Biomarker responses in juvenile brown trout (*Salmo trutta)* exposed to tunnel wash water: a laboratory and field study

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Acknowledgements

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” *Det er så fint at vi sitter her. Om 20 år kommer vi til å tenke på dette i 70-tallsfarger* ”

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Sub-lethal effects in juvenile brown trout (*Salmo trutta)* exposed to tunnel wash water: a laboratory and field study

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Abstract

Tunnels are washed regularly to maintain road safety and increase their life span. During a tunnel wash event, highly contaminated tunnel wash water is generated and released to the environment. Tunnel wash water may be led to sedimentation ponds where particles and particle bound contaminants are allowed to settle. Still, the removal of contaminants is only partial, and release of contaminated water to local recipients is of concern. Further, a growth reduction has previously been observed in fish sampled downstream of where discharge water from Vassum sedimentation pond is released to the stream Årungenelva. This reduction may be related to release of tunnel wash water from the pond to the stream. The main aim of this thesis was to investigate sub-lethal effects caused by exposure to tunnel wash water using juvenile brown trout (*Salmo trutta*) as a model species. Brown trout was exposed to filtered (1.2 µm) tunnel wash water in a laboratory study for 25 days. In addition, fish was sampled in the stream Årungenelva downstream and upstream (reference) from where water from Vassum sedimentation pond is discharged into the river. In fish from the laboratory study, the results revealed an increased concentration of several three-ring polycyclic aromatic hydrocarbon (PAH) metabolites in bile of fish exposed to tunnel wash water. This was however not observed for metabolites of the four-ring PAH pyrene or the five-ring PAH benzo[a]pyrene. In addition, an effect on the phase I enzyme Cytochrome P450 1A (CYP1A) was observed. Elevated activity of this enzyme, (measured as 7-ethoxyresorufn O-deethylase (EROD) activity) in gills and liver as well as elevated CYP1A protein in liver was observed in fish exposed to tunnel wash water. This indicates uptake of bioavailable contaminants of fish exposed to filtered tunnel wash water. In fish sampled downstream of the sedimentation pond in Årungenelva the biliary concentrations of PAH metabolites was lower while the EROD activity in liver was higher compared to responses observed in fish sampled upstream from the sedimentation pond. No differences were observed in EROD activity in gills or in CYP1A protein in liver between fish sampled at the two locations in the stream. Effects observed in fish sampled upstream of the pond may be explained by the close proximity between the upstream location and the highway. The biomarker responses in fish from Årungenelva may thus indicate that both locations in the stream is affected by road related contaminants Exposure to lead was assessed by quantifying the δ-aminolevulinic acid dehydratase (ALA-D) activity in red blood cells of fish. No inhibition of enzymatic activity was observed in tunnel wash water exposed fish in the laboratory study and no difference was observed between fish sampled at the two locations in Årungenelva. The results the ALA-D

biomarker indicated that trout were not exposed to lead at any extent. In the laboratory study, tunnel wash water from two tunnels, the Granfoss tunnel and the Nordby tunnel, was included. Stronger effects were observed in several of the investigated biomarkers in fish exposed to Nordby compared to fish exposed to Granfoss tunnel wash water. The two tunnels have similar annual average daily traffic (AADT), but the Granfoss tunnel is washed with a higher frequency. Washing frequencies may thus affect concentrations and the toxicity of road-related contaminants in tunnel wash water. In fish sampled in Årungenelva, it could not be concluded that fish sampled downstream from the sedimentation pond have experienced a higher exposure to road-related contaminants compared to fish sampled upstream from the pond. The findings of the current study could thus not relate the growth reduction previously observed in Årungenelva to the exposure of road-related contaminants. Due to severe rain the sampling in Årungenelva was postponed several times. Sampling closer to a tunnel wash event might have revealed a different pattern in the biomarkers investigated in fish from the stream.

Abbreviations

AADT	annual average daily traffic
abs	absorbance
AhR	aryl hydrocarbon receptor
ALA	aminolevulinic acid
ALA-D	δ-aminolevulinic acid dehydratase
ARNT	AhR nuclear translocator
BaP	benzo[a]pyrene
BSA	bovine serum albumin
CYP1A	Cytochrome P450 1A
dH2O	distilled water
DMSO	dimethyl sulfoxide
DO	dissolved oxygen
E18	Eurpoean route 18
ELISA	enzyme linked immunosorbent assay
EROD	7-ethoxyresorufn O-deethylase
ex	exitiation
em	emission
HC-buffer	HEPES-Cortland Buffer
HDPE	hard-density polyethylene
HPLC	high performance liquid chromatography
mRNA	messenger RNA
NADPH	nicotinamide adenine dinucleotide phosphate
NIVA	Norwegian institute of water research
NMBU	University of life sciences
NORWAT	Nordic road water
NPRA	Norwegian public roads administration
-OH	-hydroxyl
PAH	polycyclic aromatic hydrocarbons
PBG	porphobilinogen
PCA	principal component analysis

TPA	triphenylamin
Tukesy's HSD	Tukey’s honestly significant difference
TWW	tunnel wash water
UiO	University of Oslo
′	minute

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1 Introduction

Road-related activities lead to the release of a complex mixture of contaminants. These contaminants originate from wear of vehicle-parts such as brakes, tires and vehicle body in addition to combustion processes and oil and petroleum spill (Meland 2010). Contaminants can also be of non-vehicle origin, such as asphalt, bitumen and road equipment (Meland 2010). Polycyclic aromatic hydrocarbons (PAHs) and various metals have been reported as major contaminants from road and vehicle activities (Maltby et al. 1995, Meland et al. 2010a). In addition, studies have reported hydrocarbons, alkylated and substituted PAHs, heterocycles, brominated compounds, organophosphates, organotins, alkylphenols and phthalates to be present in water, sediment or dust affected by road activities (Takada et al. 1991, Aryal and Lee 2009, Meland 2012a, Wei et al. 2015). Recent characterisation of road dust from Norwegian tunnels indicates presence of several of these contaminants (M. Grung personal communication, October 2015).

While road contaminants are regularly removed from road surfaces by rain and wind, road tunnels can be considered semi-closed systems where particles and contaminants released from use of vehicles to a large extent accumulates. To ensure road safety and increase the lifespan of tunnels they are regularly washed (1-12 times a year). During a tunnel wash event road-sweeping machines are used, detergents are generally applied and road surfaces, walls and signs are washed using high pressure cleaning. According to contractors, in a two-tube four-lane tunnel 60-100 L of wash water is utilized per meter of tunnel (Meland et al. 2010a). In Norway, there are more than 1000 road tunnels, with a combined length of approximately 800 km. Release of highly contaminated tunnel wash water is therefore of concern.

Due to accumulation of road pollutants between each tunnel wash event, tunnel wash water may contain higher concentrations of several contaminants than drainage water from open roads (Garmo et al. 2015). When tunnels are washed the tunnel wash water is in most cases released directly to local recipients, such as local streams or the sea. Tunnel wash water may also be led to indoor sedimentation basins or outdoor sedimentation ponds that are constructed to retain particles and particle-bound contaminants and limit the release of contaminants to local recipients (Meland 2012b). Investigation of the efficiency of a sedimentation pond has shown that between 58% and 89% of metals and PAHs were retained in the pond (COWI 2005). This means elevated levels of both metals and PAHs will be

present in pond outlet water and has been confirmed in other studies (Lundberg et al. 1999, Meland et al. 2010a). In the study by Meland et al. (2010a), the presence of metals and PAHs in outlet water from a sedimentation pond showed PAHs to be associated with the particulate and colloidal fraction, whereas metals in addition were found in the low-molecular-mass fraction (<10 kDa) (Meland et al. 2010a). In treatment of road runoff and tunnel wash water in Norway no steps are currently applied to remove contaminants in the water-soluble fraction (colloids and low-molecular-mass fraction), but different treatment systems are currently being investigated (Paruch and Roseth 2008b, a, COWI 2012, Luz 2014).

Toxicity testing has been performed with road water where particles first have been removed, to assess the toxicity of contaminants present in the water-phase. Kjølholt et al. (2001) ran tests on bacteria, algae and daphnids and found similar toxicity of water with particles and water where particles first had been allowed to settle. This indicates contaminants that caused the toxicity were present in the water phase. A similar result was observed in a study investigating the toxicity of road dust to a benthic ostracod (*Heterocypris incongruens*) (Watanabe et al. 2011), where a dust-water mixture was centrifuged for removal of particles, and the contaminants in the water phase caused mortality to the ostracod (Watanabe et al. 2011). However, filtration of road water through bioretention columns has been found to reverse acute toxicity of road runoff; seen as reduced teratogenic effects in zebra fish embryos as well as reduced mortality and/or sub-lethal effects in salmon, daphnids and mayfly nymphs (McIntyre et al. 2014, McIntyre et al. 2015) after water had passed through the bioretention columns. In brown trout exposed to highway runoff effects on the antioxidant defence system, plasma ion regulation and blood glucose levels observed in fish exposed to highway runoff were significantly reduced after water had passed through a sedimentation pond and/or was diluted with stream water (Meland et al. 2009). However, both the study investigating treatment with bioretention columns and the study investigating treatment by sedimentation ponds found some negative effects of road runoff in fish after filtration/sedimentation compared to effects seen in control fish exposed to clean water (Meland et al. 2009, McIntyre et al. 2014, McIntyre et al. 2015).

The Vassum sedimentation pond is located outside of the city of Oslo, Norway, and was constructed in the year 2000 as part of an extension of the highway E6 and the construction of two new tunnels (in addition to the already existing Nordby tunnel). Outlet water from the pond is discharged into the stream Årungenelva. Historical data of fish length of brown trout

(*Salmo trutta*) in the stream have showed 0+ fish to be significantly reduced downstream from the pond as compared to upstream in the period after the pond was constructed (Meland et al. 2010a). The cause of the growth reduction is not known, but has been suggested to be related to release of tunnel wash water to the stream. Reduced growth may be caused by reduced energy uptake as a response to changed behaviour, reduced food quality/availability or due to metabolic trade-offs between growth and detoxifying mechanisms (Meland et al. 2010a). The growth reduction is of concern with regard to the effects road runoff and tunnel wash water can pose in the environment, even after passing through a sedimentation pond. This growth reduction was the background for the current study.

Brown trout (*Salmo trutta*) is a widely distributed fish species and native to Norway (MacCrimmon and Marshall 1968, Jonsson and L'Abée‐Lund 1993). The fish may be anadromous, migrating to the sea as sea trout, or live their entire life in freshwater. Fish growing up in freshwater streams have been shown to be very stationary, as during a recapture study 85% of recaptures was done within 20 m of release points (Bohlin et al. 2002). This makes brown trout a good indicator species for investigating exposure to contaminants between closely located sites. The species has been shown to be sensitive to presence of pollutants, and is considered a suitable indicator species both in the field and the laboratory (Rodriguez-Cea et al. 2003, Rodríguez-Cea and Sanz-Medel 2004). In addition, the species is bred in hatcheries for the purposes of introducing them to the wild. Brown trout is thus a relevant target species present in streams throughout Norway, is available for use in the laboratory and a relevant model organism for ecotoxicological testing.

Investigating effects of tunnel wash water in a controlled laboratory study allows control over factors as temperature, pH and salinity, which affect bioavailability of contaminants. It also ensures a reliable control treatment, where the observed effects can be linked to the factor investigated, namely the tunnel wash water. Targeting fish in streams can give on-site information of local conditions and local exposure to contaminants. Investigating similar endpoints both in a laboratory and a field study will provide a better understanding of effects caused by road-related contaminants than each separately.

Chemical characterisation of water and sediments can provide evidence of the presence of contaminants in the environment. However, such evaluation may say little about the

bioavailability of compounds to biota. Biomarkers are considered endpoints that reveal bioavailability and uptake of potential harmful contaminants in fish (Van der Oost et al. 2003). The biomarkers used in the present study indicate exposure to specific type of contaminants and provide an early warning of exposure to environmental contaminants.

1.1 PAHs, their metabolites and Cytochrome P450 1A

PAHs are hydrophobic organic contaminants ubiquitous to the environment (Hylland 2006). They consist of two or more aromatic rings and can be of pyrogenic or petrogenic origin. Petrogenic PAHs are formed in natural process and are present in crude oil and petroleum products, while pyrogenic PAHs are formed during incomplete combustion of organic matter (Hylland 2006). Both petrogenic and pyrogenic PAHs are present in the complex mixture of road-related contaminants (Meland 2010). The lipophilicity of PAHs make them available for uptake through biological membranes, and they are taken up by aquatic organisms both through gills and food consumption (Grung et al. 2009).

Fat-soluble compounds are in general dependent on biotransformation to more water-soluble compounds before being excreted from organisms. Cytochrome P450 (CYP) is a superfamily of haem proteins that mediate such metabolism and metabolise a range of fat-soluble compounds from endogenous compounds, such as steroids and fatty acids, to drugs and environmental contaminants (Uno et al. 2012). The CYP1A family has been heavily studied as it is involved in important phase I reactions, including the epoxidation of coplanar chemicals such as PAHs (Whyte et al. 2000). PAH epoxides may be hydrolysed by epoxide hydrolase to phenols or dihydrodiols, which may be further conjugated with glucuronic acid or sulphate (Xu et al. 2005, Xue and Warshawsky 2005). As PAHs are readily metabolised by vertebrates, the parent PAHs do not accumulate in fish at the degree to which they are present in the environment (Whyte et al. 2000, Van der Oost et al. 2003, Ariese et al. 2005), and metabolised PAHs are excreted from fish through bile or urine (Law et al. 1994). Hydroxylated PAHs in bile samples can thus be measured directly as a biomarker of exposure and determination of PAH metabolites in bile is an established method for assessing recent PAH exposure to fish (Ariese et al. 2005, Grung et al. 2009).

PAHs and other coplanar chemicals can induce CYP1A by acting as ligands binding to the aryl hydrocarbon receptor (AhR). The receptor goes through a confirmation change and

translocate to the nucleus where binding to the AhR nuclear translocator (ARNT) protein promotes transcription of CYP1A mRNA (Whitlock Jr 1999). The most potent CYP1A inducers are certain dioxins, dioxin like compounds (e.g. some polychlorinated dibenzofurans) and polychlorinated biphenyls, in addition to several PAHs (e.g. benzo[k]fluoranthene and benzo[a]pyrene) (Denison and Heath-Pagliuso 1998). While persistent halogenated aromatic hydrocarbons are poor substrates for CYP1A and will accumulate in fish, PAHs on the other hand are readily metabolized in fish and induction of CYP1A increases their metabolism and excretion (Bols et al. 1999, Uno et al. 2001). Still, epoxides are formed as intermediates of phase I metabolism and certain epoxides have electrophilic properties and can bind a variety of endogenous molecules. Formation of adducts from the binding of epoxides to DNA is among the reactions that make some PAHs potent carcinogens (Hendricks et al. 1985, Baird et al. 2005). Coplanar chemicals may therefore induce their own toxicity through AhR mediated CYP1A induction (Shimizu et al. 2000). Exposure to PAHs and other CYP1A agonists may be related to effects such as immunotoxicity, DNA damage and adducts, lesions and tumours in tissue and effects on the reproductive system, and such effects may be activated through the AhR-pathway (Whyte et al. 2000, Logan 2007).

There are many ways to detect changes in CYP1A (Nilsen et al. 1998). One is the enzyme activity of 7-ethoxyresorufin *O*-deethylase (EROD), which is a catalytic assay measuring the conversion of 7-ethoxyresorufin to resorufin. CYP1A mediate this conversion, and the amount of resorufin can be measured fluorometrically (Burke and Mayer 1974). As compounds such as fluoranthene, some nonylphenols, some polybrominated diphenyl ethers and several metals (e.g. Cd, Cu) are known to be CYP1A inhibitors (Lee et al. 1996, Willett et al. 1998, Benedetti et al. 2007), assessing CYP1A induction only through EROD activity can be a challenge when dealing with complex mixtures. Therefore, it is of value to determine the amount of CYP1A proteins in tissue. This can be done through an enzyme linked immunosorbent assay (ELISA) using CYP1A specific antibodies (Goksøyr 1991).

1.2 Lead and δ-aminolevulinic acid dehydratase (ALA-D)

In the 1920s tetraethyllead ($C_8H_{20}Pb$) was added to fuels as an antiknock agent. As evidence of the deleterious effects of lead increased, the use of leaded gasoline was phased out in the 1970s (Nriagu 1990) and marketing was prohibited for member states in the European Union

(EU) as late as 2000 as stated in *Directive 98/70/EC relating to the quality of petrol and diesel fuels and amending Council Directive 93/12/EEC L 350/58*[1]. Lead was also prohibited in materials and vehicles put on the EU market after the year 2003[2] as stated *in Directive 2000/53/EC on end-of life vehicles [2000] L 269/34* and *Commission decision amending Annex II to Directive 2000/53/EC on end-of-life vehicles (notified under document C (2010) 972) [2010] OJ L 48/12*. Although the concentration of lead in road pollution have decreased after the phase-out of leaded gasoline (Kayhanian et al. 2012), lead is still found in road runoff and tunnel wash water in Norway (Paruch and Roseth 2008b, Meland et al. 2010a, Meland et al. 2010b) and might pose a risk to biota.

An established biomarker for lead exposure is the activity of the δ-aminolevulinic acid dehydratase (ALA-D) (ICES 2004). ALA-D is an enzyme in the haem biosynthesis pathway that catalyses the formation of porphobilinogen (PBG) from two molecules of aminolevulinic acid (ALA). ALA-D is a metalloenzyme, and is inhibited by lead as lead replaces zinc in the active seat of the enzyme (Simons 1995). This replacement inhibits binding of the substrate (ALA) to the active seat, and reduces haem production. Haem is the cofactor of various enzymes including catalases, peroxidases, cytochromes and haemoglobin. Haemoglobin carries oxygen around the body, and supressed production of haem can lead to anaemia (Johansson-Sjöbeck and Larsson 1979). Further, lead exposure has been shown to cause a range of deleterious effects such as spinal deformities in fish (Holcombe et al. 1976) and neurotoxicity in mammals (Davis et al. 1990). Measuring the ALA-D activity in blood is thus a way to indicate possible harmful lead exposure.

1.3 Aims and hypothesis

The main aim of this thesis was to investigate sub-lethal effects in juvenile brown trout (*Salmo trutta*) following exposure tunnel wash water.

This was investigating by setting up a controlled laboratory study with four different treatments: clean water (control treatment), water containing lead and benzo[a]pyrene (positive control treatment) and filtered tunnel wash water from two different tunnels

[1] Some exceptions were made: e.g. it was permitted to continue marketing of leaded petrol until the year 2005, for member states where a ban would result in severe socioeconomic problems.

[2] With several exceptions e.g. in spare parts for older cars and in equipment where lead could not be easily substituted.

(Granfoss and Nordby tunnel wash water treatments). In addition fish was sampled in the stream Årungenelva both upstream (reference) and downstream of the point where discharge water from Vassum sedimentation is released into the stream. Biomarkers chosen to address the aim includes PAH metabolites in bile, EROD activity in gills, CYP1A protein in liver, EROD activity in liver and ALA-D activity in red blood cells. The following research questions were addressed:

1. Did the response in biomarkers investigated differ between the four treatments within each sampling day in the laboratory study?
2. Did the response in biomarkers investigated change as a response of time within each treatment in the laboratory study?
3. Was there any difference in the biomarker responses in fish sampled downstream compared to upstream from the sedimentation pond in Årungenelva?

In addition, correlation between the biomarkers and an overall pattern in the data was investigated using a principal component analysis.

Dybwad (2015) investigated gene expression biomarkers in gills and liver of the same trout as used in the current thesis. In addition, she investigated EROD activity in gills of sticklebacks exposed in the same aquaria in the laboratory study. Another aim was to discuss the results of the current study in light of the results observed by Dybwad (2015). Further, to evaluate the contamination pressure caused by release of discharge water from Vassum sedimentation pond to the stream, a final aim was to compare biomarker responses observed in fish held under controlled exposure conditions in the laboratory study with responses observed in fish from Årungenelva.

2 Materials and methods

2.1 Tunnels and study site

2.1.1 The Granfoss and Nordby tunnels (laboratory study)

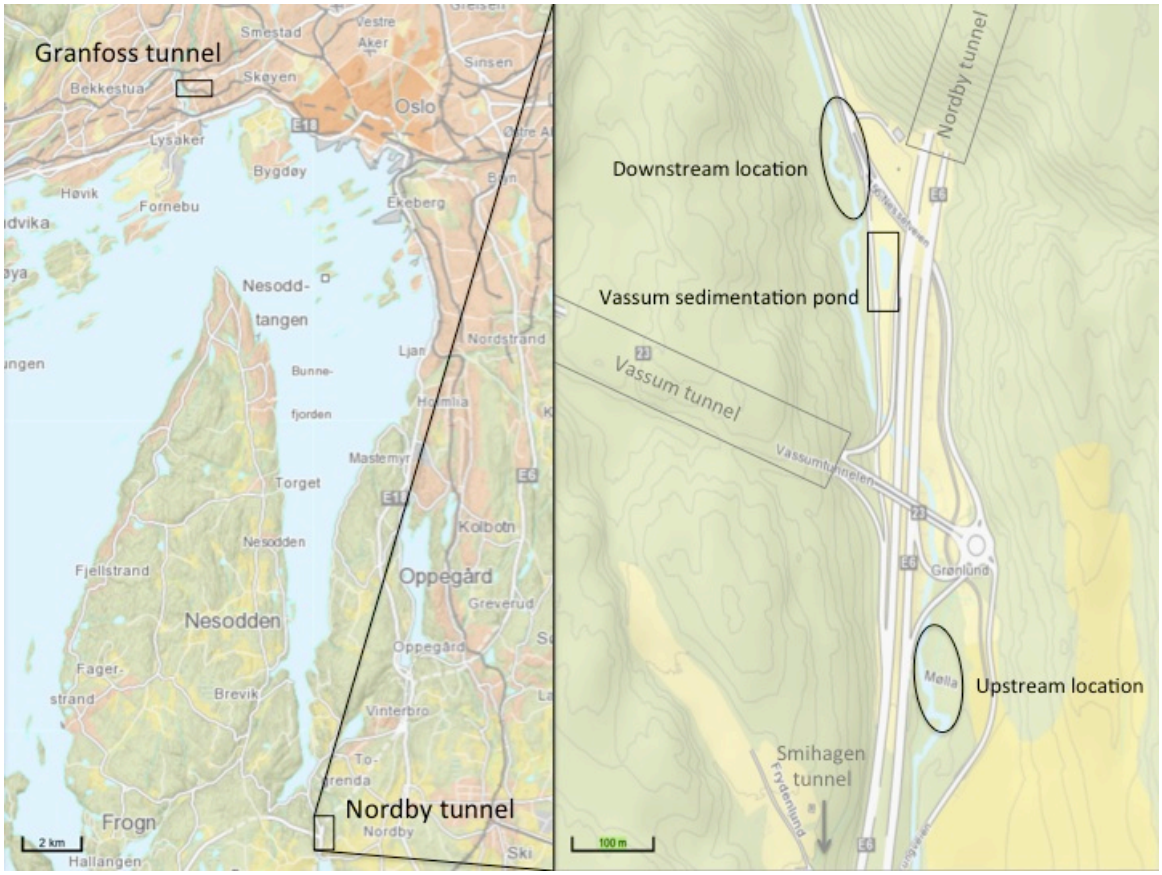

Figure 2.1 Left: a map of the locations of the two tunnels where the tunnel wash water used in the laboratory study was collected. Right: a map of the stream Årungenelva giving the two locations where fish were sampled, the location of the Vassum sedimentation pond and the location of the tunnels that drain water to the sedimentation pond (Smihaugen tunnel outside of map), and. Map from www.norgeskart.no. Figure modified from Dybwad (2015).

Tunnel wash water used in the laboratory study was collected from two tunnels, the Nordby and the Granfoss tunnel (Figure 2.1). The Nordby tunnel is located on the European route 6 (E6) in Akershus, Norway. The tunnel is 3 850 m long and has an annual average daily traffic (AADT) of 32 600 vehicles/day (Torp and Meland 2013). The Granfoss tunnel is located on the national highway 150 (Rkv 150), and consists of two tunnels. The eastern and western tunnel is 1 179 and 1 019 m respectively, and lies on each side of the boarder between Oslo and Bærum municipality. The tunnel had an AADT of 30 278 vehicles/day in 2013. Both the

Granfoss and the Nordby tunnel are two-tube four-lane tunnels. The Nordby tunnel and the Granfoss tunnel are washed four and ten times each year[3], respectively.

2.1.2 Årungenelva (field study)

The stream Årungenelva is 3.43 km and located in Ås and Frogn municipalities (Vann-nett 2015). It runs from the lake Årungen to Bunnefjorden, which is a fiord arm in the Oslo fiord. The stream is inhabited by several fish species e.g. trout, eel, roach, perch, pike, scrub and three spined sticklebacks. It is also spawning ground for sea trout and salmon from the Oslo fiord (Pura 2011).

Vassum sedimentation pond has its outlet into the stream (Figure 2.1). The sedimentation pond collects water from 1.7 ha open road as well as tunnel wash water from three different tunnels; the Nordby tunnel, the Smiehagen tunnel (AADT 38 290) and the Vassum tunnel (AADT 11 300) (Meland et al. 2010a). In 2013 and 2014, each of the tunnels was washed approximately four times each year, resulting in the Vassum sedimentation pond receiving tunnel wash water approximately once per month.

2.2 Laboratory study

2.2.1 Study species

Summer old (hatched in March 2013) brown trout (*Salmo trutta*) were obtained from Bjørklangen hatchery (Akershus, Norway). In November 2013 the fish were transported by car to the University of Oslo. During transport the fish were held in plastic bags placed in buckets with ice in the bottom to keep the water cold. At the University animal facility the fish was held in 750-L tanks having a flow through system of tap water, a 12:12 h light-dark photoperiod and was fed commercial pellets (Spirit Ørret 75 – 3.0 mm, Skretting) three times a week. In January 2014, three weeks prior to the exposure start, the fish was transferred to aquaria for acclimation. During the acclimation period and throughout the experiment the trout was fed boiled Greenland shrimp (0.6 mg per fish) twice a week. Remaining food in the tanks after feeding was removed to ensure good water quality. Trout was starved for minimum 48 hours prior to sampling to avoid evacuation of the bile. During the experimental period one fish appeared ill and was killed.

[3] Washing frequencies in the years 2013-2015.

2.2.2 Collection of tunnel wash water

The tunnel washes were conducted during the night between the 14th and 15th of November 2013 and 7th and 8th of January 2014, in the Nordby and the Granfoss tunnel respectively. Both washes were conducted without the use of soap. The water was pumped from the drainage system of the tunnels into 20-L hard-density polyethylene (HDPE, resin identification code 2) containers (Emballator Plast Mellerud) and a total of 340 L of water was collected from each tunnel wash event. Prior to sampling of tunnel wash water the containers had been filled with clean tap water for a week to remove possible compounds that could be released from the polyethylene to the water. The tunnel wash water was transported to the University of Oslo and stored at -20°C.

2.2.3 Preparation of treatment water

Four different treatments were included in the experiment; tunnel wash water from the Nordby and Granfoss tunnels, as well as a control and a positive control treatment. Tap water was used in the two control treatments (and also in the aquaria throughout the acclimation period).

To minimize confounding factors between treatments, the water for each treatment was prepared in the same way. The tunnel wash water was thawed, and water for each of the four treatments mixed separately in a 400-L tank. The water for all treatments was adjusted to have the same pH and salinity. The pH was adjusted to 7 by adding HCl/NaOH. Salinity was adjusted by adding NaCl to a concentration of 890 ± 10 ppm. This corresponds to the salinity measured in the water collected from the Nordby tunnel. One µg/L benzo[a]pyrene (BaP) and 150 µg/L lead (Pb) was added to the positive control water. BaP was added from a stock solution of 1000 µg/L BaP in dimethyl sulfoxide (DMSO) and Pb from a stock solution of 17.59 g/L lead(II) nitrate ($Pb(NO_3)_2$) in dH_2O. Final concentration of DMSO and NO_3 in the positive control water was 1095 µg/L and 89.5 µg/L, respectively. The water in all four treatments was filtered to remove particles. A peristaltic pump was used to pump the water through a 142 mm filter holder from Merck Millipore. Filters used were 12.5 or 15 cm in diameter Glass Microfiber Filters (GF/C™) with 1.2 µm pore size (Sigma Aldrich). During filtration, the water was transferred back into the 20-L plastic containers, which in the meantime had been rinsed carefully with tap water. The containers were stored in the freezer

(-20°C) until they were removed to thaw three days before the water was to be used in the experiment.

2.2.4 Setup

There were included four replicate aquaria per treatment. At the start of the acclimation period each aquarium contained four trout and as the experiment was conducted in cooperation with Ingvild Marie Dybwads master project (Dybwad 2015), they also contained eight three-spined sticklebacks (*Gasterosteus aculeatus*). Small hatching chambers (Marina Fish net breeder, 16x12.5x13 cm) were placed inside the aquaria to keep the sticklebacks separate from the trout. A filter pump (Pick Up 45, Eheim) ensured circulation and cleansing of the water. The water was aerated through a piece of chalk connected to an air-diffuser (APS 300, Tetra Tec) (Figure 2.2). Lids covered each aquarium and were held in place by stones to prevent fish from escaping. Treatments were randomly allocated to aquaria and black plastic plates were placed in between them to avoid visual contact between fish in different aquaria.

The experiment was semi-static and water was changed five times during the exposure period of 25 days. The 20-L whole glass aquaria contained 15 L of water. During a water-exchange 80% of the old water was removed from the aquaria before they were refilled with new water from the containers using a peristaltic pump. The experiment was conducted under light and

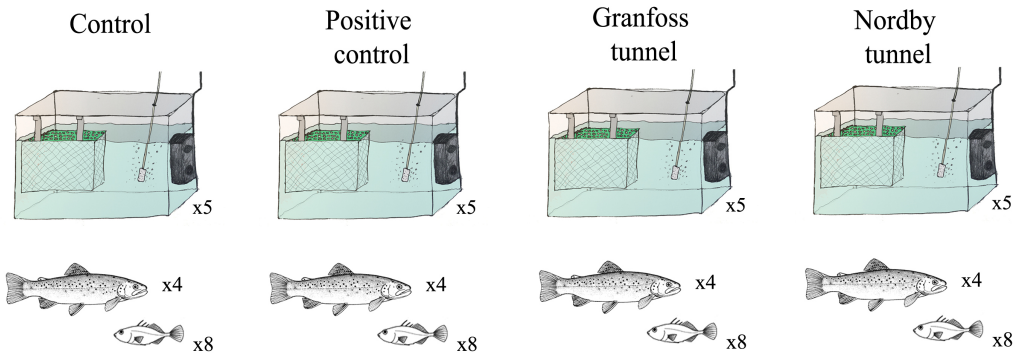

Figure 2.2 Sketch of the aquaria used in the laboratory study. Aquaria contained four brown trout and a nesting chamber with eight three-spined sticklebacks at the start of the experiment. In addition they contained an air diffuser and a filter pump.

temperature controlled conditions with a 12:12 h light-dark photoperiod and aquaria were placed in flow-through water baths (6°C).

After 5 and 25 days of exposure one fish was sampled from each aquaria, giving a total of five replicates for each treatment and each sampling point. In addition, one fish was sampled from each aquarium before the start of exposure (at day 0).

2.3 Field study

Juvenile brown trout was caught by electrofishing downstream and upstream from Vassum sedimentation pond, and was conducted by Thrond Haugen and Eivind Wollert Solberg from the University of Life Sciences (NMBU).

The fish was caught on the 21st of November 2014. Fish collected downstream from the pond was brought to the University of Oslo the same day for sampling, while the fish collected upstream from the pond was left in the stream in a keep-net at the place were they were caught, and collected the following day. The fish were transported to the University of Oslo in plastic bags placed in buckets with ice in the bottom to keep the water cold. At the University of Oslo the fish was placed in a cold room (5°C) and the water was aerated until the fish was sampled.

2.4 Fish dissection

All fish were dissected on ice-cold metal plates. To avoid cross contamination, the dissection equipment was properly rinsed in 70% rectified spirit and distilled water between dissecting each fish. Different dissection equipment was used for different tissues.

The fish were killed by a blow to the head. Blood was sampled immediately using heparinised insulin syringes (diameter 0.3 mm). The blood was transferred to Eppendorf tubes and stored on ice for 30-60 minutes before the blood samples were centrifuged in a table-centrifuge for 5 minutes to separate the plasma from the blood cells. The blood cells were frozen in liquid nitrogen to be used in analyses of ALA-D. Trout weight and length to caudal fork measurements were taken (Table 2.1). One gill arch was taken from the right side of each fish and stored in ice cold HEPES-Cortland Buffer for EROD analysis in gills, which

was conducted later the same day. Another gill arch was sampled and flash-frozen for analyses of gene expression (Dybwad 2015). Abdomen was cut open and the gall bladder was removed whole and put on Eppendorf tubes to be analysed for PAH-metabolites. Gall bladders were stored on ice in a dark box until the end of the sampling-day when they were all moved to the freezer (-20°C) for long-term storage. The liver was removed and cut in two. The posterior part was sampled to quantify CYP1A protein (using an ELISA) and EROD activity. The anterior part was used for gene-expression analyses (Dybwad 2015). Liver samples were flash frozen in liquid nitrogen.

All samples frozen on liquid nitrogen were transferred to a freezer holding -80°C. Trout samples from the laboratory study from day 5 were lost due to an error[4]. As a consequence, only analyses of the PAH metabolites in bile and EROD activity in the gills could be performed on fish sampled at day 5.

2.5 Water quality

Water quality parameters, metal concentrations and PAH concentrations were analysed by the Norwegian Institute of Water Research (NIVA), which laboratory is accredited in accordance to NS-EN ISO/IEC 17025. Water quality parameters investigated, reference methods used and the parameters limit of quantification are listed in Table 2.2. The limit of quantification corresponds to blank plus six times the standard deviation of the blank. Samples for metal analyses were collected on 50 mL acid prewashed polyethylene bottles (Naglene). To remove the acid solution the bottles were washed three times with sample-water prior to sample collection. Water for analysis of PAHs and water quality parameters was sampled on 2-L baked glass bottles and 1-L polyethylene bottles, respectively.

Samples collected for water quality parameters and metal analyses were sampled from 3 random aquaria of each treatment. From each aquaria one sample was collected before a water exchange (of water that had been in the aquaria for five days) and one sample was collected after a water exchange. This was done to get an overall picture of the exposure concentrations. This gave a total of six replicates for each treatment. For the Nordby treatment two replicates were taken after the water exchange, giving a total of 5 replicates.

[4] Stickleback samples form day 5 and 10 were also lost (Dybwad 2015).

Tabell 2.1 Length (cm), weight (g) and condition factor (*K*) of fish sampled in the laboratory and the field study. Condition factor (*K*) was calculated for each fish by the following formula: *K* = weight (g) / length (cm^3)

Laboratory study		Weight (g)		Length (cm)		Condition (*K*)	
Day	**Treatment**	Mean	SD	Mean	SD	Mean	SD
0	Control	12.9	3.6	11.1	0.9	0.9	0.1
	Positive control	11.1	3.8	10.9	1.4	0.8	0.1
	Granfoss	8.9	3.2	10.3	1.7	0.8	0.2
	Nordby	11.3	1.5	10.3	0.8	1.0	0.1
5	Control	13.8	6.4	11.2	1.5	1.0	0.1
	Positive control	15.6	3.1	11.8	0.5	1.0	0.1
	Granfoss	10.8	4.8	10.1	1.5	1.0	0.1
	Nordby	10.1	2.2	10.2	0.6	0.9	0.1
25	Control	14.6	4.7	11.2	1.0	1.0	0.1
	Positive control	12.3	1.6	10.8	0.3	1.0	0.1
	Granfoss	11.5	2.9	10.8	0.3	0.9	0.1
	Nordby	11.4	4.2	10.6	1.3	0.9	0.04
	Field study						
	Upstream	13.3	7.8	10.4	1.9	1.1	0.1
	Downstream	9.4	3.2	9.2	1.0	1.2	0.1

One mixed sample of water that had been in the aquaria for five days was taken for each treatment to be analysed for PAHs. In the field study, one sample for metal concentrations and water quality parameters was collected downstream and upstream from the sedimentation pond. In addition general water quality parameters as pH, temperature, dissolved oxygen (DO), salinity and general hardness were measured regularly during the laboratory study. In Årungenelva pH, temperature, conductivity, DO and turbidity was measured at the day of sampling. All measurements in water are presented in Table 2.3.

2.6 Quantification of PAH metabolites in bile

2.6.1 Re-suspension and standardization of bile from the field study

Bile samples from the field study had dried up inside the Eppendorf tubes (at -20°C), and bile samples had to be re-suspended. Twenty µL dH_2O was added to the tubes containing samples. Gall bladders were cut open with a scalpel and the samples were vortexed with a Whirlmixer 3x 5-10 seconds. The samples were put on ice for 20 minutes and vortexed again before the gall bladders were removed. Resuspended bile samples were transferred to new tubes, carefully making sure no parts of the gall bladders followed. The original volume of bile before samples dried in was not known, and it was necessary to standardize the resuspended samples. Concentration of the bile pigment biliverdin was quantified by

Table 2.2 Water quality parameters investigated, the method used and the parameters limit of quantification.

Analyses variable		**Reference method**	**Limit of quantification**	**Unit of measure**
Name	**Abbreviation**			
pH	pH	NS 4720		
Total organic carbon	TOC	NS-ISO 8245	0.1	mg C/L
Total phosphor	Tot P	NS 4724	1	µg P/L
Ammonium	NH_4^+	ISO 3696:1987	5	µg N/L
Nitrate	NO_3^-	NS-EN ISO 10304-1	1	µg N/L
Total nitrogen	Tot N	NS 4743	10	µg N/L
Chloride	Cl	NS-EN ISO 10304-1	0.1	mg/L
Aluminium	Al	EN ISO 17294-2	1	µg/L
Cadmium	Cd	EN ISO 17294-2	0.004	µg/L
Copper, Nickel,	Cu, Ni, Cr	EN ISO 17294-2	0.05	µg/L
Chromium				
Iron	Fe	EN ISO 17294-2	0.3	µg/L
Lanthanum*	La	EN ISO 17294-2	0.001	µg/L
Lead	Pb	EN ISO 17294-2	0.01	µg/L
Antimony	Sb	EN ISO 17294-2	0.02	µg/L
Tungsten*	W	EN ISO 17294-2	0.5	µg/L
Zinc	Zn	EN ISO 17294-2	0.2	µg/L
Naphthalene	Nap,	Internal NIVA method	0.01	µg/L
Acenaphthylene,	Ancle,	(Grimmer and Böhnke		
Acenaphthene,	Acne,	1975)		
Fluorene,	Fle,			
Phenanthrene,	Phe,			
Anthracene,	Ant,			
Fluoranthene,	Flu,			
Pyrene,	Pyr,			
Benzo[a]anthracene,	BaA,			
Chrysene,	Chrtr,			
Benzo[b]fluoranthene,	BbF,			
Benzo[k]fluoranthene,	BkF,			
Benzo[a]pyrene,	BaP,			
Dibenzo[a,h]anthracene	Dah3A			
Indeno[1,2,3-cd]pyrene,	IcdP,		0.002	µg/L
Benzo[g,h,i]perylene	BghiP			

*Method not accredited.

Table 2.3. Variables measured in water in the laboratory study and the field study. Naphthalene was the only PAH detected. - = variable not investigated, u = uncertainty in the result of the analysis performed.

		Laboratory study								Field study	
		Control		Positive control		Granfoss		Nordby*		Up-	Down-
Parameter	Unit	Mean	SD	Mean	SD	Mean	SD	Mean	SD	stream	stream
Al[a]	µg/L	16.1	9.99	16	6.7	4.8	3.36	13.4	10.18	-	-
Cd[a]	µg/L	0.08	0.07	0.05	0.01	0.1	0.01	0.1	0.02	0.03	0.02
Cr[a]	µg/L	-	-	-	-	-	-	-	-	u1.1	u1.1
Cu[a]	µg/L	2.18	0.67	3.66	1.23	9.21	1.57	14.38	1.28	3.53	3.51
Fe[a]	µg/L	15	5.48	15	5.48	32.2	10.74	31.8	12.7	-	-
La[a]	µg/L	0.029	0.005	0.012	0.007	0.015	0.013	0.053	0.035	-	-
Ni[a]	µg/L	u0.15	0.06	u0.19	0.08	6.06	0.12	4.19	0.12	2.78	2.78
Pb[a]	µg/L	0.14	0.05	53.93	15.53	0.13	0.09	0.1	0.07	0.68	0.73
Sb[a]	µg/L	0.19	0.1	0.18	0.09	3.46	0.16	5.08	0.23	-	-
W[a]	µg/L	<0.5	0	<0.5	0	2.3	0.12	16.2	0.45	-	-
Zn[a]	µg/L	4.4	0.34	5.1	0.58	55.9	5.14	192	19.43	5.7	6.6
Naphthalen[a]	µg/L	0.02	-	0.018	-	0.034	-	0.015	-	-	-
Tot P[a]	µg/L	-	-	-	-	-	-	-	-	90	91
Tot N[a]	µg/L	2142.5	1266.6	1999.2	1103.7	3590	1456.1	6174	1215.3	3220	3200
NH_4[a]	µg/L	1985.0	1410.4	1703.3	1166.5	2448.3	1692.5	3172	1440.3	-	-
NO_3[a]	µg/L	236.7	8.2	263.3	5.2	466.7	36.7	1180	44.7	-	-
TOC[a]	mg/L	2.3	0.3	2.3	0.2	6.5	0.4	15.2	0.5	9.2	9.5
Cl[a]	mg/L	480	12.8	477.2	10.8	437.2	6	424.4	12.5	-	-
pH[ab]		7.4[b]	-	7.5[b]	-	8[b]	-	8[b]	-	7.5[a]	7.5[a]
Temperature[dc]	C°	7.7[c]	1	7.5[c]	0.8	8[c]	0.8	8.2[c]	0.9	6.83[d]	6.82[d]
General hardness[e]	°dH	2	0.5	2.6	0.4	6.5	0.6	4.6	0.8	-	-
Salinity[c]	ppm	885.5	164.8	895.2	89.4	914.1	82.4	904.9	127.5	-	-
Conductivity[d]	µs/cm	-	-	-	-	-	-	-	-	148.1	142.5
DO[c]	%	95.2	2.1	95	1.7	95	1.7	94.4	1.9	-	-
DO[d]	mg/L	-	-	-	-	-	-	-	-	11.2	10.2
Turbidity[d]	NTUs	-	-	-	-	-	-	-	-	29.0	28.1

[a] Analyses conducted as described in **table 2.2**. n= 6/5* in group the laboratory study and n = 1 in groups in the field study. [b] Parameters were measured with PHM 92 LAB pH METER (Radiometer Copenhagen); pH values from the laboratory study is the median value. [c] Parameters were measured with Multiparameter Probe (Oakton**).** [d] Parameters were measured with EXO2 Multiparameter Sonde. [e] General hardness was measured using a kit from Tetra*test* kit. [bc] n = average of regular measurements throughout the study period. [de] n = 1.

measuring the absorbance of resuspended samples at 380 nm. As the pigment biliverdin has one of its major peaks at this wavelength (Doumas et al. 1987). Two µL of resuspended samples were added to a Take3 Micro-volume plate (BioTek) in quadruplicates. Resuspended samples were frozen (-20°C) until preparation of samples for HPLC.

2.6.2 Preparation of bile samples

The preparation of bile samples was conducted according to the method described by Krahn et al. (1992) and modified by Grung et al. (2009). Some modifications of the preparation protocol were made due to low volumes of bile in the current study. Gall bladders from fish from the laboratory study were thawed on ice in darkness. Bile was removed by piercing each gall bladder with a capillary tube and samples were transferred to new tubes using a rubber tube connected to a syringe. Samples of re-suspended bile from the field study were transferred to new tubes using a pipette. To each bile sample, 10 µL of internal standard containing triphenylamin (TPA) (16.2 µg/mL TPA in a solvent of 80% methanol and 1% ascorbic acid) was added and weight noted. In addition 40 µL dH_2O and 4 µL glucuronidase aryl sulfatase was added to each sample. The amount of glucuronidase aryl sulfatase was set according to the median weight of the bile of fish from the laboratory study. The samples were mixed well and incubated at 37°C for 1 h in a heating cabinet (Termax). To stop the reaction, 80 µL methanol was added. The samples were centrifuged at 4000 *g* for 10 min and the supernatant was carefully transferred to HPLC vials (Waters). The samples were stored (-20°C) until high performance liquid chromatography (HPLC) was performed by Merete Grung at NIVA.

2.6.3 High performance liquid chromatography (HPLC)

The deconjugated hydroxy PAHs (OH-PAHs) were separated into individual OH-PAHs using high performance liquid chromatography fluorescence (HPLC/f) detection (Ariese et al. 2005). Reversed phase HPLC was performed using a Vydac 201TP5415 (5µm particle size, 4.6 x 250 mm) HPLC C18 column with precolumn and an acetonnithril:water gradient (mobile phase 1 - 40:60% w/w acetonnithril-water, mobile phase 2 – 100% acetonnithril). Fluorescence excitation/emission (ex/em) wavelengths settings was programed to detect the PAH metabolites (Table 2.4). A calibration-step with 5 concentrations of PAH metabolite standards (1-OH-naphthalene, 1-OH-phenanthrene, 1-OH-pyrene and 3-OH-benzo[a]pyrene (BaP) was included before running the samples. In addition a calibration solution was run for

every 10th sample to check for possible instrument drift. The injection volume of each sample was 75 µL and the column temperature was 30°C.

The concentration of the different OH-PAHs was decided from the area of the integrated peaks in the chromatograms relative to the area and known concentration of TPA: concentration of OH-PAH = (area OH-PAH /area TPA) x TPA concentration. Hydroxy naphthalene concentrations were not quantified, as the detection spectrum for HPLC/f is very noisy in the two-ring area. The amount of 1-OH-pyrene, 1-OH-phenanthrene and 3-OH-BaP could be decided precisely, as a standard was included for these metabolites. In addition, three additional peaks were integrated as phenanthrene equivalent metabolites from the chromatograms in the area where thrre-ring PAHs are normally detected. This was investigated for bile samples of fish from the laboratory study. The three additional peaks were separated after 8.9 minutes (′), 9.4′ and one double peak after 10.1/10.2′ (Figure 2.3). Through the rest of the thesis these metabolites will be referred to as 8.9′, 9.4′ and 10.1/10.2′ PAH hydroxyl metabolites (8.9′-OH, 9.4′-OH and 10.1/10.2′-OH metabolites).

For bile samples from fish sampled in the field the TPA standard could not be used. To quantify the amount of the different hydroxy PAHs in these samples, individual standard curves for 1-OH-pyrene and 1-OH-phenanthrene were constructed from the calibration samples, which included 5 different concentrations of each hydroxy standard. Due to the re-suspension of bile samples from the field study and the differences in calculation of metabolite concentrations a comparison of PAH metabolite concentrations between the laboratory and field study samples were not possible. PAH-metabolites are expressed as ng/g bile and ng/resuspended bile/abs380 in the laboratory and field study, respectively.

Table 2.4. Time periods (in minutes), specific excitation/emission (ex/em) wavelengths used and the PAH-metabolite standards detected.

Minutes (′)	ex/em (nm)	Standards (time detected)
0 – 8	325/385	1-OH-napthalene (ca. 5.9′)
8 – 11.5	251/364	1-OH-phenenthrene(ca. 9.8′)
11.5 – 14	246/384	1-OH-pyrene (ca. 12.2′)
14 – 21.5	300/360	Triphenylamin (ca. 18.8′)
21.5 - 30	282/375	3-OH-benzo(a)pyrene (ca. 23.4′)

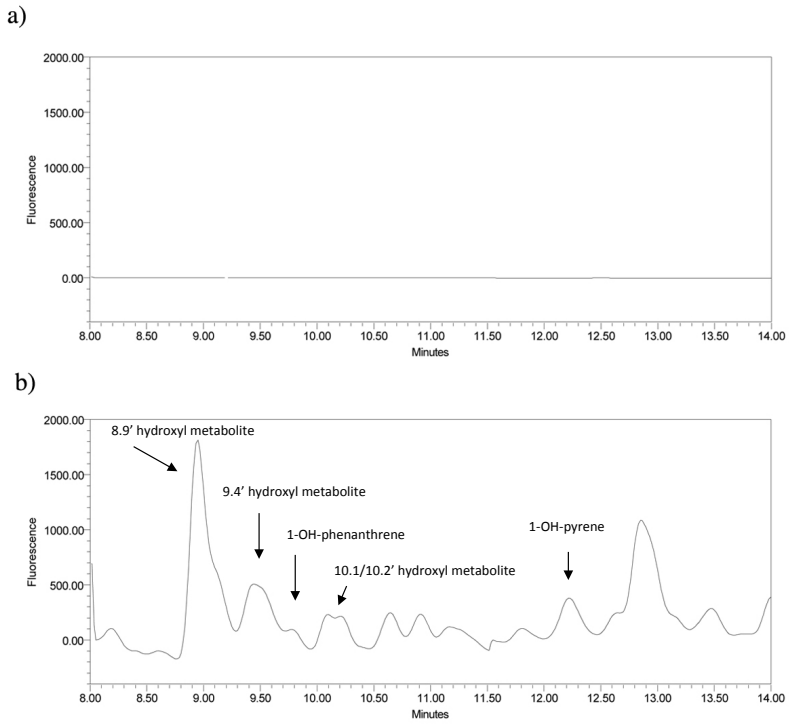

Figure 2.3 Chromatogram section of metabolites separated in the three-ring area. a) Chromatogram of a blank sample and b) chromatogram of a sample from the Nordby treatment.

2.7 EROD activity in gills

Gills were assayed for 7-ethoxyresorufin *O*-deethylase (EROD) activity as described by Jönsson et al. (2002). The assay was performed on the day fish was dissected on gill arches that were stored in ice-cold HEPES-Cortland (HC) buffer (0.38 g KCl, 7.74 g NaCl, 0.23 g $MgSO_4 \cdot 7H_2O$, 0.17 g $CaCl_2$, 0.33 g $H_2NaPO_4 \cdot H_2O$, 1.43 g HEPES and 1 g Glucose in dH_2O to a total volume of 1 L, adjusted to pH 7.7). Gill filaments were cut from approximately half of one arch to be used in the assay. These were placed in 12 or 24-well plates containing 500 µL room tempered reaction buffer (HC-buffer containing 1 µM 7-ethoxyresorufin and 10 µM dicumarol) for pre-incubation. The reaction buffer was replaced with 700 µL fresh reaction buffer and gill filaments were incubated for 30-80 minutes before triplicates of 200 µL reaction buffer for each sample was transferred to a black Nunc™ 96-well plate. A standard series of seven concentrations of resorufin (1.56 - 200 nM resorufin) was included on each plate. The standard series was made from a stock solution of resorufin (1 mM resorufin in DMSO) diluted in reaction buffer. Fluorescence was measured with a plate reader (SynergyNX BioTek) at 530/590 nm ex/em. The remaining gill tissue was stored in the freezer (-20°C) in the 12/24 well plates for quantification of gill tissue at a later point.

A picture was taken of each well containing gill filaments and assessment of the amount of gill tissue was performed with a picture analysis. A digital ruler tool was constructed in Adobe Photoshop CS5 and the amount of gill tissue was assessed in mm^2. Prior to this pictures were randomized and a code was constructed by drawing random numbers (Rstudio). EROD activity was expressed as pmol resorufin/min/gill tissue.

2.8 CYP1A protein and EROD activity in liver

2.8.1 Isolation of hepatic microsomes

Microsomes were isolated from liver tissue as described by Dignam (1990). The liver samples were kept on ice at all times during the procedure to minimize loss of enzymatic activity. Livers were thawed on ice and transferred to tubes containing approximately 50 ceramic beads (Precellys 24 Soft Tissue homogenizing 1.4 mm ceramic beads, Bertin Technologies). Each tube was filled with homogenisation buffer (potassium-phosphate buffer pH 7.8 containing 0.15 M KCl, 0.1mM dithritiol and 5% w/w Glycerol) to a total volume of 750 µL. Livers were homogenised using Precellys® 24 (Bertin Technologies) at 6000 rpm, 3x 10 seconds with 5 seconds break in between. Temperature was maintained at 4°C (Cryolys, Bertin Technologies). The homogenates were transferred to new Eppendorf tubes using Pasteur pipettes and centrifuged for 30 min on 10 000 *g* at 4°C (Heraeus Multifuge 3 S-R, Kendo Laboratory Products). The supernatants were carefully transferred to a centrifugation tube, and centrifuged at 100 000 *g* for 60 minutes at 4°C (Sorvall MTX150 Micro-Ultracentrifuge, Thermo Fischer Scientific). The supernatants, which now consisted of cytosol, were removed. Two hundred µL microsomal buffer (Potassium-phosphate buffer pH 7.8 containing 0.15 M KCL and 20% w/w glycerol) was added to the remaining microsomal pellets. The pellets and the buffer were transferred to Eppendorf tubes and the pellets were homogenised for minimum 3x 5 seconds on ice using the motorised pistil for Eppendorf tubes (VWR). The homogenates were aliquoted as follows: 10 µL to protein analyses, 20 µL to ELISA and the rest to EROD. The samples were immediately frozen and stored at -80°C.

2.8.2 Protein analyses

Protein analyses were performed as described by Lowry et al. (1951). Samples were thawed on ice and diluted 5 - 30 times in Tris buffer solution (0.1 M, pH 8). A two-step dilution series with 4 concentrations of protein (Bovine gamma globulin, Sigma) diluted in Tris buffer was prepared (0.2 - 1.6 mg/mL). Ten µL of blank (Tris-buffer), standard dilution series,

diluted samples and reference sample were added in triplicates to a 96-well plate. 25 µL of Reagens A (BioRad) and 200 µL of Reagens B (BioRad) were added to each well using a multi-pipette. The plate was incubated for 15 minutes at room temperature before the absorbance was read with SynergyNX BioTek plate reader at 750 nm.

2.8.3 EROD activity

CYP1A activity was measured using the 7-Ethoxyresorufin *O*-deethylase (EROD) activity assay as described by Burke and Mayer (1974) and modified by Eggens and Galgani (1992). All work was conducted under subdued lighting. Samples of the hepatic microsomal fraction were thawed on ice and diluted in phosphate buffer (0.1 M potassium-phosphate buffer, pH 8.0) to concentrations between 1 - 1.5 mg/mL protein. The remaining steps were conducted at room temperature. A reaction buffer was prepared from a stock solution of 7-ethoxsyresorufin (2.59 mM 7-ethoxsyresorufin in DMSO) and phosphate buffer. The reaction buffer had a final concentration of 3 µM 7-ethoxsyresorufin. A standard dilution series was prepared from a stock solution of resorufin sodium salt (1 mM resorufin sodium salt in DMSO) and reaction buffer. The dilution series had 7 concentrations ranging from 0.01 to 7.29 µM resorufin. Two hundred and seventy-five µL resorufin dilution series (including blank - reaction buffer only) were added in duplicates, 50 µL of diluted samples and reference sample were added in six replicates and 50 µL phosphate buffer (blank) were added in eigth replicates to a black Nunc^{TM} 96-well plate. To correct for quenching, 10 µL of 0.32 µM resorufin-standard was added to three of the six replicates of samples and reference samples. Two-hundred µL reaction buffer was added to each well with blank, sample and reference sample using a multi-pipette. Twenty-five µL of 2.4 mM NADPH-solution (120 µL 50 mM NADPH stock solution in 2.380 mL phosphate buffer) was added to all wells with blank (phosphate buffer), samples and reference sample. The plates were immediately read with SynergyNX BioTek plate reader in 8 steps with 39 seconds intervals at 530/590 nm excitation/emission and with shaking between each reading. EROD activity was expressed as pmol resorufin/min/mg protein.

2.8.4 CYP1A protein (ELISA)

Relative levels of CYP1A protein was quantified through an indirect enzyme-linked immunosorbent assay (ELISA) for CYP1A as described by Goksøyr (1991) with some modifications. Samples of hepatic microsomal fraction were thawed on ice. Samples were

diluted to a concentration of 10 µg/mL protein in coating buffer (1 carbonate-bicarbonate capsule (Sigma) in 100 mL of dH_2O). Hundred µL diluted samples were added in quadruplicates, and 100 µL of blank (coating buffer only) were added in eight replicates of to a Nunc^{TM} immunosorb 96-well plate. To each plate run a reference sample was added quadruplicates. The plates were covered with tape and incubated over night (4°C).

Plates were washed 3 times (2 x 30 sec + 90 sec) with TTBS (0.2 M Tris buffer at pH 8.5 containing 0.5 M NaCl and 0.05% Tween20) using an automatic plate washer (ScanWasher300). Three hundred µL blocking solution (1% w/w bovine serum albumin (BSA) in TTBS) was added to each well using a multi-pipette. The plates were incubated at room temperature for 60 minutes and washed 3 times with TTBS. To four of eigth replicate wells containing the blank (coating buffer) 100 µL of antibody buffer (0.1 % w/w BSA in TTBS) was added. Hundred µL of primary antibody solution (rabbit anti-fish CYP1A primary antibody (Biosense Laboratories) diluted 1000x in anti-body buffer) was added to the four remaining wells containing the blank (coating buffer) and to each well containing samples and reference sample. The plates were covered with tape and incubated overnight (4°C).

Plates were washed 3 times with TTBS and 100 µL secondary antibody solution (goat anti-rabbit IgP conjugated with HRP (Sigma) diluted 3000x in antibody-buffer) was added to all wells. Plates were covered with tape and incubated for 6-7 hours (4°C). The plates were washed 5 times (4x 30 sec + 90 sec) with TTBS. Room-tempered colour-development buffer (TMB Plus, Kem-EN-Tech) was added to all wells. Plates were incubated for 12-15 minutes for colour to develop, before 50 µL of stop solution (1.5 M SO_4H_2) was added to each well. The absorbance was read with SynergyNX BioTek plate reader at 450 nm. Samples were standardized against the reference sample between plates, and CYP1A protein was expressed as relative CYP1A quantity (abs450).

2.9 δ-aminolevulinic acid dehydratase (ALA-D) activity in red blood cells

2.9.1 ALA-D assay

The δ-aminolevulinic acid dehydratase (ALA-D) activity assay was performed as described by Hodson (1976) and modified by ICES (2004). The protocol was further modified by

Nakagawa et al. (1995) and Erdahl (2014) to do not include the environmental contaminant mercuric chloride. Blood samples were thawed on ice and diluted to a total volume of 500 µL with dilution buffer (0.1 M Sodium phosphate buffer, pH 7 with 0.5% v/v Triton-X). Samples were homogenised using a hand held pistil homogenizer for Eppendorf (VWR). Samples were centrifuged at 10 000 *g* at 4°C for 15 minutes (Heraeus Multifuge 3 S-R, Kendo Laboratory Products). The supernatant was transferred to a new Eppendorf tube using a pasteur pipette and mixed with a Whirlmixer. Fifty µL of each sample was added in six replicates to new Eppendorf tubes. The rest of the sample was stored at -20°C to be analysed for protein content. To three of the replicate tubes, 200 µL ALA-reagent (5.51 mM δ-aminolevulinic acid in dilution buffer thawed from -80°C freezer daily) was added, while 200 µL of dilution buffer (blank) was added to the remaining three tubes. Up to this point, samples had been kept on ice. Samples were mixed with a Whirlmixer and incubated at 25°C for 120 minutes in a heating cabinet (Termax). To end the reaction, 300 µL precipitation buffer (10% w/v trichloracetic acid in ddH_2O) was added to each tube. After five minutes on the bench samples were centrifuged at 2500x *g* for five minutes. A standard solution of porphobilinogen (PBG) of 40 µg/mL was prepared fresh daily from a stock solution (1 mg/mL PBG). A dilution series of 7 concentrations of PBG (1 - 40 µg/mL) was prepared from the standard solution, and added in duplicates of 150 µL to a 96-well plate (Nunct^{TM}). Hundred and fifty µL of the three replicates of sample and ALA as well as sample and blank was added in duplicates to a 96-well plate. A reference sample treated the same way as the other samples was also included on each plate. In the end 150 µL of Ehrlichs reagent (6 mL ddH_2O, 20 mL perchloric acid, 86 mL 99,7% acetic acid and 2 g ρ-dimethylaminobenzaldehyde - made fresh daily) was added to each well with a multi-pipett. The plates were mixed gently by hand and left on the bench to incubate for 15 minutes before absorbance was read with SynergyNX BioTek plate reader at 550 nm. ALA-D activity was expressed as ng PGB/min/mg protein.

2.9.2 Protein analysis

The blood samples had coagulated and was homogenised as good as possible before 50 µL of homogenate were transferred to a new tube. Two hundred µL of 1 M NaOH was added to each tube and samples were mixed and left to incubate for 30 minutes at 60°C in a heat block (AccuClock, Labnet). To neutralise, 300 µL of 1.67 M HCl was added to each tube, which

resulted in an 11x dilution. Samples were either not diluted or diluted further 2-4 times before protein analysis was performed as described in section 2.8.2.

2.10 Statistical analyses

The statistical program R Studio, version 0.98.1091, was used for all the statistical analysis (RCoreTeam 2015). Packages used include: "car" (Fox and Weisberg 2011), "dunns.test" (Dinno 2015) and "vegan" (Oksanen et al. 2015). The significance level α was set to $p = 0.05$ for all tests conducted.

Before tests were run homogeneity of variance was investigated using Levene's test (Levene 1960). When the assumption of equal variance was fulfilled either on non-transformed or log10-transformed data, differences between group means were investigated with a Students two-sample *t*-test or a one-way ANOVA, depending on the number of group means compared. If a significant *p*-value was detected in the ANOVA, a Tukey's honestly significant difference (Tukey's HSD) post-hoc test was applied (Tukey 1949). If the argument of equal variance was still not fulfilled after log10-transformation, a non-parametric Wilcoxon rank-sum test (Wilcoxon 1945) or Kruskal-Wallis one-way analysis of variance (Kruskal and Wallis 1952) was performed. If a significant *p*-value was detected in the Kruskal Wallis test, a Dunns post hoc test with Bonferroni correction was applied. Groups in the field study was in addition tested for normality using Shapiro Wilks test (Shapiro and Wilk 1965), before parametric tests were run. When parametric tests were applied on data from the laboratory study, it was assumed a normal distribution in each group, as group sizes (n=3-5) were considered to small to make a reliable evaluation of normal distribution. In figures, different letters were used to depict significant differences when comparing more than two groups (Tukey's HSD/Kruskal Wallis test), while asterisk were used to depict significant differences when two groups were compared (Students-*t*/Wilcoxon).

In data from the laboratory study, several tests were performed on the same biomarker (one one-way ANOVA for each sampling day (three total) and one t-test/one-way ANOVA for each treatment over time (four total)). Running many tests, including using the same group in two individual tests (e.g. control treatment day 0 used both when comparing differences between groups at day 0, and when investigating effects on the control treatment as a

response of time), can result in a larger probability of making a type I error than the significance level α. Conducting several tests was chosen over conducting one two-way ANOVA as this was considered the best to answer the research questions investigated, and as conducting a two-way ANOVA increases the risk of type II error by including non-relevant comparisons. To compensate for the increased risk of type I errors a Bonferroni correction of the α-level is possible. This was considered but not applied, as lowering the significance level also reduces the statistical power, increasing the probability of making a type II error in each individual test, and because it does not seem to be a consensus of when such a correction should be applied (Cabin and Mitchell 2000). Due to these choices, the number of significant *p*-values reported may be considered liberal rather than conservative.

A principal component analysis (PCA) was conducted to observe the overall trend in the dataset and to investigate correlations between biomarkers. Points that are located close together are similar in regard to the variation explained by the two axes plotted. The response of the variables increases in the direction of the arrows, and as the length of the arrows increases the more of the existing variation in the variable is described by the plot. In addition, arrows that are correlated (a small angle between them) show a similar pattern between observations, while arrows placed in a 90 degrees angle share none of the same variation. Before running the PCA, the variables included in the multivariate analysis were normalised by autoscaling to a corresponding Z score. Z is a dimensionless parameter and after conversion each variable will have a $\mu=0$ and $s=1$ in addition to preserving the original shape of the data (Shaw 2003). PCA is sensitive to missing values, resulting in removal of observations (fish in the current study) from the dataset when the response of at least one biomarker was missing.

3 Results

3.1 PAH metabolites in bile

The biliary concentration of 8.9′-OH metabolite was higher in fish from the Granfoss (G) and the Nordby (N) treatments compared to fish from the control (C) and the positive control (P) treatments, as well as in fish from the Nordby treatment compared to fish from the Granfoss treatment day 5 of exposure (ANOVA: G-C $p = 0.02$, G-P $p = 0.02$, N-C $p < 0.0001$, N-P $p < 0.0001$ and N-G $p = 0.003$; Figure 3.1 a). The same was observed in fish sampled at day 25 of exposure (ANOVA on log10-transformed data: G-C $p = 0.02$, G-P $p = 0.03$, N-C $p = 0.0002$, N-P $p = 0.0004$ and N-G $p = 0.05$; Figure 3.1 a). In addition, the biliary concentration of 8.9′-OH metabolite increased as a response of time in fish from the Nordby treatment, with higher biliary concentration in fish sampled at day 5 (D5) and day 25 (D25) compared to fish sampled at day 0 (D0) (Kruskal Wallis: D5-D0 $p = 0.02$ and D25-D0 $p = 0.01$, Figure 3.1 a). The biliary concentration of 9.4′-OH metabolite was higher in fish sampled from the Nordby treatment compared to fish sampled from the control and the positive control treatments at day 5 of exposure (ANOVA: N-C $p = 0.02$ and N-P $p = 0.01$, Figure 3.1 b). In addition, the biliary concentration of 9.4′-OH metabolite increased as a response of time in fish from the Nordby treatment, with higher biliary concentration in fish sampled at day 5 compared to fish sampled at day 0 (ANOVA: D5-D0 $p = 0.02$, Figure 3.1 b). The biliary concentration of 1-OH-phenanthrene was higher in fish from the Nordby treatment compared to fish from the positive control treatment at day 5 of exposure (ANOVA: N-P $p = 0.05$, Figure 3.1 c). The biliary concentration of 10.1/10.2′-OH metabolite was higher in fish from the Nordby treatment compared to fish from the control and the positive control treatments at day 5 of exposure (ANOVA: N-C $p = 0.01$ and N-P $p = 0.007$, Figure 3.1 d). At day 25 of exposure, the biliary concentration of this metabolite was higher in fish sampled from the Granfoss and the Nordby treatments compared to fish sampled from the control and the positive control treatments (ANOVA on log10-transformed data: G-C $p = 0.003$, G-P $p = 0.002$, N-C $p < 0.0001$ and N-P $p < 0.0001$; Figure 3.1 d). In addition, the biliary concentration of 10.1/10.2′-OH metabolite increased as a response of time in fish sampled from the Nordby treatment, with higher concentration in fish sampled at day 5 and day 25 compared to fish sampled at day 0 (ANOVA: D5-D0 and D25-D0 $p < 0.0001$, Figure 3.1 d).

No differences were observed in biliary concentrations of 1-OH-pyrene (Figure 3.1 e). Fish sampled from the positive control treatment at day 25 had a higher biliary concentration of 3-

OH-benzo[a]pyrene (BaP) compared to fish sampled from the control treatment and the Granfoss treatment (ANOVA on log10-transformed data: P-C $p = 0.004$ and P-G $p < 0.02$, Figure 3.1 f). Further there was an increase in biliary 3-OH-BaP in the positive control treatment as a response of time, with higher biliary concentration in fish sampled at day 25 compared to fish sampled at day 0 and day 5 (ANOVA: D25-D0 $p = 0.009$ and D25-D5 $p = 0.02$, Figure 3.1 f).

For fish sampled in Årungenelva a difference was observed in the biliary concentration of 1-OH-phenanthrene between sampling locations, with lower biliary concentration in fish sampled at the downstream location compared to fish sampled at the upstream location (*t*-test: $p = 0.001$, Figure 3.2 a). The same was observed for the biliary concentration of 1-OH-pyrene in fish sampled in Årungenelva (*t*-test: $p = 0.05$, Figure 3.2 b). 3-OH-BaP was not detected in bile samples in fish from Årungenelva.

3.2 EROD activity in gill filaments

The EROD activity in gills was higher in fish sampled from the Nordby and the Granfoss treatments compared to fish sampled from the control and the positive control treatments at day 5 (ANOVA: G-C, G-P, N-C and N-P $p < 0.0001$; Figure 3.3 a). The same pattern was observed in fish sampled at day 25 of exposure (ANOVA: G-C, G-P, N-C and N-P $p < 0.0001$; Figure 3.3 a). EROD activity was increased as a response of time in all four treatments. Fish sampled from the Granfoss treatment had higher activity in fish sampled at day 5 compared to fish sampled at day 0 (ANOVA: D5-D0 $p = 0.001$, Figure 3.3 a). Fish sampled from the Nordby treatment had higher EROD activity in fish sampled at day 5 and 25 compared to fish sampled at day 0 (ANOVA: D5-D0 $p = 0.001$ and D25-D0 $p = 0.05$, Figure 3.3 a). Fish sampled from the control treatment had higher EROD activity in fish sampled at day 5 compared to fish sampled at day 0 and 25 (ANOVA: D5-D0 $p = 0.0007$ and D5-D25 $p < 0.0001$, Figure 3.3 a). This was also observed for fish sampled from the positive control treatment (ANOVA on log10-transformed data: D5-D0 $p = 0.01$ and D5-D25 $p = 0.005$; Figure 3.3 a). No difference in EROD activity in gills was observed between fish sampled at the two locations in Årungenelva.

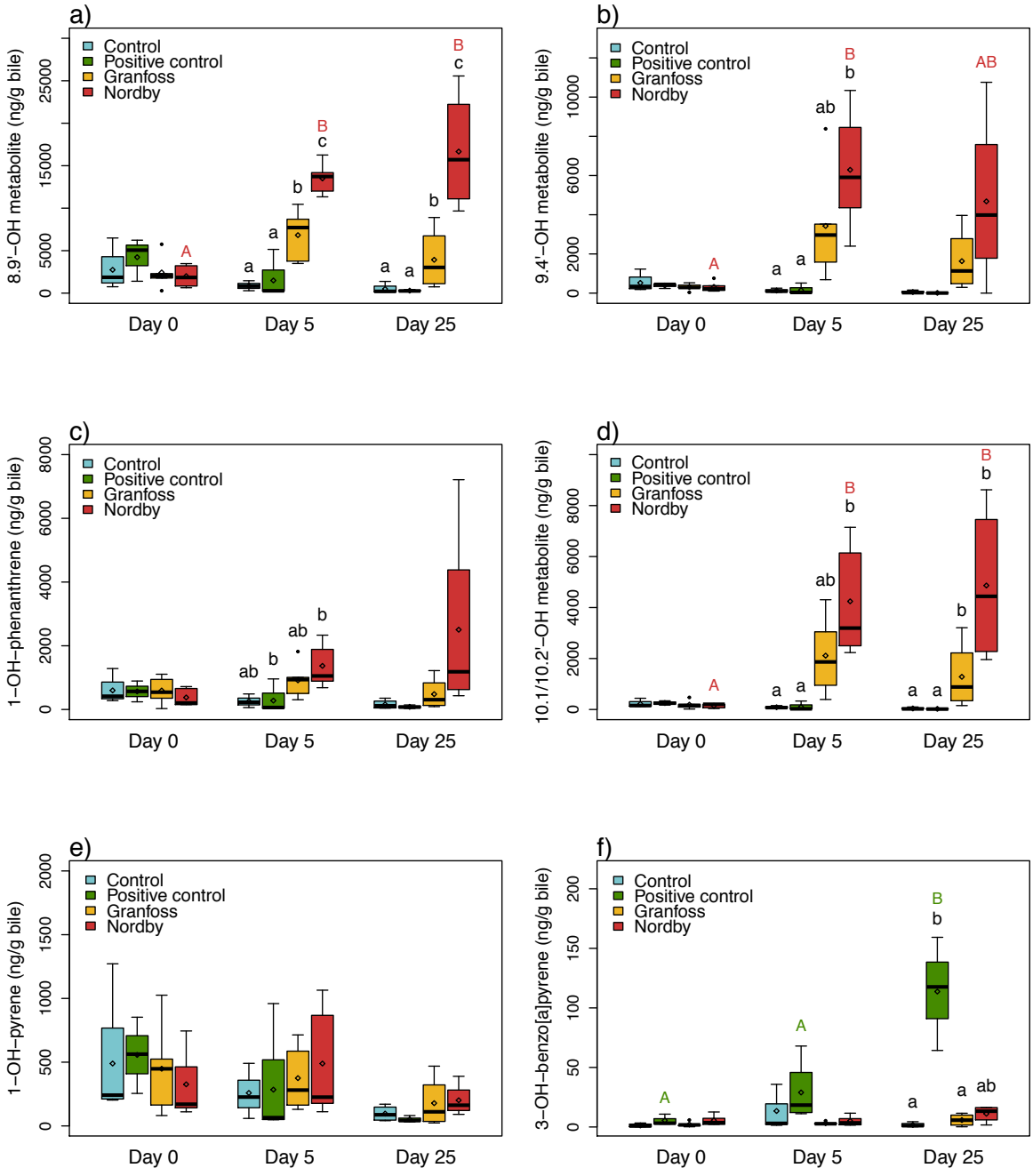

Figure 3.1 PAH metabolites measured in bile of trout from the laboratory study. a) 8.9′-OH, b) 9.4′-OH, c) 1-OH-phenanthrene, d) 10.1/10.2′-OH, e) 1-OH-pyrene and f) 3-OH-BaP. Different lowercase letters indicates significant differences between treatments within each sampling day. Different uppercase case letters indicate significant differences within a treatment during the sampling period. Diamond (♢) gives the position of the mean value in each group. The line represents the median value in each group, and the box is defined by the 1st and 3rd quartiles (area where 25 % of the observations below and above the median are found). Whiskers extend to the most extreme data point that is no more than 1.5 times the interquartile range. Observations that exceed or fall below the range of the whiskers are shown as filled dots. n = 3-5 in each group.

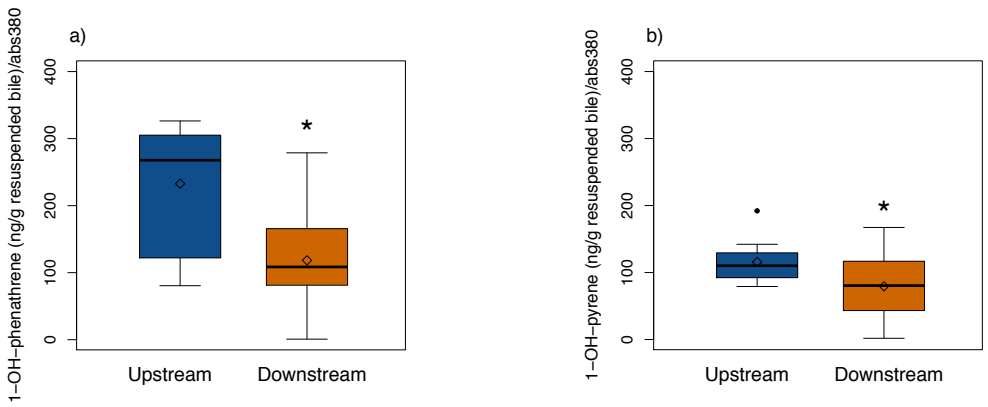

Figure 3.2 PAH metabolites measured in bile of trout from the stream Årungenelva. a) 1-OH-phenanthrene and b) 1-OH-pyrene. Asterisk (*) indicates a significant difference between groups. Diamond (♢) gives the position of the mean value in each group. The line represents the median value in each group, and the box is defined by the 1st and 3rd quartiles (area where 25 % of the observations below and above the median are found). Whiskers extend to the most extreme data point that is no more than 1.5 times the interquartile range. Observations that exceed or fall below the range of the whiskers are shown as filled dots. n = 10 upstream and 20 downstream.

3.3 CYP1A protein in liver

CYP1A protein was higher in fish sampled from the Granfoss and Nordby treatments compared to fish sampled from the control and positive control treatments, as well as in fish sampled from the Nordby treatment compared to fish sampled from the Granfoss treatment at day 25 of exposure (ANOVA: G-C, G-P, N-C, N-P and G-N $p < 0.0001$; Figure 3.3 b). Further, CYP1A protein was increased as a response of time in fish sampled from the Granfoss treatment, with higher CYP1A protein in fish sampled at day 25 compared to fish sampled at day 0 (*t*-test: $p < 0.0001$, Figure 3.3 b). The same was observed in fish sampled from the Nordby treatment (*t*-test: $p < 0.0001$, Figure 3.3 b) and in fish sampled from the control treatment (*t*-test: $p = 0.03$, Figure 3.3 b). No difference was observed in CYP1A protein between fish sampled downstream and upstream of the sedimentation pond in Årungenelva.

3.4 EROD activity in liver

EROD activity in liver was higher in fish sampled from the Nordby treatment compared to fish sampled from the control and the positive control treatment at day 25 of exposure (ANOVA: N-C and N-P $p = 0.008$, Figure 3.3 c). The EROD activity in the Granfoss treatment increased as a response of time, with higher concentrations at day 25 compared to day 0 of exposure (*t*-test: $p = 0.02$, Figure 3.3 c). The same was observed in fish sampled from the Nordby treatment (*t*-test: $p = 0.02$, Figure 3.3 c) and in fish sampled from the positive control treatment (*t*-test: $p = 0.01$, Figure 3.3 c). EROD activity in liver of fish

sampled in Årungenelva was higher at the downstream location compared to the upstream location (*t*-test: $p = 0.01$, Figure 3.3 c).

3.5 ALA-D activity in red blood cells

The ALA-D activity was lower in fish sampled from the positive control treatment compared to fish from the control, the Granfoss and the Nordby treatments sampled at day 25 (ANOVA: P-C, P-G and P-N $p < 0.0001$; Figure 3.3 d). The ALA-D activity was reduced in the positive control treatment as a response of time, with lower activity at day 25 compared to day 0 (*t*-test: $p = 0.002$, Figure 3.3 d). No difference was observed in ALA-D activity between fish sampled at the two locations in the Årungenelva (Figure 3.3 d).

3.6 Correlation between markers

A principal component analysis (PCA) was applied on data from the laboratory study, including fish sampled at day 0 and 25 (Figure 3.4 a). The first two principal component axes explained 67% of the variation in the data (47% and 20% for PC1 and PC2, respectively). Fish from the Granfoss and Nordby treatments sampled at day 25 clustered in two separate groups to the left in the plot. The EROD activity in gills and liver and the CYP1A protein in liver as well as 1-OH-phenanthrene and the phenanthrene equivalent metabolites (8.9′-OH, 9.4′-OH and 10.1/10.2′-OH metabolites) were negatively correlated with axis 1. This indicates high activity and concentration of these markers in fish exposed to tunnel wash water sampled at day 25. The PCA indicates that fish from the Nordby treatment had higher response in these markers than fish fro the Granfoss treatment. All fish sampled at day 0, as well as fish from the control and positive control treatments sampled at day 25 were located to the right along PC1, with low responses in the aforementioned markers. The ALA-D and 1-OH-pyrene markers were positively correlated, while 3-OH-BaP was negatively correlated with PC2. There was generally a large variation between fish clustering along PC2. Fish from the positive control treatment were separated from the other fish along this axis, and had high biliary concentration of 3-OH-BaP metabolite and low activity of ALA-D as well as low biliary concentration of 1-OH-pyrene.

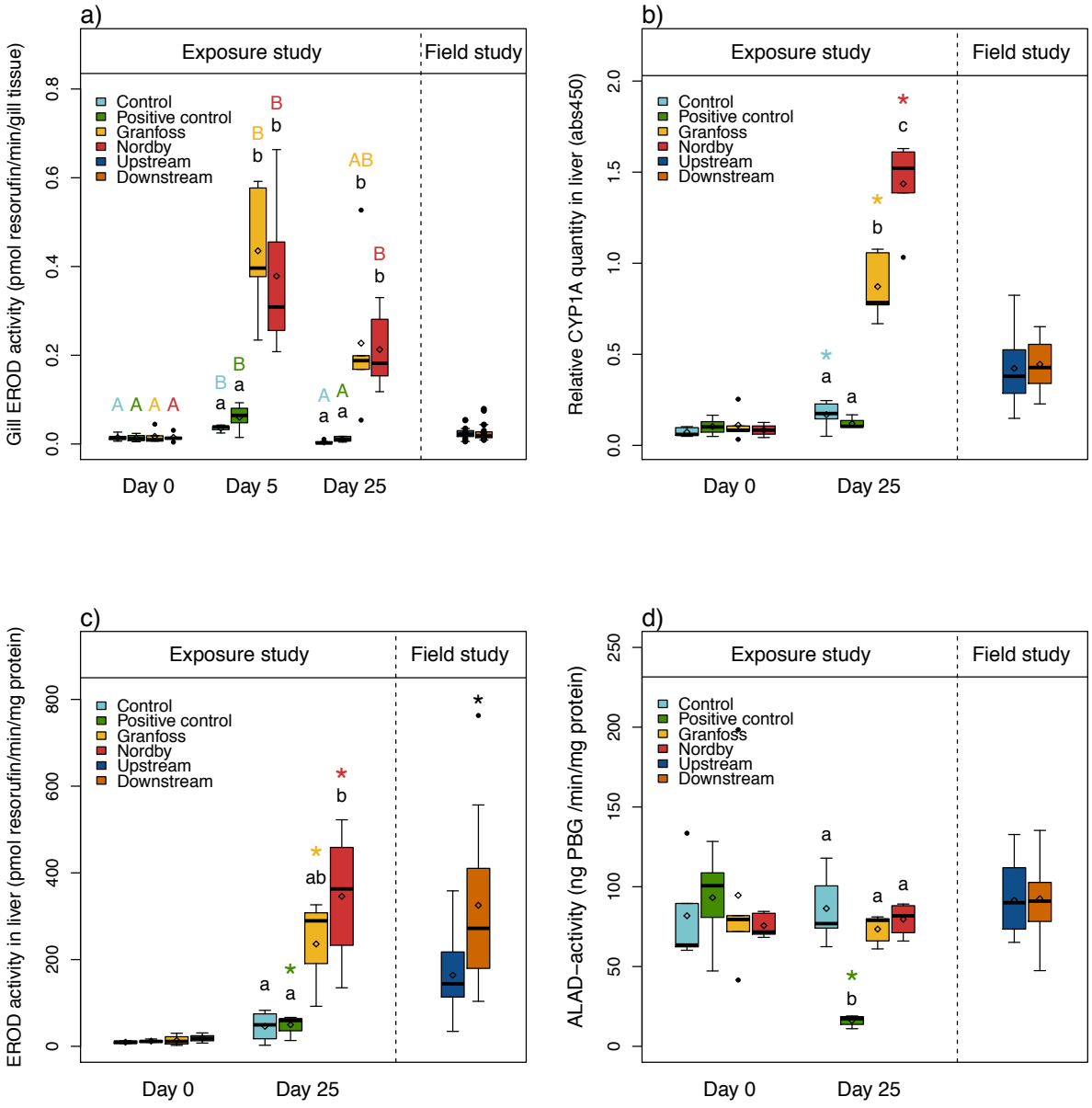

Figure 3.3 Responses investigated biomarkers. a) EROD activity in gill filaments, b) Relative CYP1A quantity in liver, c) EROD activity in liver and d) ALA-D activity in red blood cells. Different lowercase letters indicates significant differences between treatments within each sampling day. Different uppercase case letters indicate significant differences within a treatment during the sampling period (EROD in gills). Asterisk (*) indicates a significant difference when two groups where compared; between day 0 and day 25 in the exposure study (CYP1A protein, EROD in liver, ALA-D) and between the sampling locations in the field study. Diamond (♢) gives the position of the mean value in each group. The line represents the median value in each group, and the box is defined by the 1st and 3rd quartiles (area where 25 % of the observations below and above the median are found). Whiskers extend to the most extreme data point that is no more than 1.5 times the interquartile range. Observations that exceed or fall below the range of the whiskers are shown as filled dots. n = 3-5 in each group in the laboratory study, 11 upstream and 20 and downstream.

The first two principal component axes explained 58% of the variation in the data (37% and 21% for axis 1 and 2, respectively), when a PCA was applied for biomarker responses investigated in fish from Årungenelva (Figure 3.4 b). A separation between fish sampled at the two locations was observed along the first principal component axis. A large variation between fish was observed along this axis, but in general fish from the downstream location was placed to the left in the plot and fish at the upstream location to the right. The markers that contributed to this separation was the PAH metabolites (positively correlated with PC1) and the EROD activity in liver (negatively correlated with PC1). Fish from the downstream location had high EROD activity and low concentration of the PAH metabolites in bile, while the opposite was observed for fish from the upstream location. CYP1A protein in liver was also partly showed to contribute to the separation between the groups, with higher response in fish sampled at the downstream location. No separation of fish according to sampling location was observed along PC2, and the loadings representing the markers of EROD activity in gills and ALA-D does not seem to contribute to the clustering observed in the biplot.

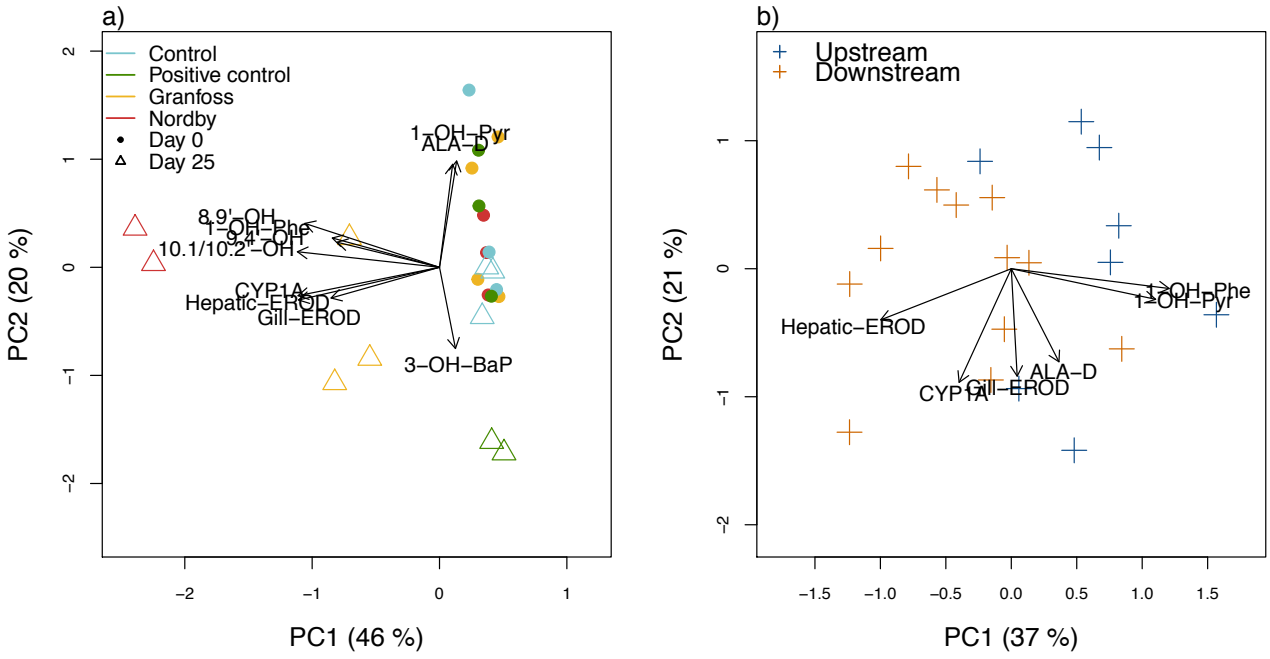

Figure 3.4. Principal component analysis (PCA) of biomarkers investigated in brown trout in a) the laboratory study and b) the field study. Each point represents the ordination scores for one individual fish and loadings (arrows) representing the variables (biomarkers) included in the ordination. Arrows represent the biomarker investigated (the abbreviations used in the figure are showed in the parenthesis): 8.9′-OH metabolite (8.9′-OH), 9.4′-OH metabolite (9.4′-OH), 1-OH-phenanthrene (figure a) and 1-OH-phenanthrene/abs380 (figure b) (1-OH-Phe), 10.1/10.2′-OH metabolite (10.1/10.2′-OH), 1-OH-pyrene (figure a) and 1-OH-pyrene/abs380 (1-OH-Pyr), 3-OH-benzo[a]pyrene (3-OH-BaP), EROD activity in gills (Gill-EROD), CYP1A protein in liver (CYP1A), EROD activity in liver (hepatic-EROD), ALA-D activity in red blood cells (ALA-D).

4 Discussion

The main aim of this study was to investigate sub-lethal effects caused by exposure tunnel wash water in juvenile brown trout (*Salmo trutta*). This was investigated through a controlled laboratory study and by sampling fish in a stream influenced by discharge water from a sedimentation pond.

4.1 PAH metabolites

In the laboratory study, one of the main effects observed on tunnel wash water exposed trout was higher concentrations of 1-OH-phenanthrene and the three-ring PAH metabolites quantified as phenanthrene equivalents (8.9′-OH, 9.4′-OH and 10.1/10.2′-OH metabolites). This was observed compared to the concentrations in fish sampled from one or both of the control treatments at least one of the sampling days in the laboratory study. PAHs are reported to be a major part of road pollution (Meland 2010) and most PAHs accumulate in bile in a dose-dependent manner (Grung et al. 2009), thus the results indicates that higher concentration of bioavailable three-ring PAH parent compounds were present in tunnel wash water compared to water in the two control treatments.

The phenanthrene equivalent metabolites cannot be accurately identified as the only three-ring PAH standard generally available is 1-OH-phenanthrene. The phenanthrene equivalent metabolites identified could be different phenanthrene metabolites (e.g. different phenols or dihydrodiols). The main metabolite of phenanthrene in crustaceans, elasmobranchs and mammals has been found to be 9,10-dihydro-9,10-dihydroxy (9,10-DHD) phenanthrene (Chaturapit and Holder 1978, Nordqvist et al. 1981, Solbakken and Palmork 1981), while 1,2-dihydro-1,2-dihydroxy (1,2-DHD) phenanthrene has been found to be the most prevalent metabolite in various teleost fish species (rainbow trout, flounder, cod and coalfish) exposed to phenanthrene *in vivo* (Solbakken et al. 1980, Solbakken and Palmork 1981, Goksøyr et al. 1986). Phenanthrene does not appear to induce CYP1A to any extent (Billiard et al. 2004). However, in hepatocytes exposed to phenanthrene *in vitro,* contrasting concentrations of different phenanthrene metabolites have found between fish that were pre-exposed to CYP1A inducers *in vivo* and fish that were not pre-exposed to such inducers (Goksøyr et al. 1986, Goksøyr et al. 1987, Pangrekar et al. 2003). A shift from 9,10-DHD phenanthrene in un-exposed fish towards 1,2-DHD phenanthrene in inducer-exposed fish was observed in rainbow trout and to some extent in cod (*Gadus morhua*) by Goksøyr et al. (1986,1987). This

pattern is however not consistent between studies and species, as a shift from dihydrodiols (e.g. 1,2-DHD and 9,10-DHD) to phenol metabolites has been observed in brown bullhead (*Ameriurus nebulosus*) (Pangrekar et al. 2003). These findings indicate that the prevalence of different metabolites can change in the presence and absence of CYP1A inducers. This is relevant when comparing the concentration of phenanthrene metabolites in bile between fish that are exposed in varying degree to CYP1A inducers. These studies may further explain why a more apparent increase was observed in the phenanthrene equivalent metabolites than the increase observed for 1-OH-phenanthrene (see appendix D), and suggests that more than one metabolite of phenanthrene should be quantified when investigating PAH metabolites in fish exposed to complex mixtures, such as road pollution. The unidentified metabolites could also belong to other three-ring PAHs. The HPLC separates metabolites according to their water solubility and anthracene is a three-ring PAH with similar K_{ow} as phenanthrene (Table 4.1). This PAH have been found to be metabolised by fish (Roubal et al. 1977) and the unidentified metabolites could thus also be metabolites of anthracene.

In studies investigating total concentrations of phenanthrene, anthracene, pyrene and benzo[a]pyrene in water influenced by road pollution, pyrene have been found to be more prevalent than the others, followed by phenanthrene or BaP and with anthracene as the least prevalent (Table 4.1). Still, no significant differences were observed for metabolites of pyrene or BaP in bile of tunnel wash water exposed fish compared to fish sampled from the control treatment. In two studies the presence of the aforementioned PAHs were investigated in the dissolved fraction (filtered at <0.45 or 0.7 μm) (Table 4.1). In these studies phenanthrene was reported as the most prevalent PAH followed by pyrene, and with anthrace.ne and benzo[a]pyrene either at low concentrations or below the detection limit (Table 4.1). Thus, due to the higher lipophilicity of the higher weight PAHs, pyrene and BaP may have been bound to particles in tunnel wash water to a greater extent, and subsequently been removed from water during filtration (1.2 μm in the current study). In addition, the storage and homogenisation of exposure water in plastic containers in the current study may also have lowered the concentration of PAHs (see chapter 4.5). In addition, bioavailability of PAHs depends on the presence of humic substances in water. While the bioavalability of BaP have been found to be reduced by increased humic substances, this was not observed for the three-ring PAH anthracene (Spacie et al. 1983). Together, these factors may explain why only 1-OH-phenanthrene and the phenanthrene equivalent metabolites, and not pyrene and BaP metabolites were increased in bile of tunnel wash water exposed fish. Further, this may

imply that other higher weight PAHs, in addition to pyrene and BaP, may have been taken up at low concentrations in fish exposed to tunnel wash water in the current laboratory study.

The metabolites quantified as phenanthrene equivalent metabolites (8.9′-OH, 9.4′-OH and 10.1/10.2′-OH metabolites) also increased in tunnel wash water exposed trout (significant for the Nordby treatment) in fish sampled at day 5 and day 25 compared to fish sampled at day 0. No difference was observed between fish sampled at day 5 and day 25. An increased concentration of PAH metabolites has been observed as a response of time in fish that were not fed (Collier and Varanasi 1991). As fish in the current study were fed and the gall bladder is emptied regularly during feeding, similar concentrations at day 5 and 25 was expected and was further in accordance with a study investigating PAH metabolites in bile exposed over a long duration with regular feeding (Grung et al. 2009).

In contrast to our study, the equivalent metabolites of pyrene and BaP in addition to phenanthrene equivalent metabolites were increased in the bile of coho salmon (*Oncorhynchus kisutch*) exposed to road runoff filtered through bioretention columns with

Table 4.1. Molecular weight (MW) and water-octanol coefficient (K_{ow}) for phenanthrene (Phe), anthracene (Ant), pyrene (Pyr) and benzo[a]pyrene (BaP) and their total concentration in water affected by road pollution (TWW = tunnel wash water, SW = stormwater, RR = road runoff). n.m = not measured. LOQ = limit of quantification.

			Byman (2012)	Meland et al. (2010a)	Paruch and Roseth (2008a)	Zgheib et al. (2011)		Zhang et al. (2008)	
	MW[a] (g/mol)	K_{ow}[a]	Total (µg/L)	Total (µg/L)	Total (µg/L)	Total (µg/L)	Dissolved[c] (µg/L)	Total (µg/L)	Dissolved[d] (µg/L)
Phe	178.2	4.52	1.31	0.034	0.17	0.09 - 0.17	0.025-0.11	0.52	0.11
Ant	178.2	4.56	n.m.	0.013	n.m.	0.016-0.096	< LOQ	0.069	0.024
Pyr	228.3	5.08	1.88	0.23	0.61	0.1 - 1.22	0.015-0.02	0.72	0.086
BaP	252.3	6.2	0.40	0.018	0.36	0.041 - 0.31	< LOQ	0.18	0.0045
Rep. (n)			47	4	1	6		9	
Water			TWW	TWW	TWW	SW[e]		RR	
Country			Sweden	Norway	Norway	France		China	

[a] Values taken from Haynes (2015)
[b] Water solubility at 25°C
[c] Dissolved fraction equals < 0.45 µm
[d] Dissolved fraction equals < 0.70 µm
[e] Stormwater also contains water from roofs and impervious surfaces in the urban landscape

soil or soil+plants, compared to salmon exposed to clean water passing through the same columns (McIntyre et al. 2015). Bioretention columns possess a similar function as sedimentation ponds and are used as a method for removal of road-related contaminants. In the study by McIntyre et al. (2015) the concentration of PAHs measured in water was similar between treatments, thus the PAH metabolites measured in bile was a more sensitive method for assessing exposure to PAHs than investigating concentrations in water (McIntyre et al. 2015). This indicates water-borne PAHs to be available for uptake at very low concentrations and further that concentrations of pyrene and BaP were similar in water between all four treatments in the laboratory study of the current study.

Sedimentation ponds are constructed to retain road related contaminants, and a large extent of contaminants are retained in such ponds (COWI 2005). However, during a tunnel wash event elevated concentration of several PAHs have been measured in outlet water from Vassum sedimentation pond to the stream Årungenelva (Meland et al. 2010a). The concentrations of 1-OH-phenanthrene and 1-OH-pyrene in bile of fish from Årungenelva in the current study were higher in fish sampled at the upstream compared to the downstream location, and thus opposite of what was expected. The sampling of fish in Årungenelva had to be postponed several times due to severe rain events (see Appendix C). Due to this, two weeks had passed between sampling of fish in the stream and the last episode where Vassum sedimentation pond received tunnel wash water. In sheepshead minnows (*Cyprinodon variegatus*) exposed through water, the time needed to eliminate 95% of the bioaccumulated pyrene and phenanthrene was 2 – 4 days after fish had been transferred to clean water (Jonsson et al. 2004). Thus, the results of PAH metabolites in the fish sampled in Årungenelva in the current study may have been different if fish were sampled immediately following a tunnel wash event. The higher exposure of PAHs at the upstream compared to the downstream location can be explained by the close proximity between the upstream location and the motorway, leading to splashing and contamination from the road to the river. In addition, a bridge treated with creosote crosses the stream further up from the upstream location. As creosote is a material with high concentration of PAHs (Vo et al. 2015), contamination from the bridge to the upstream location may be present. 3-OH-BaP was below detection limit for all samples in Årungenelva, indicating low uptake of BaP in Årungenelva.

To summarise according to the research questions addressed there was a higher concentration of 1-OH-phenanthrene (only between the Nordby and the positive control treatment at day 5)

and in the metabolites quantified as phenanthrene equivalents at day 5 and/or day 25 in fish exposed to either one or both of the tunnel wash water treatments (question 1). This indicates presence of bioavailable three-ring PAHs in tunnel wash water at higher concentrations than in control water. In addition, there was an increase in the phenanthrene equivalent metabolites as a response of time in fish exposed to the Nordby treatment (question 2), and the exposure concentration of PAHs seem to be maintained throughout the 25 days of exposure. As opposed to this, tunnel wash water exposed fish did not accumulate metabolites of 1-OH-pyrene or 3-OH-BaP in bile at a higher degree than did fish exposed to control water or fish sampled at day 0 (question 1 and 2). The reason why an increase was not observed for the latter two PAH metabolites may be removal of these PAHs during storage or filtration of water. In Årungenelva there was higher concentration of 1-OH-phenanthrene and 1-OH-pyrene in bile of fish sampled at the upstream location compared to fish sampled at the downstream location (question 3). This may be caused by splashing of contaminants from the road to the upstream location or other local sources of PAHs. Sampling closer to a tunnel wash event might have revealed another pattern.

4.2 CYP1A

Higher EROD activity in gills was observed from fish sampled in both tunnel wash water treatments at day 5 and in the Nordby treatment at day 25, compared to fish from the control treatment. In addition, higher CYP1A protein and EROD activity in liver was observed in fish from at least one of the tunnel wash water treatments compared to the control treatment at day 25 of exposure. Dybwad (2015) investigated expression of CYP1A mRNA in gills and liver of the same brown trout as investigated in the current study. The findings of CYP1A mRNA were in accordance with results of CYP1A protein and EROD activity in fish from the laboratory study observed in the current study (Table 4.2). Increased hepatic gene expression of CYP1A has also previously been found in brown trout exposed to tunnel wash water (Meland et al. 2010b, Meland et al. 2011). CYP1A protein and EROD activity will generally increase following an increase in the expression of CYP1A mRNA as a result of binding of CYP1A agonists to the Ah-receptor (Piskorska-Pliszczynska et al. 1986, Billiard et al. 2002). Results of the current study and the study by Dybwad (2015) thus indicate presence of CYP1A agonists in both tunnel wash water treatments.

Higher EROD activity in gills was also observed as a response of time of trout exposed to tunnel wash water, and a similar high activity between day 5 and 25. Higher CYP1A protein and EROD activity was also observed between tunnel wash water exposed fish sampled at day 25 and fish sampled at day 0. EROD activity thus remained high in gills throughout the 25 days of exposure, and CYP1A protein and EROD activity in liver was elevated above basal after 25 days of exposure to tunnel wash water.

EROD activity in gills, CYP1A protein in liver and EROD activity in liver was also higher in fish exposed to control and positive control water at exposure start, compared to fish sampled at day 0. This indicates that other unknown factors affected CYP1A in the current study. This could be attributed to factors such as stress, but stress was not found to induce hepatic CYP1A in arctic charr (*Salvelinus* alpinus) (Jørgensen et al. 2001). The increase in EROD activity and CYP1A protein in fish from the control treatment is thus not known. It should be noted the increase in the markers related to CYP1A was low in fish exposed to control and positive control water as compared to the increase seen in fish exposed to tunnel wash water. In general, similar response in these markers was observed between fish exposed to control and positive control water. Thus, the positive control treatment did not seem to work as intended, in regard to markers related to CYP1A (will be discussed in section 4.5).

In Årungenelva an unclear picture of exposure to CYP1A agonists was observed between fish sampled at the two locations in the stream. There was no difference in EROD activity in gills between fish sampled at the two locations and this was further observed in expression of CYP1A mRNA (Dybwad 2015). Abrahamson et al. (2007) found EROD activity in gills to be a sensitive marker of detecting exposure to water-born CYP1A agonists. Thus, the results may indicate a similar exposure to water-born CYP1A agonists between the two locations in the stream. However, for markers related to hepatic CYP1A fish had higher expression of CYP1A mRNA upstream, similar CYP1A protein in fish from the two locations, but higher EROD activity in fish sampled downstream. It has previously been reported poor correlation between CYP1A mRNA and EROD activity (Kammann et al. 2008), and the results may indicate a complex exposure scenario between CYP1A inducers and inhibitors in the stream.

4.3 CYP1A inducers and inhibitors in tunnel wash water

As PAHs are reported as important contaminants in road runoff these can be expected to be a main reason for any induction. In the current study, and as observed in the PCA biplots, the markers related to CYP1A were correlated with 1-OH-phenanthrene and markers representing the phenanthrene equivalent metabolites in fish from the laboratory study. Earlier studies have found phenanthrene to be a weak inducer of CYP1A at very high concentrations. This has been observed at aqueous exposure concentrations ranging from 100 to 800 µg/L in embryos of marine medaka (*Oryzias melastigma*) (Mu et al. 2012, Mu et al. 2014) and in sediment spiked with 544 ng g^{-1} phenanthrene in European sea bass (*Dicentrarchus labrax*) (Martins et al. 2015). However, studies with rainbow trout found no induction of CYP1A caused by exposure to phenanthrene *in vitro* or *in vivo* (Bols et al. 1999, Billiard et al. 2004) and are in accordance with the low affinity observed between phenanthrene and the AhR (Billiard et al. 2002). In the field study, and as observed in the PCA biplot, the markers of phenanthrene and pyrene metabolites showed a negative correlation of that observed for EROD activity. However, they showed a similar trend of that observed for CYP1A mRNA in the same fish (Dybwad 2015). As with phenanthrene; pyrene was unable to induce EROD activity in a study on rainbow trout (Bols et al. 1999). While BaP has been found to induce CYP1A in trout (Bols et al. 1999, Levine and Oris 1999), the concentration of 3-OH-BaP in fish of the current study was either low (laboratory study) or not detected (field study), and BaP was not correlated with markers of CYP1A in the PCA biplots (laboratory study). Thus, single exposure to BaP is not expected to have caused the observed CYP1A induction. Still, investigation of EROD activity in brown trout after exposure to PAH mixtures *in vivo* has indicated PAHs to have additive effects, and the effects was found to be synergistic when weak inducers were co-exposed with strong PAH inducers (Basu et al. 2001). PAHs as retene, BaP, benzo[k]fluoranthene, benzo[b]fluoranthene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene have been detected in road pollution (Meland 2012a, Wei et al. 2015), and have been found to induce CYP1A (Bols et al. 1999, Billiard et al. 2004, Han et al. 2013). Thus, low exposure to several PAHs may have caused an Ah-dependent effect on CYP1A in the fish investigated in the current study. In addition, several heterocycles and alkylated PAHs are known CYP1A agonists (Barron et al. 2004) and are expected to be present in road pollution (Takada et al. 1991).

In a recent study investigating the AhR potency of contaminants in roadside snow, the authors found that up to 9% of the observed AhR activity measured in a rat hepatoma cell line could be attributed to PAHs (Muusse et al. 2012). Other CYP1A agonists, including PCBs and dioxins was not detected in samples, and the authors suggested the discrepancy between the total and explained AhR potency in samples to be caused by the presence of other, yet unidentified, AhR agonists in the environment (Muusse et al. 2012). This may be true in the current study. Further, a first-pass effect in gills (involving parent compounds being metabolised in gills and hindering transport of CYP1A agonists to the liver) have been suggested for PAHs (Levine and Oris 1999). The induction observed in both liver and gills of the current study may thus indicate high concentration of PAHs (or other similar compounds) or the presence of dioxin-like compounds that are poor substrates for CYP1A and subsequently will be transported with the blood to the liver, and hence cause induction of CYP1A in this organ.

In fish sampled in Årungenelva, the variable responses observed in hepatic markers of CYP1A may indicate presence of confounding factors in fish sampled in Årungenelva. Factors such as sex, feeding status and age can lead to different responses in markers of CYP1A (Whyte et al. 2000) and might explain some of the variation observed in the current study. However, fish in the current study was juvenile and Rodríguez-Cea and Sanz-Medel (2004) found no significant differences in EROD activity between sexes or between fish of age 0+ and 1+.

Another factor that can explain the differences in the response between the markers of CYP1A include inhibition of e.g. EROD activity, and may be expected in complex mixtures of environmental contaminants, such as tunnel wash water. Compounds found to inhibit EROD activity was reviewed by Whyte et al. (2000), and include among others metals. The authors however concluded that inhibition of EROD activity was observed at exceedingly high concentration of the investigated inhibitors. Other studies have reported that the ability of metals to inhibit EROD activity may be related to oxidative stress caused by exposure to metals (Viarengo et al. 1997, Risso-de Faverney et al. 2000). Gene expression markers (metallothionein (MT), glutathione-S-transferase (GST) and γ-glutamylcysteine synthetase (GCS)) investigated by Dybwad (2015) suggests fish exposed to tunnel wash water experienced oxidative stress in the laboratory study. For fish from Årungenelva some markers that may be related to oxidative stress was increased upstream compared to

downstream (glutathione peroxidase (GPx) and peroxisome proliferator activated receptor γ (PPARγ)), while other genes were increased upstream compared to downstream (MT, heat shock protein 70 (HSP70) and HSP90). In addition to metals, other contaminants have been found to be able to inhibit EROD activity. Contaminants with such properties that have been found at elevated concentrations in water and sediment affected by road activities include fluoranthene (Willett et al. 2001), nonylphenol (Vaccaro et al. 2005), alkylphenol mixtures (Hasselberg et al. 2004), phthalates (Agus et al. 2015) and detergents (Sen and Semiz 2007). Thus, EROD activity may be inhibited to some extent in both fish exposed to tunnel wash water the laboratory study and in fish sampled from Årungenelva. This may explain the more apparent effect observed for CYP1A protein than EROD activity for fish in the laboratory study, and the inconsistent responses observed for hepatic CYP1A in fish sampled in Årungenelva.

To summarise according to the research questions addressed there was higher EROD activity in gills of fish from both tunnel wash water treatments at day 5 and day 25 compared to the two control treatments (question 1), indicating presence of bioavailable CYP1A agonists in the filtered tunnel wash water. There was also observed increased activity in gills as a response of time (question 2), and high activity at both day 5 and 25 indicates maintained high EROD activity in gills through the exposure study. In addition, higher CYP1A protein and EROD activity in liver was observed in fish from at least one of the tunnel wash water treatments compared to fish from the control treatment at day 25 of exposure (question 1) and the responses of these markers were elevated in fish exposed to tunnel wash water at day 25 compared to fish sampled at day 0 (question 2). This may indicate uptake of bioavailable CYP1A agonist, transport of these contaminants to the liver and induction of CYP1A in liver tissue. The less strong response in EROD activity compared to CYP1A protein may indicate some inhibition of EROD activity occurred in fish in the current study. Metals or other organic compounds that are known CYP1A inhibitors and are recorded to be a part of the complex mixture of environmental contaminants in road pollution may have caused this. In Årungenelva there was observed higher hepatic CYP1A activity in fish sampled downstream, but no differences was observed for the other markers of CYP1A investigated (question 3). Seen together with results on gene expression observed by Dybwad (2015), a complex interaction between CYP1A inducers and inhibitors may be present in the stream.

4.4 Lead and δ-aminolevulinic acid dehydratase activity

In contrast to responses observed for PAH metabolites and CYP1A, no effect was observed for ALA-D activity in red blood cells of trout exposed to tunnel wash water in the laboratory study. Further, no difference was observed in ALA-D activity between fish from the two locations in Årungenelva. The results were in accordance with the low concentrations of lead measured in water of the laboratory study (~0.1 µg/L in the control and the two tunnel wash water treatments) and in Årungenelva (~0.7 µg/L). The positive control treatment worked as intended with regard to lead exposure, and an inhibition was observed at concentrations of 53.9 ± 15.5 µg/L Pb in fish sampled from this treatment at day 25 of exposure.

A study on brown trout exposed to stream water affected by metal contamination from a shooting range observed reduced ALA-D activity in these fish (Heier et al. 2009). The concentrations of lead in water of that study ranged between 20.7-45.9 µg/L Pb (Heier et al. 2009) and are in accordance with concentrations found to affect ALA-D activity in the positive control treatment of the current study. Inhibition of ALA-D has been observed after exposure to 10 and 13 µg/L Pb (Hodson et al. 1978, Johansson-Sjöbeck and Larsson 1979), and the inhibition has been found to be prolonged for 7 weeks after ended exposure at concentrations as low as 10 µg/L (Johansson-Sjöbeck and Larsson 1979). Concentration of lead in discharge water from Vassum sedimentation pond to Årungenelva was found to be ~10 µg/L Pb during a tunnel wash event in a previous study, of which approximately one forth was found in the low molecular mass fraction (<10 kDa) (Meland et al. 2010a). Dilution with stream water will however reduce concentrations further. In another study, concentrations of lead in tunnel wash water from the Nordby tunnel were measured at concentration of almost 30 µg/L (Meland et al. 2010b). However, virtually all of this lead was bound to particles larger than 0.45 µm (Meland et al. 2010b). Other studies have also found lead to be highly bound to particles (Meland et al. 2009, Zgheib et al. 2011). Due to particle binding lead that may have been present in tunnel wash water used in the laboratory study are expected to have been removed during filtration of water (1.2 µm). In the field study, exposure of lead through food consumption is also possible. However, as ALA-D activity in fish from Årungenelva was similar to that observed in control fish of the laboratory study the results do not indicate that fish sampled in Årungenelva have been exposed to lead at any significant extent. Together, this may indicate that the treatment

facilities of tunnel wash water currently available are sufficient to avid exposure of harmful concentrations of lead to biota.

Another factor which may have affected ALA-D activity in the present study is the presence of high concentrations of zinc in road pollution (e.g. concentration measured in water in the current study and by Meland (2012a) and Paruch and Roseth (2008b)). High prevalence of zinc in road pollution might protect against ALA-D inhibition, as there is a competition between lead and zinc in binding to the enzymes active seat (Simons 1995) and as inhibition of ALA-D by lead has been reversed after addition of zinc *in vitro* (Finelli et al. 1975). Other metals, such as copper and mercury have not been found to effect ALA-D activity (Hodson et al. 1977).

Gene expression of ALA-S was investigated in the gills and liver of the same trout as in the current study (Dybwad 2015). ALA-S is the first enzyme in the haem synthesis pathway, and its enzymatic activity has been found to increase in fish exposed to lead in a time-dependent manner (Haffor and Al-Ayed 2003). However, no effect was observed for ALA-S in fish exposed to lead in the positive control treatment in the laboratory study (Dybwad 2015). To the contrary, ALA-S was up-regulated in the gills of tunnel wash water exposed fish compared to fish sampled at day 0 in the laboratory study (Dybwad 2015). Up-regulation of hepatic ALA-S has previously been observed in brown trout exposed to tunnel wash water (Meland et al. 2011). Considering the gene expression of ALA-S observed by Dybwad (2015) alongside the results of ALA-D activity in fish sampled from the positive control in the current study, the effect observed on ALA-S activity by Haffor and Al-Ayed (2003) may not have been regulated through an increased gene expression, but may rather be regulated by other mechanisms (e.g. the free haem pool). The increase in ALA-S gene expression after exposure to tunnel wash water was found in concert with an increase in CYP1A (Meland et al. 2011, Dybwad 2015). As haem is a cofactor of cytochrome P450, the increase of ALA-S mRNA might be a consequence of up-regulation of CYP1A (Iba et al. 1999) rather than exposure to lead.

To summarise according to the research questions addressed there was observed no inhibition of ALA-D activity in any of the two tunnel wash water treatments at day 25 compared to the control treatment (question 1), or in any of the tunnel wash water treatments as a response of time (question 2). However, an inhibition of activity was observed in the positive control

treatment (question 1 and 2). This was expected due to addition of lead in water of this treatment. In Årungenelva, there was no difference in ALA-D activity between fish from the two sampling locations (question 3). This indicates low presence of lead in filtered tunnel wash water, and there was no evidence of uptake of harmful lead by fish in Årungenelva.

4.5 Environmental significance of tunnel wash water

In the results of three-ring PAH metabolites and markers of CYP1A, and as illustrated by the PCA biplot of fish from the laboratory study, a stronger effect of tunnel wash water was observed in fish exposed Nordby as compared to fish exposed Granfoss tunnel wash water. Even though detergents were not used during the tunnel wash events, foaming was observed in the aquaria in the laboratory study, particularly in the Nordby treatment. This could be due to leftover detergents on the walls or in the drainage system of the tunnel. Detergents cause increased solubility of lipophilic contaminants, and might have led to increased availability of PAHs and CYP1A agonists in the Nordby tunnel wash water. In addition, even though the annual average daily traffic load (AADT) is similar between the two tunnels, the Granfoss tunnel have been washed ten and the Nordby tunnel four times each year during the last year. When tunnel wash water was collected, it was 20 and 86 days since last wash in the Granfoss and Nordby tunnel, respectively. The analyses of metals in water together with the observed biomarker responses indicate an overall higher exposure of road related contaminants in fish exposed to Nordby compared to Granfoss tunnel wash water. Gene expression markers of CYP1A, metallothionein (MT), δ-aminolevlinic acid synthase (ALAS), glutathione-S-transferase (GST) and vitellogenin (VT) in liver and/or gills of the same fish as investigated in the current study further supported this (Dybwad 2015). In this regard it is suggested that more prevalent washes can reduce the acute toxicity related to exposure of contaminants related to road and vehicle activities in tunnel wash water. However, tunnel wash water released to the environment will be further diluted with recipient water, and the concentrations of water-borne contaminants will thus be reduced in local streams. Also, the total load of road related contaminants to recipients will not be reduced with increased washing frequencies. The tunnel wash water normally also contains large volumes of detergents shown to cause adverse acute mortality to amphibians (Johansen 2013). These are factors that need to be considered when washing frequencies are decided.

When considering the results of fish sampled in Årungenelva the upstream location might be a poor reference when investigating effects related exposure to road related contaminants. In the study by Dybwad (2015), markers related to biotransformation of coplanar chemicals (CYP1A), metal excretion (MT) and stress (heat shock protein (HSP) 70 and 90) was higher at the upstream location as compared to the downstream location, and in the current study elevated PAH metabolites in bile was observed in fish upstream from the pond. In addition, several gene expression markers (CYP1A, MT, ALAS, GST, γ-glutamylcysteine synthetase (GCS) and VT) was as high or higher at both locations in Årungenelva compared to the expression observed in fish exposed to tunnel wash water in laboratory study (not tested statistically) (Dybwad 2015). Although levels of hepatic CYP1A protein and EROD activity in fish in the current study was not compared directly between fish in the laboratory study and fish in the field study, the levels of these markers in fish from both locations in Årungenelva appeared to be higher as compared to levels in fish sampled at day 0 and to control water in the laboratory study. In total, this might indicate fish from both locations in Årungenelva to be affected by road-related activities. However, genetic differences and exposure histories may affect how gene transcription and/or enzyme activity respond to exposure of environmental contaminants (Logan 2007). Thus, the observed levels of the investigated biomarkers in fish sampled in the field and laboratory study may not be comparable. The inclusion of another reference location in the field study (e.g. fish from a river unaffected by anthropogenic activities) might have made it possible to make conclusions of the exposure status in Årungenelva. Seen in light of this, the results of the current study do not support a possible link between exposure to road-related contaminates and the growth reduction of 0+ fish sampled downstream of Vassum sedimentation pond that have been previously reported by Meland et al. (2010a). However, tunnel wash water also contains detergents, and markers included in the current have mainly investigated exposure organic contaminants as metals and PAHs. Further, exposure to tunnel wash water during sensitive life stages (e.g. fish eggs and embryos) might give effect at later stages in life. In a recent study, researchers found reduced weight and length of zebrafish embryos at the age of 5 and 8 months, after a four-day exposure to PAH contaminated sediments (4.4 µg/g total PAHs) during embryonic development (Vignet et al. 2014). Thus, the results of the current study are not sufficient to disprove tunnel wash water released to the stream to be responsible for the growth reduction of juvenile brown trout observed in the stream. The ecological relevance of the release of tunnel wash water is thus, with the evidence currently available, not known.

Together, the results of the current study show that the contaminants present in tunnel wash water after filtration, and possibly after a retention time in a sedimentation pond, are bioavailable for uptake in fish. Further, this indicates that the treatment facilities of tunnel wash water currently applied in Norway do not remove all environmental contaminants present in tunnel wash water and road runoff. The acute toxicity of road-related contaminants in tunnel wash water might be reduced with increased washing frequencies, but washing frequencies need, in addition to results of the current study, to be decided in light of results of other studies investigating effects of e.g. detergents.

4.6 Handling of tunnel wash water and uncertainties in lab-methods

Homogenisation and storage of tunnel wash water may have affected toxicity of treatment water used in the laboratory study. In the current study the treatment water was homogenised and stored in various plastic containers, in addition to being stored at -20°C. A study by McIntyre et al. (2014) investigated whether different treatments of the road runoff affected its toxicity before investigating effects of road runoff on biota. The concentrations of PAHs and metals in road runoff was not found to be affected by freezing (-20°C), but short-time storage for homogenisation of water in a HDPE cistern (volume: 1135 L) lead to a decrease of total PAHs from 4.1 to 1.6 µg/L (McIntyre et al. 2014). Similar reduction of PAHs and other fat-soluble contaminants may be expected in the current study. The concentrations of all PAHs (except naphthalene) were below the detection limit in the current study, and may have been caused by the aforementioned aspect. In addition, the water sampled for PAH analyses had already been in the aquaria for five days prior to sampling. A study found concentrations of PAHs in tanks containing one fish to be decrease to less than 5% of nominal concentrations during the first 24 h (Basu et al. 2001), and storage of water samples for 7 days in darkness (4°C) showed a 96% drop in PAH concentrations (McIntyre et al. 2014). This may thus explain the lack of detected PAHs in treatment water of the current study. In addition, lead in the positive control treatment was measured at a concentration of 53.9 ± 15.5 µg/L Pb, while 150 µg/L Pb was added. This reduction may be caused by removal of lead during filtration.

Both filtration and storage in plastic containers may have reduced benzo[a]pyrene (BaP) added to the positive control treatment. The positive control treatment was included to ensure

that responses in the investigated markers would be detected if present. As induction of CYP1A in gills and liver has been found at concentrations of <1 µg/L BaP (Levine and Oris 1999a), the lack of response in CYP1A protein and EROD activity, as well as mRNA in the same fish (Dybwad 2015), may have been caused by the factors mentioned. Another factor may be the co-exposure between BaP and lead in the positive control. Effects of *in vitro* lead exposure was however neither observed for CYP1A mRNA, concentration or activity in a Antarctic fish species (Benedetti et al. 2007), but a reduction in EROD activity was observed in crucian carp (*Carassius carassius*) at exposure concentration of 50 µg/L Pb (Ding et al. 2014).

Some analytical variation in quantification of PAH metabolites in bile of fish from the Årungenelva is expected, as bile samples were resuspended and the concentration of PAH metabolites had to be standardised against biliverdin. Biliverdin is a pigment that accumulates in bile over time (Collier and Varanasi 1991). The pigment has thus been used to standardize the time since last evacuation of bile and consequently the amount of PAH accumulated as a response of feeding status (Collier and Varanasi 1991). Standardization of PAH metabolites to biliverdin levels have however been found to add more error to bile metabolite data (Aas et al. 2000). Care should therefore be taken before drawing any strong conclusions on PAH exposure between the two locations in Årungenelva. PAH metabolites are considered a biomarker where concentrations can be compared between studies (Hylland et al. 2012), but as preparation of samples was different between the laboratory and field study, a direct comparisons could not be made in the current study.

In the current study, the activity of ALA-D (ng PBG min^{-1} mg^{-1} protein) was much higher than has been observed in other studies. The ALA-D activity in uncontaminated areas are reported to lie between 15-25 ng PBG min^{-1} mg^{-1} protein, for various saltwater species (ICES 2004). In the current study the ALA-D concentration ranged from 42 to 198 ng PBG min^{-1} mg^{-1} protein (not included fish sampled in the positive control treatment at day 25). The protocol used was without the use of the environmental toxic substance mercuric chloride, in accordance with procedures described by Nakagawa et al. (1995) and Erdahl (2014). Erdahl (2014) reported the method without mercury to be significantly lower than the method with mercury on blood from Atlantic cod, but the correlation between the methods was significant ($R^2 = 0.8$). Explanations for the much higher activity in the current assay are not known, but might be related to species differences. In the current study, the dilution buffer used in the

assay had a pH of 7.0, however a pH of 6.2 is proposed used when investigating ALA-D activity in freshwater fish species (ICES 2004). In addition, large variations were observed between technical replicates when running the assay. It is however not known if these variations can be linked to the observed high activity of the ALA-D enzyme. Still, the significant reduction in activity in the positive control treatment indicates inhibition of ALA-D activity would be detected if present.

5 Conclusions

Juvenile brown trout were exposed for 5 and 25 days to filtered (1.2 µm) tunnel wash water from two different tunnels, the Granfoss and the Nordby tunnel. PAH metabolites in bile confirmed that lower weight three-ring PAHs were available for uptake at increased concentrations in tunnel wash water compared to control water. In addition, elevated and similar concentrations of two of four investigated three-ring PAH metabolites was observed in the Nordby treatment at day 5 and 25, and indicates maintained high uptake of the three-ring PAH parent compounds throughout the 25 days of exposure. No significant differences were observed for metabolites of the four-ring PAH pyrene or the five-ring PAH benzo[a]pyrene (BaP) in fish exposed to tunnel wash water. This may be caused by removal of these PAHs when water was filtered, or adherence of these PAHs to various plastic containers during homogenisation and storage.

Higher EROD activity in gills as well as CYP1A protein and EROD activity in liver indicate elevated exposure of CYP1A agonists to fish sampled from the tunnel wash water treatments compared to fish sampled from the control treatment. In addition, higher responses in these markers were observed in tunnel wash water exposed fish compared to fish sampled at day 0, indicating elevated levels of CYP1A throughout the 25 days of exposure. An increase was also observed in fish exposed to control water compared to fish sampled before exposure start, indicating other unknown factors affected CYP1A in fish from the current study. However, this increase in CYP1A was low compared to the increase observed in tunnel wash water exposed fish. Further, a more apparent effect was observed for CYP1A protein compared to EROD activity in liver of fish exposed to tunnel wash water. This may be due to presence of contaminants able to inhibit the EROD activity in tunnel wash water.

Brown trout were also sampled in the stream Årungenelva both downstream and upstream from where discharge water from the Vassum sedimentation pond is released to the stream. Compared to fish sampled upstream fish sampled downstream had lower biliary concentrations of 1-OH-phenanthrene and 1-OH-pyrene and higher EROD activity in liver. No differences were observed in EROD activity in gills or in CYP1A protein in liver between fish sampled at the two locations in the stream. Biomarker responses investigated in fish sampled in Årungenelva indicate road-related contaminants may be present at both locations

in the stream. Varying responses between markers of CYP1A may indicate an exposure to both CYP1A inducers and inhibitors in the stream.

No effects was observed on ALA-D activity in red blood cells in fish exposed to tunnel wash water in the laboratory study, or between fish sampled at the two locations in Årungenelva. This indicates low exposure to bioavailable lead, and may indicate that the treatment facilities of tunnel wash water currently available are sufficient to avid exposure of lead to biota.

More apparent effects were observed in fish from the Nordby compared to the Granfoss treatment in the laboratory study. The Granfoss and Nordby tunnels are washed four and twelve times a year, respectively. More frequent washing of tunnels may thus reduce the acute toxicity of tunnel wash water. A previous study has reported a growth reduction in fish caught downstream of the Vassum sedimentation pond. The authors have hypothesised this to be caused by tunnel wash water released to the stream. The current study could not validate a higher exposure to contaminants in fish sampled at the downstream location, thus the reason for the growth reduction observed in fish downstream of the sedimentation pond is still unknown. Due to the lag between the last tunnel wash event and sampling of fish in Årungenelva another exposure pattern in fish between the two locations may have been revealed if fish were sampled closer to a tunnel wash event in the current study. Together, the results of the current study indicate that contaminants present in the water phase of tunnel wash water are bioavailable for uptake in fish. Further, the treatment facilities of tunnel wash water currently applied in Norway do not remove all environmental contaminants present in tunnel wash water and road runoff. The ecological relevance of the release of tunnel wash water is, however, with the evidence currently available not known.

6 Further perspectives

In this study, biomarkers related to exposure to road related contaminants could not verify a stronger contamination pressure downstream compared to upstream from Vassum sedimentation pond. Thus, there are still uncertainties on whether tunnel wash water released from the pond to the stream have caused the previously observed growth reduction of juvenile brown trout downstream of Vassum sedimentation pond. It is suggested that further research should be applied on exposure of tunnel wash water to sensitive life stages (e.g. eggs and embryos). Effects of tunnel wash water containing detergents should be investigated. Investigations should be applied on environmental relevant concentrations of contaminants and detergents. The current study has indicated that a combination between controlled laboratory study and field investigations are valuable when investigating effects of contaminants on biota.

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Appendix

Appendix A

Table A1 An overview of all the chemicals used, the producer, product number and in which context they were used.

Chemical	Producer	Product	Used in
4-(Dimethylamino)benzaldehyde	Sigma-Aldrich	D2004	h
5-Aminolevulinic acid hydrochloride, approx.	Sigma-Aldrich	A3785	h
Acetic acid	Sigma-Aldrich	33209	h
Acetonnitril	VWR Prolab	20060.290	c
β-Glucuronidase/aryl sulfatase from Helix pomata	Sigma-Aldrich	9001-45-0	b
Benzo[a]pyrene	Sigma	B1760	i
Bovint serum albumin	Sigma-Aldrich	A7030	f
CaCl2	Sigma-Aldrich	C1016	a
Carbonate-bicarbonate	Sigma	C3041	f
DC™ Protein assay, reagent A	BioRad	500-0113	g
DC™ Protein assay, reagent B	BioRad	500-0114	g
Dicumarol	Sigma-Aldrich	66766	a, i
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D4540	a, e
Dithiotreitol (DDT)	Fluka	03.12.83	d
Glucose	Sigma	G5400	a
Glycerol	Sigma	G5516	d
Goat anti-rabbit IgG conjugated with HRP	Sigma	A0545	f
H_2SO_4	Merck	1.00731.1000	f
HEPES	AppliChem	A1069	a
K_2HPO_4	Sigma	P-5379	d, e
KCl	Merck	1.04936.1000	a,d,e
KH_2PO_4	Sigma-Aldrich	P5379	d, e
L-Ascorbic acid	Sigma-Aldrich	255564	b
Methanol	Sigma-Aldrich	32213N	b
$MgSO_4$ x $7H_2O$	Sigma-Aldrich	M1880	a
Na_2HPO_4 x H_2O	Sigma-Aldrich	71504	h, a
NaCl	Merck	1.06404.1000	a, e, f
NaH_2PO4	Sigma-Aldrich	30412	h
OH-PAH std.			c
$Pb(NO_3)_2$	Sigma	22862-1	i
Perchlroic acid, 70%	Prolabo	589.293	h
Prophobilinogen (PGB)	Sigma-Aldrich	P1134	h
Protein standard (Bovine gamma globulin)	Sigma-Aldrich	P5369	g
Rabbit anti-fish CYP1A antibody	Biosense Laboratories	C02401201-500	f
Resorufin ethyl ether	Sigma-Aldrich	E3763	a, e
Resorufin sodium salt	Sigma-Aldrich	R3257	a, e
TMB Plus	Kem-En-Tech	4395L	f
Trichloracetic acid (TCA)	Merck	1.00807.1000	h
Triphenylamine	Aldrich	T81604	b

Triton-X-100	Sigma-Aldrich	9002-93-1	h
Trizma base	Sigma-Aldrich	T1503	f, g
Trizma HCl	Sigma-Aldrich	T3253	f, g
Tween-20	Sigma-Aldrich	P1379	f
β-NADPH reduced tetra sodium salt	Sigma-Aldrich	N1630	e

a= EROD(gills), b= bile preparation, c=HPLC, d=preparation of liver microsomes, e=EROD(liver), f=ELISA, g=proteinanalyses, h=ALA-D, i=positive control water

Appendix B

Table A2 Weight, Condition, length and biomarker responses of individual fish from the exposure study and the field study.

Sampling day	Aquarium	Treatment	Wei-ght (g)	Cond-ition (*K*)	Len-gth (cm)	8.9'-OH	9.4'-OH	1-OH-phe	10.1/10.2'-OH	1-OH-pyr	3-OH-BaP
0	1	Granfoss	6.0	0.85	8.9	2297.50	409.72	943.86	160.80	524.29	5.85
0	2	Control	18.2	0.96	12.4	1637.58	266.94	395.35	130.47	219.47	3.33
0	3	Granfoss	6.7	0.92	9.0	5751.05	526.37	1106.84	472.85	1025.41	1.77
0	4	Nordby	12.8	0.89	11.3	3476.47	758.94	718.99	204.32	744.85	3.62
0	5	Nordby	13.0	0.98	11.0	836.46	141.61	203.85	66.58	141.74	2.20
0	6	Granfoss	7.1	0.75	9.8	1775.33	274.25	542.53	194.83	448.02	1.84
0	7	Positive	14.3	0.92	11.6	5059.33	480.73	568.32	248.35	562.89	3.15
0	8	Control	12.8	0.96	11.0	6491.19	1227.05	1284.95	439.91	1271.58	0.18*
0	9	Positive	14.9	0.86	12.0	6232.21	445.37	893.71	338.94	852.65	10.77
0	10	Nordby	9.6	1.16	9.4	3223.12	215.25	153.09	204.18	170.08	12.59
0	11	Control	13.1	0.93	11.2	751.06	186.67	278.35	123.44	202.93	1.52
0	12	Positive	5.5	0.80	8.8	NA	NA	NA	NA	NA	NA
0	13	Granfoss	12.4	0.58	12.9	283.61	38.43	28.71	18.54	81.41	0.18*
0	14	Nordby	10.8	0.96	10.4	1844.31	375.32	658.60	206.46	463.24	7.28
0	15	Nordby	10.5	1.18	9.6	633.68	103.91	152.63	37.47	110.57	2.17
0	16	Positive	11.1	0.66	11.9	1398.92	238.56	240.82	171.84	254.94	2.46
0	17	Positive	9.7	0.97	10.0	NA	NA	NA	NA	NA	NA
0	18	Granfoss	12.5	0.99	10.8	2056.22	288.47	353.17	93.89	163.82	1.04
0	19	Control	8.0	0.82	9.9	NA	NA	NA	NA	NA	NA
0	20	Control	12.5	0.94	11.0	2089.21	415.89	436.46	177.94	263.59	0.18
5	1	Granfoss	9.7	1.03	9.8	7717.89	3521.74	937.86	3047.46	586.43	3.15
5	2	Control	12.5	0.91	11.1	287.08	46.44	60.22	33.95	58.92	1.43
5	3	Granfoss	3.4	0.71	7.8	10454.50	8381.63	1818.57	4305.99	713.79	5.22
5	4	Nordby	13.5	1.07	10.8	13728.04	8452.52	1886.27	6140.83	867.29	1.82
5	5	Nordby	8.3	0.96	9.5	11335.08	2398.07	684.12	2499.53	176.98	1.54
5	6	Granfoss	11.5	1.00	10.5	3767.36	1583.71	504.15	949.50	163.01	2.23
5	7	Positive	18.6	1.07	12.0	253.98	22.26	32.08	19.92	51.78	11.16
5	8	Control	11.4	0.86	11.0	832.40	96.54	226.79	73.23	226.29	3.03
5	9	Positive	15.9	0.83	12.4	294.04	34.25	54.31	30.30	77.83	23.60
5	10	Nordby	11.1	0.86	10.9	16256.80	5906.09	1052.57	3193.46	225.53	3.44
5	11	Control	25.0	0.97	13.7	1457.71	252.74	488.00	152.67	490.78	35.81
5	12	Positive	11.1	0.84	11.0	5147.92	514.61	959.59	331.75	959.48	67.97
5	13	Granfoss	16.2	0.96	11.9	3492.40	685.92	306.01	393.05	129.96	2.70
5	14	Nordby	8.3	0.86	9.9	12010.67	4354.14	889.08	2232.86	111.26	7.02
5	15	Nordby	9.3	0.98	9.8	14186.08	10338.51	2333.26	7146.75	1064.69	11.58
5	16	Positive	14.3	0.94	11.5	NA	NA	NA	NA	NA	NA
5	17	Positive	18.3	1.08	11.9	257.71	38.24	69.94	19.10	48.53	13.14
5	18	Granfoss	13.0	1.06	10.7	8700.72	2964.11	1008.39	1865.43	280.90	2.45

5	19	Control	9.3	0.93	10.0	NA	NA	NA	NA	NA	NA
5	20	Control	10.9	1.09	10.0	NA	NA	NA	NA	NA	NA
25	1	Granfoss	14.8	1.06	11.2	1480.05	669.86	164.85	533.68	46.74	0.18*
25	2	Control	17.9	1.03	12.0	136.91	14.35	42.22	18.21	41.00	0.81
25	3	Granfoss	8.4	0.76	10.3	NA	NA	NA	NA	NA	NA
25	4	Nordby	15.5	0.99	11.6	12533.94	4405.41	819.37	2590.02	173.60	16.29
25	5	Nordby	6.1	0.90	8.8	NA	NA	NA	NA	NA	NA
25	6	Granfoss	9.8	0.80	10.7	8902.89	3965.07	1220.14	3208.19	468.69	8.41
25	7	Positive	14.7	1.02	11.3	255.36	1.36*	31.71	9.16	31.71	117.63
25	8	Control	20.7	1.06	12.5	145.97	16.39	54.96	20.97	49.97	1.37
25	9	Positive	12.9	1.03	10.8	422.72	35.30	142.68	51.35	81.10	159.26
25	10	Nordby	15.8	0.94	11.9	9668.89	3562.62	432.34	1958.81	151.05	1.69
25	11	Control	9.4	0.89	10.2	270.20	30.91	174.71	23.10	124.91	4.42
25	12	Positive	11.4	1.02	10.4	210.00	5.79	74.42	15.94	41.76	64.26
25	13	Granfoss	10.2	0.83	10.7	739.72	295.34	90.12	152.46	23.33	3.02
25	14	Nordby	9.1	0.91	10.0	18884.91	1.20	7212.75	8619.11	90.70	10.21
25	15	Nordby	10.3	0.89	10.5	25564.18	10755.26	1549.40	6288.46	389.24	16.34
25	16	Positive	10.5	0.86	10.7	NA	NA	NA	NA	NA	NA
25	17	Positive	11.9	0.89	11.0	NA	NA	NA	NA	NA	NA
25	18	Granfoss	14.2	1.07	11.0	4575.90	1594.64	450.14	1231.16	174.84	11.53
25	19	Control	14.1	1.00	11.2	1380.28	157.69	354.31	98.90	170.29	0.18*
25	20	Control	11.1	1.02	10.3	NA	NA	NA	NA	NA	NA

Årungenelva		**Location**	**Wei-ght (g)**	**Cond-ition (*K*)**	**Len-gth (cm)**	**8.9'-OH**	**9.4'-OH**	**1-OH-phe**	**10.1/10.2'-OH**	**1-OH-pyr**	**3-OH-BaP**
	1	Downstream	13.4	1.23	10.3	NA	NA	NA	NA	NA	NA
	2	Downstream	8.5	1.13	9.1	NA	NA	NA	NA	NA	NA
	3	Downstream	6.5	1.27	8.0	NA	NA	NA	NA	NA	NA
	4	Downstream	7.3	1.31	8.2	NA	NA	NA	NA	NA	NA
	5	Downstream	12.5	1.05	10.6	NA	NA	NA	NA	NA	NA
	6	Downstream	9.8	1.14	9.5	NA	NA	NA	NA	NA	NA
	7	Downstream	6.3	1.38	7.7	NA	NA	NA	NA	NA	NA
	8	Downstream	10.1	1.17	9.5	NA	NA	NA	NA	NA	NA
	9	Downstream	12.9	1.25	10.1	NA	NA	NA	NA	NA	NA
	10	Downstream	9.3	1.19	9.2	NA	NA	NA	NA	NA	NA
	11	Downstream	10.1	1.21	9.4	NA	NA	NA	NA	NA	NA
	12	Downstream	5.3	1.12	7.8	NA	NA	NA	NA	NA	NA
	13	Downstream	8.0	1.09	9.0	NA	NA	NA	NA	NA	NA
	14	Downstream	11.9	1.00	10.6	NA	NA	NA	NA	NA	NA
	15	Downstream	12.8	1.21	10.2	NA	NA	NA	NA	NA	NA
	16	Downstream	11.6	1.31	9.6	NA	NA	NA	NA	NA	NA
	17	Downstream	12.7	1.34	9.8	NA	NA	NA	NA	NA	NA
	18	Downstream	6.9	1.26	8.2	NA	NA	NA	NA	NA	NA
	19	Downstream	10.7	1.25	9.5	NA	NA	NA	NA	NA	NA
	20	Downstream	5.4	1.14	7.8	NA	NA	NA	NA	NA	NA
	21	Downstream	5.5	1.03	8.1	NA	NA	NA	NA	NA	NA
	1	Upstream	29.1	1.04	14.1	NA	NA	NA	NA	NA	NA

	2	Upstream	23.8	1.06	13.1	NA	NA	NA	NA	NA	NA
	3	Upstream	4.7	1.00	7.8	NA	NA	NA	NA	NA	NA
	4	Upstream	9.9	1.08	9.7	NA	NA	NA	NA	NA	NA
	5	Upstream	8.0	1.06	9.1	NA	NA	NA	NA	NA	NA
	6	Upstream	10.1	0.98	10.1	NA	NA	NA	NA	NA	NA
	7	Upstream	9.1	1.09	9.4	NA	NA	NA	NA	NA	NA
	8	Upstream	9.8	1.25	9.2	NA	NA	NA	NA	NA	NA
	9	Upstream	17.7	1.20	11.4	NA	NA	NA	NA	NA	NA
	10	Upstream	10.3	1.13	9.7	NA	NA	NA	NA	NA	NA
	11	Upstream	13.6	0.97	11.2	NA	NA	NA	NA	NA	NA

* Values below quantification limit. Values set to half of the detection limit

Table A2 Continious

Sampling day	Aquarium	Treatment	1-OH-phe/ abs380	1-OH-pyr/ abs380	Gill EROD	Hepatic EROD	CYP1A protein	ALA-D
0	1	Granfoss	NA	NA	0.0096	NA	0.106	41.53
0	2	Control	NA	NA	0.0104	NA	0.097	63.46
0	3	Granfoss	NA	NA	0.0064	14.1	0.083	81.91
0	4	Nordby	NA	NA	0.0151	7.5	0.043	70.28
0	5	Nordby	NA	NA	0.0308	30.9	0.126	68.28
0	6	Granfoss	NA	NA	0.0067	2.2	0.078	198.47
0	7	Positive	NA	NA	0.0077	13.3	0.101	100.66
0	8	Control	NA	NA	0.0172	6.6	0.056	133.58
0	9	Positive	NA	NA	0.0050	9.8	0.049	128.40
0	10	Nordby	NA	NA	0.0042	NA	NA	83.38
0	11	Control	NA	NA	0.0063	11.9	0.050	60.19
0	12	Positive	NA	NA	0.0190	9.2	0.166	80.82
0	13	Granfoss	NA	NA	0.0446	8.4	0.033	71.83
0	14	Nordby	NA	NA	0.0115	18.1	0.087	71.62
0	15	Nordby	NA	NA	0.0130	NA	0.080	84.55
0	16	Positive	NA	NA	0.0118	16.8	0.073	47.24
0	17	Positive	NA	NA	0.0239	11.3	0.130	108.72
0	18	Granfoss	NA	NA	0.0186	30.1	0.254	79.49
0	19	Control	NA	NA	0.0268	6.6	0.103	62.37
0	20	Control	NA	NA	0.0132	12.9	0.060	89.56
5	1	Granfoss	NA	NA	0.5918	NA	NA	NA
5	2	Control	NA	NA	0.0324	NA	NA	NA
5	3	Granfoss	NA	NA	0.5770	NA	NA	NA
5	4	Nordby	NA	NA	0.3088	NA	NA	NA
5	5	Nordby	NA	NA	0.6634	NA	NA	NA
5	6	Granfoss	NA	NA	0.3963	NA	NA	NA
5	7	Positive	NA	NA	0.0478	NA	NA	NA
5	8	Control	NA	NA	0.0427	NA	NA	NA
5	9	Positive	NA	NA	0.0805	NA	NA	NA
5	10	Nordby	NA	NA	0.2080	NA	NA	NA

5	11	Control	NA	NA	0.0395	NA	NA	NA
5	12	Positive	NA	NA	0.0930	NA	NA	NA
5	13	Granfoss	NA	NA	0.3771	NA	NA	NA
5	14	Nordby	NA	NA	0.4552	NA	NA	NA
5	15	Nordby	NA	NA	0.2558	NA	NA	NA
5	16	Positive	NA	NA	0.0148	NA	NA	NA
5	17	Positive	NA	NA	0.0644	NA	NA	NA
5	18	Granfoss	NA	NA	0.2342	NA	NA	NA
5	19	Control	NA	NA	0.0386	NA	NA	NA
5	20	Control	NA	NA	0.0247	NA	NA	NA
25	1	Granfoss	NA	NA	0.1990	289.4	1.077	66.05
25	2	Control	NA	NA	0.0022	66.8	0.246	117.94
25	3	Granfoss	NA	NA	0.0538	NA	0.772	61.06
25	4	Nordby	NA	NA	0.1821	522.4	1.629	NA
25	5	Nordby	NA	NA	0.1535	134.9	1.386	87.10
25	6	Granfoss	NA	NA	0.1683	92.3	0.668	78.99
25	7	Positive	NA	NA	0.0043	66.6	0.168	11.05
25	8	Control	NA	NA	0.0017	83.0	0.227	62.45
25	9	Positive	NA	NA	0.0065	13.4	0.103	16.43
25	10	Nordby	NA	NA	0.2810	NA	1.611	89.19
25	11	Control	NA	NA	0.0106	32.4	0.175	100.57
25	12	Positive	NA	NA	0.0100	58.3	0.105	NA
25	13	Granfoss	NA	NA	0.5271	326.4	1.057	81.10
25	14	Nordby	NA	NA	0.1179	331.1	1.522	66.00
25	15	Nordby	NA	NA	0.3300	395.0	1.032	76.39
25	16	Positive	NA	NA	0.0171	NA	NA	18.18
25	17	Positive	NA	NA	0.0174	60.6	0.102	19.01
25	18	Granfoss	NA	NA	0.1878	NA	0.784	79.95
25	19	Control	NA	NA	0.0022	NA	0.145	74.09
25	20	Control	NA	NA	0.0051	2.5	0.050	76.90

Årungenelva		**Location**	**1-OH-phe/ abs380**	**1-OH-pyr/ abs380**	**Gill EROD**	**Hepatic EROD**	**CYP1A protein**	**ALA-D**
	1	Downstream	81.16	56.02	0.0249	543.0	0.436	81.00
	2	Downstream	104.47	51.90	0.0749	763.0	0.528	90.96
	3	Downstream	81.33	48.86	0.0301	260.6	0.427	47.45
	4	Downstream	28.25	31.51	0.0109	272.1	0.370	NA
	5	Downstream	278.77	167.26	0.0197	NA	0.624	63.64
	6	Downstream	175.35	154.28	0.0244	556.6	0.481	97.26
	7	Downstream	90.16	94.09	0.0146	154.2	0.408	126.31
	8	Downstream	134.65	80.10	0.0170	NA	0.598	96.25
	9	Downstream	83.82	61.53	NA	338.2	0.555	81.12
	10	Downstream	15.65	4.30	0.0177	159.7	0.295	67.52
	11	Downstream	118.35	80.93	0.0090	347.8	0.406	89.17
	12	Downstream	118.02	29.28	0.0169	103.8	0.228	135.30
	13	Downstream	235.79	116.36	0.0143	NA	0.340	97.92
	14	Downstream	84.93	89.22	0.0097	NA	0.611	NA

15	Downstream	189.62	131.42	0.0138	225.2	0.594	77.34
16	Downstream	112.69	83.42	0.0187	381.2	0.417	62.18
17	Downstream	155.92	117.40	0.0142	410.4	0.652	128.44
18	Downstream	NA	NA	0.0434	205.1	0.299	107.53
19	Downstream	0.80	1.87	0.0194	179.8	0.291	135.12
20	Downstream	256.74	151.85	0.0793	160.6	0.301	93.42
21	Downstream	22.11	37.66	0.0437	466.2	0.490	79.16
1	Upstream	305.10	126.87	0.0084	237.1	0.239	75.70
2	Upstream	275.74	107.71	0.0544	217.6	0.614	132.75
3	Upstream	326.34	192.06	0.0533	34.5	0.267	111.95
4	Upstream	NA	NA	0.0245	116.0	0.304	88.30
5	Upstream	80.56	93.11	0.0253	143.1	0.353	65.17
6	Upstream	322.52	142.17	0.0211	113.6	0.470	67.10
7	Upstream	259.73	129.43	0.0349	77.3	0.380	96.31
8	Upstream	121.66	112.59	0.0220	NA	0.551	73.44
9	Upstream	279.14	92.33	0.0057	197.0	0.148	91.69
10	Upstream	122.05	79.15	0.0127	358.5	0.498	NA
11	Upstream	235.13	81.60	0.0209	145.4	0.824	113.27

Appendix C

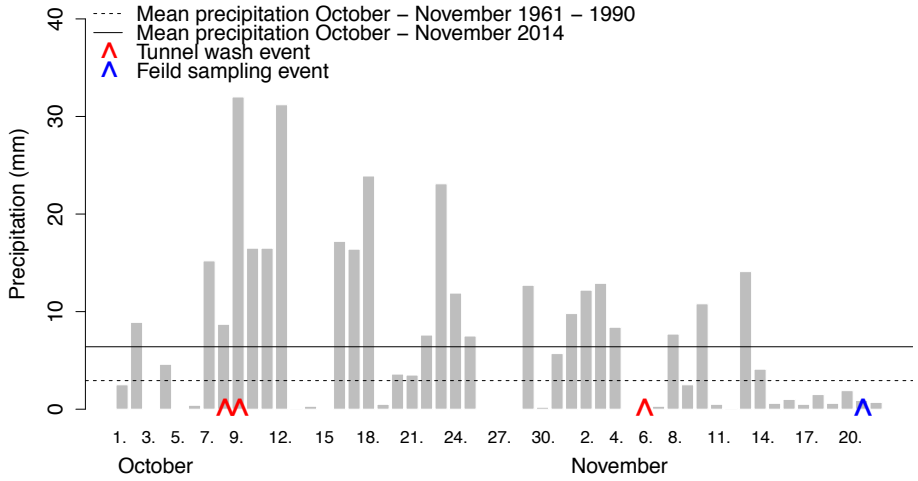
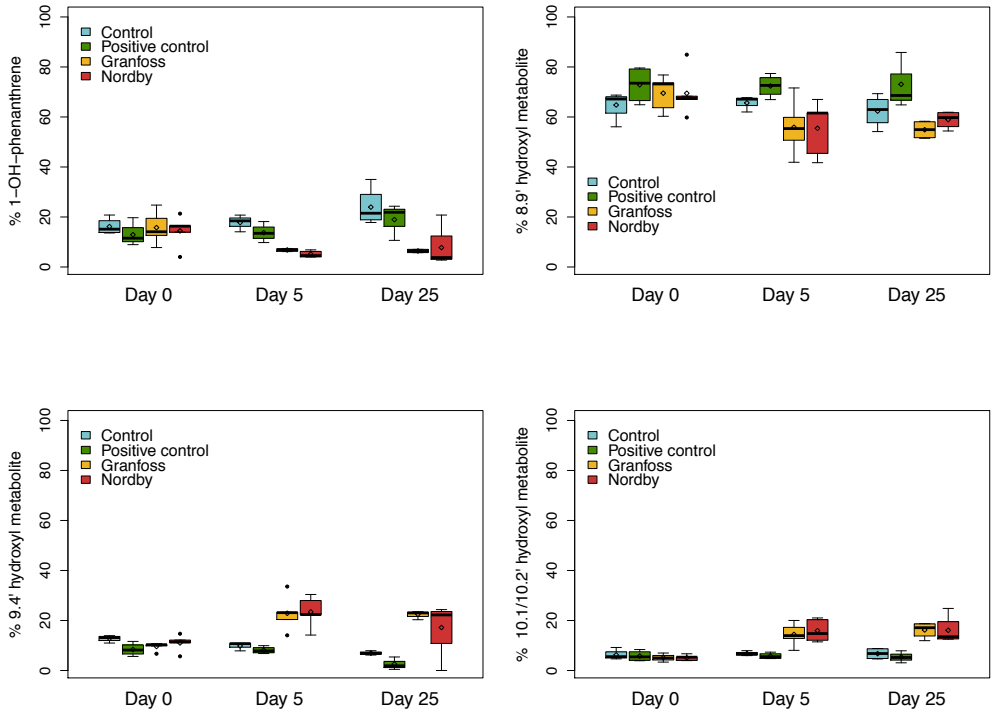


Figure A2. Total daily precipitation in Ås observed by FAGKLIM at Søråsfeltet representing the conditions by the stream Årungenelva and Vassum prior to the sampling in Årungenelva 21st of November 2014. Mean precipitation in October – November 1961-1990 was 6.4 mm/day, while precipitation in October – November 2014 was 2.9 mm/day. Last tunnel wash events prior to sampling was in the Nordby tunnel the 8th and 9th of October and in the Vassum tunnel the 6th of November . Figure modified from Dybwad (2015).

Appendix D



Figur A1 Percentages of each metabolite relative to total (total = Σ(1-OH-Phe, 8.9′-OH, 9.4′-OH, 10.1/10.2′-OH)).