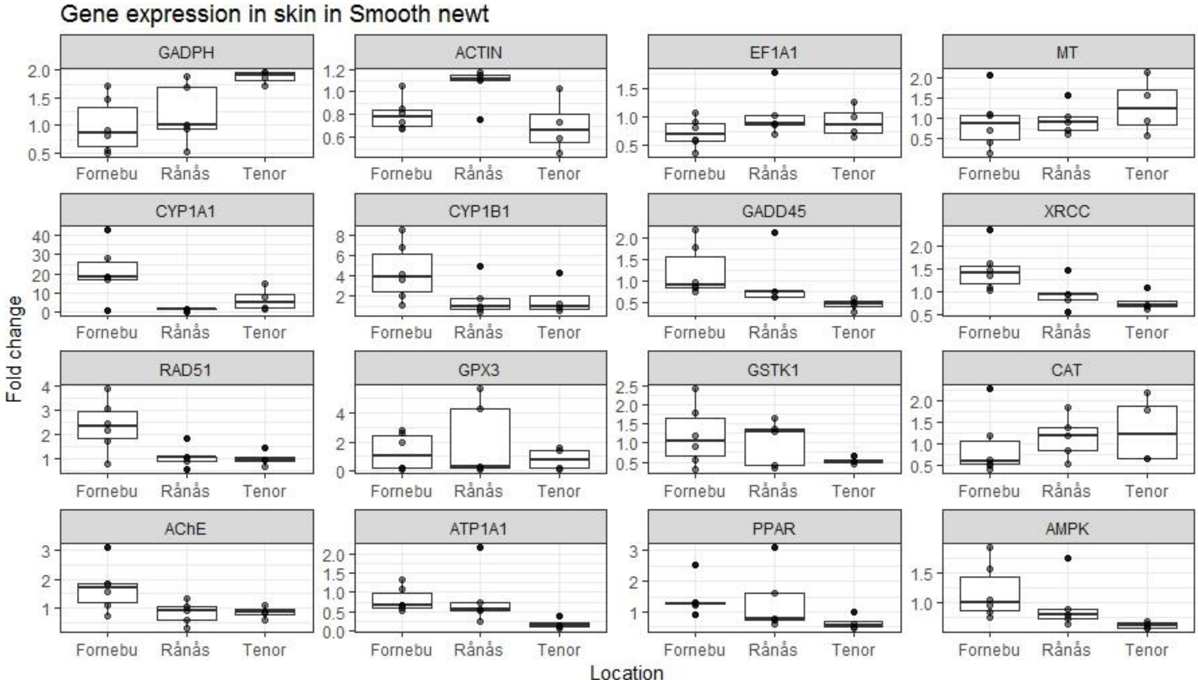
Location	Copper (µg/kg)	Chromium (µg/kg)	Nickel (µg/kg)	Zinc (µg/kg)	Arsenic (µg/kg)	Lead (µg/kg)	Cadmium (µg/kg)	TOC
Berle (R)	24	26	28	110	8.1	56	1.2	19
Bommestad East	11	10	6.5	60	2.9	10	0.15	2
Borre	13	16	17	42	3.8	7.3	0.088	0.2
Eikeberg	23	25	25	88	3.9	12	0.019	2
Emmerud	35	38	28	200	5.7	27	0.58	10
Fevang	62	27	21	340	3.9	16	0.17	8.7
Finstad	14	25	17	80	4.2	16	0.27	4
Fornebu	100	65	68	380	6.9	21	0.33	12
Helland	19	23	25	61	5.3	10	0.17	0.4
Holmane	26	17	13	96	4.1	65	0.072	1.2
Holt	45	47	43	250	6	17	<0.01	2.3
Idrettsveien	56	26	27	530	19	19	0.29	17
Lundeby	12	14	9.4	57	3	13	0.17	3.9
Neset	12	12	8.5	50	1.2	3.8	<0.01	0.1
Nøklegård	5.7	1.3	2.8	8.6	<0.5	4.2	<0.01	0.6
Nøstvedt	45	26	21	220	3.9	13	0.25	7.3
Nygård	17	8.3	11	67	2.3	6.8	0.77	28
Rånås (R)	35	7.1	12	97	5.6	17	1.3	38
Ringdal	26	19	11	110	4.3	17	0.14	4.3
Seierstad	19	14	10	110	3.6	9.5	0.16	1.8
Skinmo	34	36	32	170	5.6	14	0.2	1.7
Sky	30	20	15	180	4.4	9.8	0.04	3.4
Støttum	26	32	30	100	5.3	13	0.19	1.2
Talaldrud Junction	110	33	31	470	5.7	16	0.68	4

Taraldrud North	120	37	25	510	3.1	15	0.2	10
Talaldrud South	100	46	38	520	5.5	26	0.38	8.8
Tenor	44	30	32	180	4.1	12	0.57	3.2
Tveiten	18	21	21	71	5.8	12	<0.01	2.3
Vassum	120	35	26	860	4.1	17	0.35	9.9
Vennersrød (R)	16	28	29	63	5.5	13	0.29	3.6

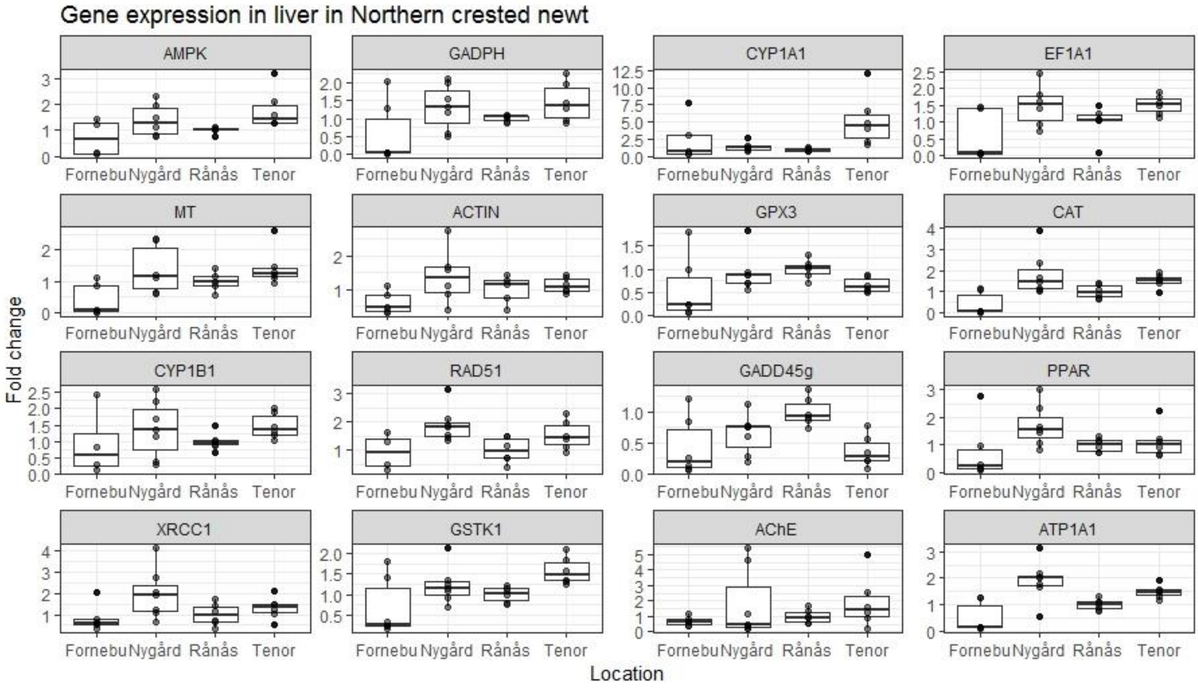
Appendix C – Additional data



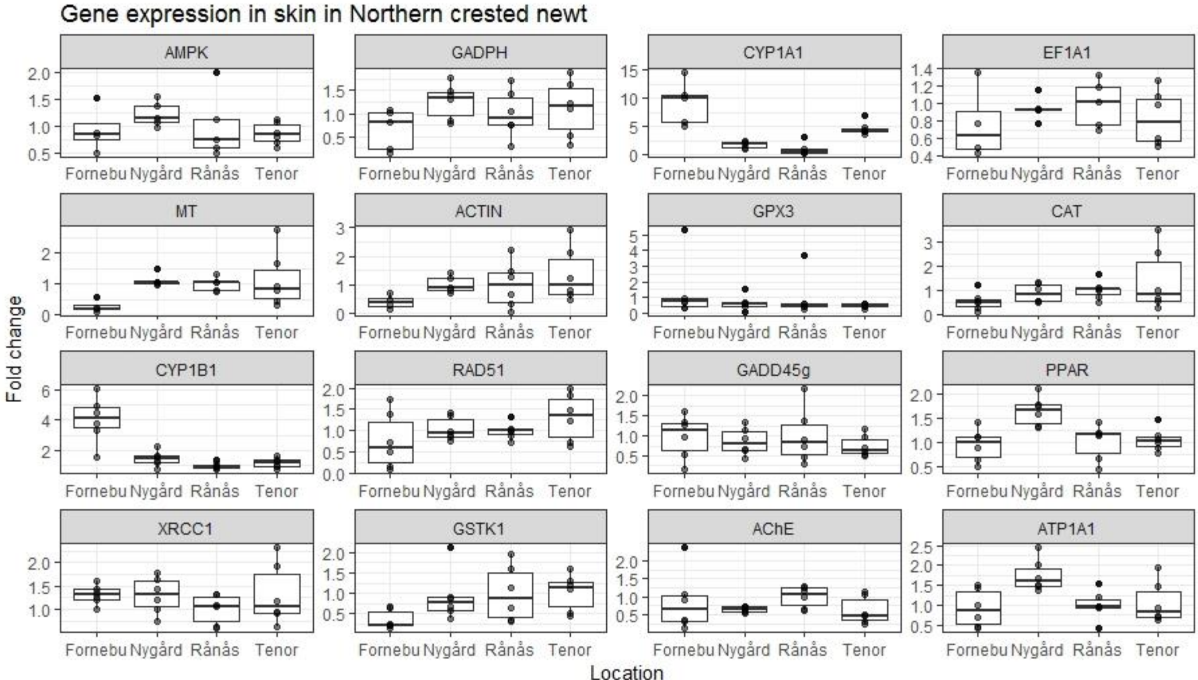
C1 – Boxplot of all genes in liver samples from smooth newt



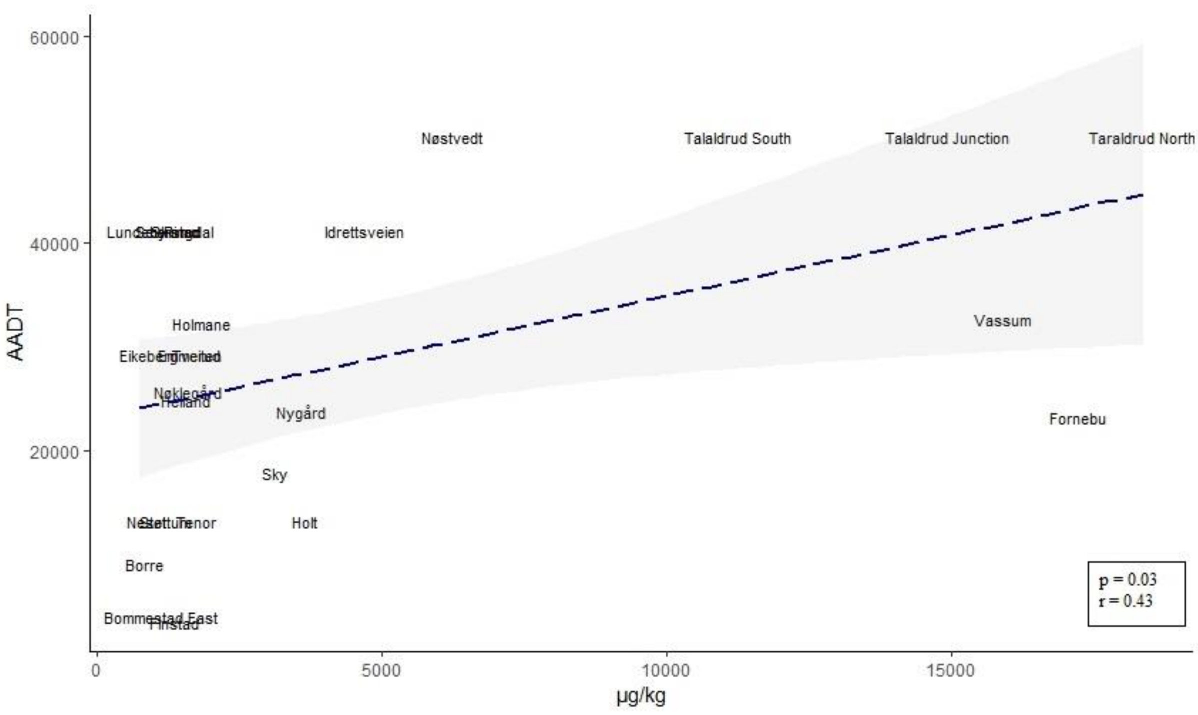
C2 – Boxplot showing fold change in all genes in skin samples from smooth newt.



C3 – Boxplot showing fold change in all genes in liver from northern crested newt



C4 – Boxplot of fold change in skin samples from northern crested newt.



C5 – AADT vs. Total PAH concentration

Appendix D – Methods and chemicals

Micronucleus Assay

Micronuclei contain chromosomal fragments enclosed in a membrane. During mitosis, these structures are sometimes not incorporated into a daughter nucleus and remain excluded. Micronuclei therefore serve as a simple indicator of chromosomal damage.

50µL of blood sample were placed on a microscope slide. The slides were tipped over yielding a thin layer of blood cells. The microscope slides were put in a humid dark chamber for 60 minutes. The slides were air-dried and kept dark until staining procedure. The slides were rinsed with PBS and stained for 5 minutes using 1ml of 1µg/ml Bizbenzimide (CAS 23491-45-4) (diluted in distilled water). Following staining procedure, the samples were rinsed in distilled water. The cells were mounted in glycerol-Mcllvaine buffer. The samples were kept dark in 4°C prior to analyses.

D1 – Chemicals used in micronucleus assay

Chemical	CAS-NO
Bizbenzimide	23491-45-4
Glycerol-Mcllvaine buffer	

Comet Assay

Comet assay is a method to analyze modified or damaged DNA in organisms which are exposed to genotoxic contaminants in aquatic environments. In this assay, blood cells are incorporated into agarose and lysed to liberate the DNA. The cells are then subjected to electrophoresis which makes the fragmented DNA migrate. The DNA are stained with a fluorescent dye and visualized using a fluorescence microscope. The broken fragments of the DNA resemble a comet tail. The length and other attributes of the tail is measured to determine the extent of DNA damage.

The blood was diluted in 200µL PBS-EDTA and kept on ice in the field. The samples were further diluted x100 in PBS-EDTA at the lab. 75mg low melting point (LMP) agarose were mixed with 10mL PBS-EDTA and heated until the liquid was clear. The agarose solution was

transferred to Eppendorf tubes and kept at heating block at 37°C. 10µL sample were gently mixed by stirring with 90µL agarose solution, 10µL of which were transferred to the hydrophilic side of a pre-cooled (4°C) GelBond® film. The films were transferred to lysis buffer in which they were kept in darkness (4°C) until the next day.

The following day the GelBond® films were rinsed in electrophoresis buffer (4°C) to unwind the DNA. The films were transferred to an electrophoresis chamber containing 1.4L fresh electrophoresis buffer. The films remained in the bath for 20 minutes before horizontal electrophoresis was conducted for 20 minutes. The electric current makes the fragmented DNA migrate towards the positive pole of the chamber. After electrophoresis the films were rinsed in neutralizing buffer at room temperature before transferred to fresh neutralizing buffer for 15 minutes. After the neutralization step, the films were rinsed in distilled water and 96% ethanol. The films were further fixed in darkness in 96% ethanol between 1,5-24 hours before airdried.

The films were stained using SYBRgold. 50µL SYBRgold were diluted in 50mL Tris-EDTA buffer (TE buffer). The solution was poured in boxes and rocked at a rocking table for 20 minutes. After the staining, the films were airdried in darkness before scored. The films were mounted on a plexiglass using distilled water and covered by a coverage glass. The slides were scored using a fluorescence microscope (Carl Zeiss Axio Scope A1, ex/em 520/610). The slides were illuminated with blue light and a real time camera (Allied Vision Technologies) was used to visualize the cells in the samples. The scoring was conducted by the program Comet Assay IV (Perceptive Instruments, version 4.2). Cells which overlapped, and cells located towards the end of the sample were avoided. 50 cells per gel were scored.

The first batch of comet assay was unfortunately unsuccessful. A second batch was conducted, the following changes was made to the protocol:

- Cells in the blood samples were counted using a Countless II machine to verify presence of blood cells.
- The blood samples were diluted in 100µL PBS-EDTA with no further dilution.
- The samples were duplicated using two different films. One of the films was treated with hydrogen peroxide before rinsed in PBS and after stored in lysis buffer and further processed as described above.

D2 – Chemicals used in Comet assay

Chemical	**CAS-NO**
NaCl (58.44 g/mol)	7647-14-5
NaOH (40.0g/mol)	1310-73-2
$Na_2EDTA \cdot 2H_2O$ (372.2 g/mol)	6381-92-6
Trizma base (121.2 g/mol)	77-86-1
Triton X-100	9002-93-1
NaOH (40,0 g/mol)	1310-73-2
Na2-EDTA (372,24 g/mol)	6381-92-6
Conc HCl (37%)	7647-01-0
Trizma-base (121.14 g/mol)	77-86-1
Trizma-HCl (157.56 g/mol)	1185-53-1