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Philosophiae Doctor (PhD) Thesis 2010:25

Ecotoxicological Effects of Highway and Tunnel Wash Water Runoff

Økotoksikologiske effekter av vegavrenning og tunnelvaskevann

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Preface and acknowledgements

This thesis is based on research conducted throughout the years 2005 to 2010 at the Norwegian University of Life Sciences, Department of Plant- and Environmental Sciences in collaboration with the Norwegian Public Roads Administration (NPRA). The research will hopefully contribute to increased knowledge about environmental impacts regarding highway and tunnel wash water runoff, and it will provide knowledge to the scientific community as well as to the NPRA and their sectorial environmental responsibility.

The ideas and the planning of the experiments were conducted by me under supervision and guidance from Professor Brit Salbu, Professor Bjørn Olav Rosseland and Professor Oddvar G. Lindholm. The doctoral thesis is based on four scientific papers which are either published or accepted for publication (Papers I, II and III) or submitted (Paper IV) to international peer reviewed journals.

First of all I would like to express my gratitude to my main supervisor Brit who admirably encouraged and helped me to finalise my plans regarding getting a PhD grant from my employer (NPRA). You have through this work provided me with constructive critics and valuable comments. Bjørn Olav, a great acknowledgement to you for sharing your knowledge regarding fish physiology and toxicology. I will also thank you for giving me the opportunity to be a guest lecturer in your course in ecotoxicology. Finally, your annual Christmas dinner has certainly been a highlight through all these years. Oddvar, you were not initially involved in the PhD-planning but joined the supervisor group in 2006. I acknowledge your contribution to Paper I.

I would also like to acknowledge Knut Erik Tollefsen and Eivind Farmen at the Norwegian Institute for Water Research (NIVA) for introducing me to the field of genomics and for many interesting discussions during the work with Papers II and IV. I am full of gratitude to Professor Reidar Borgstrøm who kindly shared his data from years of sampling in Årungselva. That was an important contribution to Paper I.

Lene, you have been a major support during these years, both as a friend and colleague. We have had a lot of interesting and valuable discussions which certainly have improved my thesis. Thanks to all other colleagues at the IPM/isotope laboratory, it has been a great time for me!

I would also like to express my gratitude to my employer, NPRA, and especially to my former and current managers, and of course, all my colleagues.

Finally, and most of all, I am very grateful for all the support and love from my wife, Åsne. I could not have done this without you!

Abstract

In Norway, the traffic loadings have shown a substantial increase during the last decades. From 1948 to 2008 the transportation load has increased from 2.5 to 60.6 million passenger km. Hence, the ever increasing traffic has without doubt a significant negative effect on the environment. For example, highway runoffs typically contain a cocktail of both organic and inorganic contaminants being able to cause detrimental effects on the aquatic ecosystem.

The present thesis, which is part of the Norwegian Public Roads Administrations ongoing work with the European Water Framework Directive, has addressed questions related to ecotoxicological effects of highway runoff. In addition, manmade runoff from tunnel wash maintenance, being far less described in the scientific literature compared to natural occurring runoffs, was included. Hence, exposure characteristics (e.g. source characterisation), environmental impacts (fish toxicity) and mitigation strategies (sedimentation ponds) were essential aspects in the present thesis.

The results presented in this thesis showed that runoff water, caused by precipitation as well as by manmade tunnel wash maintenance, were contaminated. According to various environmental quality standards (EQS) from Europe and North America, the water concentrations of metals such as aluminum (Al), cupper (Cu), iron (Fe), lead (Pb), zinc (Zn) and polycyclic aromatic hydrocarbons (PAH) such as pyrene, fluoranthene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene and indeno(1,2,3-cd)pyrene were of most concern.

To gain knowledge about the sources of the traffic related contaminants, enrichment factors (EF) for metals and ratios between various PAHs were calculated. The calculations were based on tunnel wash water runoff as this runoff has little or no impact from other anthropogenic sources (e.g. deposition of long range air pollution or other industrial emissions). The source characterization of tunnel wash water runoff revealed that metals such as Zn, Cu and antimony (Sb) originated mostly from the vehicle (tires and brakes). The high EFs of chloride (Cl) and sodium (Na) were most likely due to road salt applications, while the low EFs for Al, barium (Ba), calcium (Ca), chromium (Cr), Fe, potassium (K), magnesium (Mg) and nickel (Ni) indicated contribution from the pavement material. Finally, the PAH ratios revealed contribution from a mix of sources such as asphalt, tires and combustion.

Generally, the concentrations of both organic and inorganic contaminants were higher in untreated highway and tunnel wash water compared to concentrations measured in the pond outlets, indicating removal of contaminants from the water phase within the pond by e.g. sedimentation. However, the removal of dissolved metal species (< 0.45 μm) was less obvious, and the investigation of a tunnel wash event in the Nordby tunnel showed that 24 % of the metal masses were discharged as low molecular mass species (< 10

kDa). High concentrations of road salt and dissolved organic matter (DOC) together with oxygen depletion are in this respect important as they may contribute to increased transportation and remobilization of contaminants in the sedimentation ponds. In a biological context, poor removal of contaminants as LMM species is worrying as they are considered more mobile and bioavailable than contaminants associated with particles.

By using brown trout (*Salmo trutta*) as a model organism in the toxicity assessment of highway and manmade tunnel wash water runoff, it was demonstrated that several metals, such as Al, Cu, cobalt (Co), Fe, Pb and Sb were gill reactive. However, the metal accumulation and probably the accumulation of other contaminants as well (e.g. PAHs) were most likely modified by the high concentrations of Ca and DOC in the water. Nevertheless, the accumulation of metals in gills of exposed fish most likely provoked short term biological effects manifested by increased glucose levels in blood, being a good biomarker for general stress. Typically, this was followed by a small but notable decrease in blood plasma concentrations of Cl and Na indicating impairment of the ion regulatory system. In addition, several components associated with the antioxidant defense system were triggered in liver of exposed fish. For example, a slight increase in enzymatic activity of superoxide dismutase (SOD) and catalase (CAT) together with increased concentrations of metallothionein (MT) were measured in fish exposed to highway runoff from the Skullerud junction, while a modest up-regulation of the mRNA transcriptions of the oxidative stress biomarkers thioredoxin (TRX) and γ-glutamylcysteine synthetase (GCS) were observed in fish exposed to tunnel wash water runoff from the Nordby tunnel. The fish exposed to tunnel wash water demonstrated that PAHs and/or other organic contaminants were readily bioavailable, although normally strongly attached to particles, as a significant up-regulation of the mRNA transcription of the mixed function oxidase enzyme (phase I) cytochrome P450 1A (CYP1A) was observed.

In addition to confirming increased expression of the antioxidant defense system and CYP1A in tunnel wash water exposed fish, the DNA microarray analysis revealed that traffic related contaminants also could suppress several immunological processes, as well as inhibiting the cholesterol biosynthesis several hours after the exposure. The microarray analysis also indicated the presence of organophosphorus compounds (OP), due to the apparent up-regulation of the paraoxonase enzyme (PON) which is the main protector against OP mediated neurotoxicity. Finally, an apparent up-regulation of arsenite methyltransferase (AMT) indicated that metalloids such as arsenic (As) and Sb were bioreactive, despite that no accumulation of these metalloids were observed in the liver.

The short term fish exposure studies were conducted in undiluted or in slightly diluted runoff water (e.g. 50:50 in Skullerud pond outlet water). Hence, in a real life situation the biological short term effects might have been less pronounced due to higher dilution factors under normal hydrological conditions. However, observations of reduced growth

in the summer old sea trout population downstream the Vassum sedimentation pond during the last years, receiving tunnel wash water runoff from the Nordby tunnel, indicated in fact a long term negative biological effect. On average, individuals in the summer old sea trout population downstream the sedimentation pond were 21 % shorter than individuals from the population upstream.

In a biological context, the results in the present study have demonstrated that sedimentation ponds may not be sufficiently effective in mitigating environmental impacts from highway and tunnel wash water runoffs. Hence, more research on new and/or alternative mitigation strategies should be addressed. It would also be advisable to establish a best management practice (BMP) of existing treatment facilities (e.g. removal of contaminated sediment and/or dead plant material), and in addition, providing guidelines on how to perform tunnel wash maintenance in an environmental sustainable perspective. Issues that should be addressed in this context are e.g. water volumes, cleaning agents, washing frequencies, in addition, how to avoid washing events during vulnerable periods for aquatic organisms (e.g. during smoltification of anadromous salmonids). Finally, the obtained results emphasis the inclusion of chemical speciation of the runoff water and biological parameters in the assessment of treatment performance, being a more sustainable and reliable approach than the measurement of total concentrations only.

Sammendrag

I de siste 10-årene har det vært en betydelig trafikkvekst i Norge. Transportarbeidet har for eksempel økt fra 2,5 til 60,6 millioner passasjerkilometer i perioden fra 1948 til 2008. Den stadig stigende trafikkveksten har uten tvil en betydelig negativ effekt på miljøet, da avrenning fra vei inneholder en cocktail av både organiske og uorganiske forurensningsstoffer som kan forårsake skadelige effekter på det akvatiske økosystemet.

Denne avhandlingen, som er en del av Statens vegvesen sitt pågående arbeid med EUs Vanndirektiv, adresserer problemstillinger relatert til økotoksikologiske effekter av avrenning fra vei. Avrenning fra vasking av tunneler er også inkludert, da dette er et tema som er langt mindre omtalt i den vitenskaplige litteraturen sammenlignet med naturlig avrenning. Eksponeringskarakterisering (f.eks. kildekarakterisering), miljøeffekter (giftvirkninger på fisk) og avbøtende tiltak (sedimenteringsbassenger) har derfor vært essensielle aspekter i denne avhandlingen.

Resultatene i denne avhandlingen viste at avrenningsvann, både forårsaket av nedbør og tunnelvasking, var forurenset. I henhold til ulike europeiske og nordamerikanske miljøstandarder (EQS) var konsentrasjonen av metaller som f.eks. aluminium (Al), kobber (Cu), jern (Fe), bly (Pb) og sink (Zn), og polysykliske aromatiske hydrokarboner (PAH) som f.eks. pyren, benso(b)fluoranten, benso(k)fluoranten, benso(ghi)perylen og ideno(1,2,3-cd)pyren mest bekymringsfull.

Anrikningsfaktorer for metaller og forholdstall mellom ulike PAH-stoffer ble beregnet for å øke kunnskapen om de ulike kildene som bidrar til de trafikkrelaterte forurensningsstoffene. Beregningene ble gjort på tunnelvaskevann ettersom dette avrenningsvannet har liten eller ingen påvirkning fra andre antropogene kilder (f.eks. avsetninger som følge av langtransporterte forurensninger eller andre industrielle utslipp). Kildekarakteriseringen av tunnelvaskevannet viste at metaller som f.eks. Zn, Cu og antimon (Sb) stammet mest fra kjøretøyet (bildekk og bremser), høye anrikningsfaktorer for klorid (Cl) og natrium (Na) skyldes mest sannsynlig veisalting, mens lave anrikningsfaktorer for Al, barium (Ba), kalsium (Ca), krom (Cr), Fe, kalium (K), magnesium (Mg) og nikkel (Ni) indikerte bidrag fra veidekket. PAH-forholdstallene viste bidrag fra flere kilder som f.eks. asfalt, bildekk og eksos.

Konsentrasjonene av både organiske og uorganiske stoffer var generelt høyere i urenset avrenningsvann og tunnelvaskevann sammenlignet med konsentrasjonene målt i utløpet av rensebassengene, noe som indikerte at forurensningsstoffer le fjernet fra vannfasen rensebassenget ved f.eks. sedimentering. Rensingen av løste metallforbindelser (< 0,45 μm) var imidlertid mindre effektiv da en studie av en vaskeepisode i Nordbytunnelen avslørte at 24 % av metallene ble sluppet ut som lavmolekylære (LMM) forbindelser (< 10 kDa). Høye konsentrasjoner av veisalt og løst organisk materiale (DOC) og oksygensvinn er viktig i denne sammenhengen fordi de kan bidra til økt transport og

muligens remobilisering av forurensningsstoffer i sedimenteringsbassengene. I en biologisk sammenheng så er lav rensegrad av forurensningsstoffer som foreligger i lavmolekylære former bekymringsfullt fordi disse er antatt mer mobile og biotilgjengelig sammenlignet med partikkelassosierte forurensningsstoffer.

Ved å benytte brunørret (*Salmo trutta*) som modellorganisme i toksisitetsvurderingene av avrenningsvann fra vei og tunnelvaskevann, ble det avdekket at flere av metallene som f.eks. Al, Cu, kobolt (Co), Fe, Pb and Sb var gjellereaktive. Akkumuleringen av metaller og sannsynlig også av andre forurensningsstoffer (f.eks. PAH) ble trolig modifisert av høye konsentrasjoner av Ca og DOC i vannet. Tiltross for dette så fremprovoserte akkumuleringen av metaller i eksponert fisk biologiske korttidseffekter uttrykt ved økte glukosekonsentrasjoner i blod, noe som er en god biomarkør for generelt stress. Typisk nok så ble dette etterfulgt av en mindre, men merkbar, reduksjon i blodplasmakonsentrasjoner av Cl og Na, noe som indikerte forstyrrelse av det ioneregulatoriske systemet. I tillegg ble flere komponenter assosiert med antioksidantforsvaret i lever hos eksponert fisk trigget. For eksempel så ble det målt en moderat økning i enzymaktiviteten til superoksid dismutase (SOD) og catalase (CAT) sammen med økt konsentrasjon av metallothionein (MT) i fisk eksponert for avrenningsvann fra Skullerudkrysset, mens det for fisk eksponert for tunnelvaskevann fra Nordbytunnelen ble observert en moderat oppregulering av mRNA-transkripsjon av biomarkørene for oksidativt stress thioredoxin (TRX) og γ-glutamylcysteine synthetase (GCS). Fisken som ble eksponert for tunnelvaskevann viste også at PAH var biotilgjengelig ved at mRNA-transkripsjonen av "mixed function oxidase" enzymet cytokrom P450 1A (CYP1A) i lever ble signifikant oppregulert, selv om disse stoffene ofte er sterkt bundet til partikler.

I tillegg til å bekrefte økt ekspresjon av antioksidantforsvaret og CYP1A i fisk eksponert for tunnelvaskevann, DNA-mikromatriseanalysene viste at trafikkrelaterte forurensningsstoffer også kunne undertrykke flere immunologiske prosesser samt inhibere biosyntesen av kolesterol flere timer etter eksponering. Mikromatriseanalysen indikerte også tilstedeværelsen av organofosfater (OP). Dette på grunn av en tilsynelatende oppregulering av enzymet paraoxonase (PON) som er viktig i beskyttelsen av OP-indusert nevrotoksistet. En tilsynelatende oppregulering av arsenitt-metyltransferase (AMT) indikerte at metalloider som f.eks. arsen (As) og Sb var bioreaktive tiltross for at ingen akkumulering av disse metalloidene ble observert i lever.

Korttidseksponeringen av fisk ble gjennomført i ufortynnet og i svakt fortynnet avrenningsvann (f.eks. 50:50 fortynning av utløpsvann fra Skullerud sedimenteringsbasseng). I en virkelig situasjon vil fortynningsfaktoren under normale hydrologiske forhold være større og de biologiske korttidseffektene kunne sannsynligvis være mindre åpenbare. I de senere årene er det imidlertid blitt avdekket at den sommergamle sjøørretpopulasjonen nedstrøms Vassum sedimenteringsbasseng som mottar vaskevann fra Nordbytunnelen, har redusert vekst, noe som indikerer biologiske

langtidseffekter. I gjennomsnitt var individene i sjøørretpopulasjonen nedstrøms sedimenteringsbassenget 21 % kortere i lengde sammenlignet med individene i populasjonen oppstrøms.

Basert på disse resultatene så konkluderes det med at sedimenteringsbassenger ikke nødvendigvis er tilstrekkelig i å beskytte vann og vassdrag det overordnede svaret på hvordan beskytte vann og vassdrag mot forurensing fra vei- og tunnelvaskavrenning. Det er derfor behov for mer forskning på nye og/eller alternative rensestrategier. Det vil også kunne være tilrådelig å etablere "best management practice (BMP)" for eksisterende renseanlegg (f.eks. fjerning av forurenset sediment og/eller dødt plantemateriale), og i tillegg sørge for retningslinjer på hvordan gjennomføre tunnelvasking på en miljømessig forsvarlig måte. Problemstillinger i denne sammenhengen er f.eks. vannvolum, valg av vaskemiddel, vaskehyppighet, samt hvordan unngå vasking i perioder hvor det akvatiske dyrelivet er sårbart (f.eks. smoltifiseringsperioden til anadrome laksefisker). De presenterte resultatene viser også viktigheten av å inkludere kjemisk karakterisering av avrenningsvannet og biologiske parametere i vurderingen av rensegrad, noe som vil være en mer bærekraftig og pålitelig tilnærming sammenlignet med målinger av totale konsentrasjoner.

List of papers

I. Sondre Meland, Reidar Borgstrøm, Lene Sørlie Heier, Bjørn Olav Rosseland, Oddvar Lindholm, Brit Salbu. Chemical and ecological effects of contaminated tunnel wash water runoff to a small Norwegian stream (accepted, Science of the Total Environment).

II. Sondre Meland, Lene Sørlie Heier, Brit Salbu, Knut Erik Tollefsen, Eivind Farmen Finne, Bjørn Olav Rosseland. 2010. Exposure of brown trout (*Salmo trutta* L.) to tunnel wash water runoff – chemical characterisation and biological impact (published, Science of the Total Environment).

III. Sondre Meland, Brit Salbu, Bjørn Olav Rosseland. 2010. Ecotoxicological impact of highway runoff using brown trout (*Salmo trutta* L.) as an indicator model (published, Journal of Environmental Monitoring).

IV. Sondre Meland, Eivind Farmen, Lene Sørlie Heier, Bjørn Olav Rosseland, Brit Salbu, Knut Erik Tollefsen. Hepatic gene expression profile in brown trout (*Salmo trutta*) exposed to traffic related contaminants (submitted, Science of the Total Environment)

List of abbreviations

AADT	Annual average daily traffic
AhR	aryl hydrocarbon receptor
BLM	Biotic Ligand Model
BMP	Best management practice
CAT	Catalase
cDNA	Complementary DNA
CMC / CCC	Criteria maximum concentration / criteria continuously concentration
CYP1A	Cytochrome P450 1A
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EF	Enrichment factor
EQS	Environmental Quality Standards
ERA	Ecological (Environmental) risk assessment
FIAM	Free Ion Activity Model
GCS	γ-glutamylcysteine synthetase
GPX	Glutathione peroxidase
GSH	Glutathione
GSIM	Gill Surface Interaction Model
GST	Glutathione S-transferase
H_2O_2	Hydrogen peroxide
HMM	High molecular mass species
LMM	Low molecular mass species
Log K_{ow}	Log octanol-water partition coefficient
MAC / AA	Maximum allowable concentration / annual average value
MRC	Mitochondrion-rich cells (chloride cells)
mRNA	Messenger-Ribonucleic acid
MT	Cd/Zn-metallothionein
MT-A	Metallothionein A gene
NPRA	Norwegian Public Roads Administration
$O_2^{\cdot -}$	Superoxide radical
$^{\cdot}OH$	Hydroxyl radical
PAH	Polycyclic aromatic hydrocarbons
PCA	Principal component analysis
PCB	Polychlorinated biphenyl

PHAH	Planar halogenated aromatic hydrocarbons
PRC	Principal response curve
pRDA	Partial RDA
PVC	Pavement cells
qrtPCR	Quantitative real-time polymerase chain reaction
RDA	Redundancy analysis
RNS / RCS / RBS	Reactive nitrogen- / -chlorine- / -bromine species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SUDS	Sustainable drainage systems
TOC / DOC	Total- / Dissolved organic carbon
TRX	Thioredoxin
UDPGT	UDP-glucoronosyl transferase
VTG	Vitellogenin
WFD	European Water Framework Directive

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1 Introduction

A modern and well functioning transportation network is fundamental for a modern living, e.g. maintaining vital settlements in rural areas, ensuring proper security for the road users as well as ensuring a safe and secure flow of goods and services. In Norway for example the road network increased from approximately 45 000 km in 1948 to 93 000 km today, while the transportation load increased from 2.5 to 60.6 million passenger km in the same period (OVF 2008). This increased traffic load has undoubtedly a major negative effect on the environment and it contributes significantly not only to emissions of greenhouse gasses, but also to noise, local air, soil- and water pollution.

In 2007 the European Water Framework Directive (WFD) was implemented by law in Norway. This directive points out a new area for the administration of water bodies, by aiming to achieve good ecological and chemical quality by 2015. Diffuse or non-point pollution, including highway runoff, is in this context a major issue, and the Norwegian Public Roads Administration (NPRA) is expected to take part and to make necessary actions regarding the WFD implementation. This because a general principle in the Norwegian governmental policy is that all ministries and all their operational departments has a sectorial environmental responsibility. Although research on highway runoff issues in Norway has been done since early 1980s, the WFD is without doubt a strong incitement for the NPRA to strengthen the knowledge on these matters. The present thesis is therefore considered as part of the ongoing work by NPRA on the WFD, and addresses questions related to highway runoff in terms of exposure characteristics (e.g. source characterisation), environmental impacts (fish toxicity) and mitigation strategies (sedimentation ponds).

The first papers regarding highway runoff were published in the early 1970s, and in 1984 a Norwegian study evaluated the toxicity of highway runoff using a battery of aquatic organisms, including algae, fungi and animals from various phyla (Gjessing et al. 1984). The toxicity was characterized as modest, but the annual average daily traffic (AADT) was rather low (< 10 000 vehicles). Along with an ever increasing traffic density, highway runoff containing a cocktail of contaminants is now of great concern for regulatory authorities as well as for agencies being responsible for planning, building and maintenance of the road network.

An important aspect of highway runoff, but far less described in the scientific literature, is the manmade runoffs from tunnel washings. Washing and cleaning of tunnels are routinely performed to remove dust and dirt and thereby increasing the lifespan of the tunnels. In addition, traffic safety is enhanced by removing oil, grease and particles from the road surface which otherwise may reduce the friction. In Norway, more than 1 000 tunnels having a distance close to 800 km have been built, and many of these tunnels do not have any kind of treatment facility of the contaminated wash water.

As highway runoff has been identified as a significant source of diffuse pollution, much effort has been put on developing measures to mitigate the peak runoff volumes and to mitigate the contamination of the receiving waters (Eriksson et al. 2007). Such measures are typically launched under the concept of Best Management Practice (BMP) or Sustainable Drainage Systems (SUDS), and include facilities such as infiltration system, sedimentation ponds, wetlands and vegetated systems (Lawrence et al. 1996; Pennington et al. 2003). These systems remove pollutants by naturally occurring processes such as sedimentation, biodegradation, sorption, chemical precipitation and biological uptake (Lawrence et al. 1996; Madsen et al. 2006).

The far most applied mitigation strategy along Norwegian roads is to lead the runoff through sedimentation ponds. The first sedimentation ponds were planned and constructed during the 1990s, and during the last 10 – 15 years there has been a substantial increase in the number of treatment facilities along major roads in Norway. Currently, there are close to 150 treatment facilities in Norway, including those under construction and planning. This is still a quite small number compared to for example Sweden where over 400 facilities along their public road network have been introduced (Starzec et al. 2005). Both figures do, however, mirror the effort put on this matters. In addition, the rather large and sudden increase in sedimentation ponds in Norway is also connected to the recent established policy by the Norwegian Public Roads Administration who has developed a set of guidelines for the road planners regarding when measures against highway runoff should be considered applied or not (NPRA 2008). For example, roads expected to have AADT less than 8 000 vehicles is normally planned without any treatment facility, while roads expected to have AADT greater than 8 000 vehicles will, depending on the recipient and AADT, normally be planned and constructed with some kind of treatment facility.

The current practice in new tunnels with high traffic loadings is to treat the tunnel wash water in sedimentation ponds either inside the tunnel or outside. However, the majority of the tunnels in Norway do not have any form of treatment facility other than gully pots, which mainly removes larger particles and coarse material.

The treatment facilities performance (e.g. sedimentation ponds) in terms of reducing the contamination of the receiving waters is considered fairly high 26 – 86 % (e.g. Farm 2002; Vollertsen et al. 2006). However, these numbers are typically based on total concentrations and do not consider that contaminants may appear in various physico- and chemical forms. The removal efficiency of dissolved contaminants, which are considered more mobile and bioavailable than particle bound contaminants, may in fact be questioned. For example, Marsalek et al. (1999) documented that the toxicity in effluents discharged from two storm water treatment ponds was only slightly reduced compared to inlet toxicity measurements. BMP design and performance assessment are generally an engineering driven science (Lawrence et al. 1996) which may explain why

issues related to bioavailability often is neglected, although a major reason for utilizing BMP technology is to protect the aquatic life from chemical and physical perturbation.

Despite the fact that a lot of research has been carried out through the last decades on various topics within the collective term "highway runoff", there are still research gaps to be filled. For example, the U.S. Transportation Research Board published an extended review report aimed to identify research needs related to highway runoff management (Venner et al. 2004). Many of the identified research needs were within topics such as "*Toxicity and Bioassessment*", "*Fate and Transport of Highway Runoff Constituents*", "*Pollutant Retention*" and "*Cold Weather Studies and De-icing Agent Impacts*". Hence, the present thesis aims to bring forth some new knowledge on these topics by linking sources to biological responses with the use of advanced and novel technologies.

1.1 Objectives and structural outline of the thesis

The overall objective of the present thesis was to assess the ecotoxicological effects of naturally and manmade runoffs from open road areas and tunnels in Norway. This objective was met by addressing four sub-objectives, where the scientific work has been presented in four individually papers (Papers I – IV):

1. Identify sources by performing chemical characterisation of discharged tunnel wash water (Paper I).

2. Assess the toxicity of highway and tunnel wash water runoff by using fish as a model organism (Papers II, III and IV).

3. Assess whether sedimentation ponds reduces the toxicity of highway runoff (Paper III).

4. Exploration of the effects of traffic related contaminants on biological and molecular pathways in fish by using toxicogenomics (Paper IV).

An outline of the four individual papers is depicted in Figure 1. The two cases in the present thesis considering naturally and manmade runoffs are limited to cover chemically acute (Papers II, III and IV) and chronically (Paper I) induced effects on fish. The experiments were conducted at sites having sedimentation ponds, mainly for two reasons:

1. The exposure water utilised in the fish experiment was considered to be unaffected by anthropogenic sources other than the roads and the tunnels.

2. Knowledge about the functioning of the treatment facilities, would allow information on the contaminant removal efficiencies and toxicity reductions to be gained.

Although sediment contamination and sediment toxicity to benthic organisms are important aspects of highway runoff, these were not within the frame of the current PhD work.

Chapter 1 gives a brief introduction of the topic and in addition a brief presentation of the research objectives together with the organisation of the thesis. Chapter 2 presents a brief literature review, covering chemical and biological issues related to highway and tunnel wash water runoff. The experimental outline, field work and analytical methods are presented in Chapter 3, while Chapter 4 provides a methodological consideration. A brief summary of the four papers including the major findings is given in Chapter 5. The overall results are briefly discussed in Chapter 6. Finally, Chapter 7 draws conclusions and future perspectives based on the main findings.

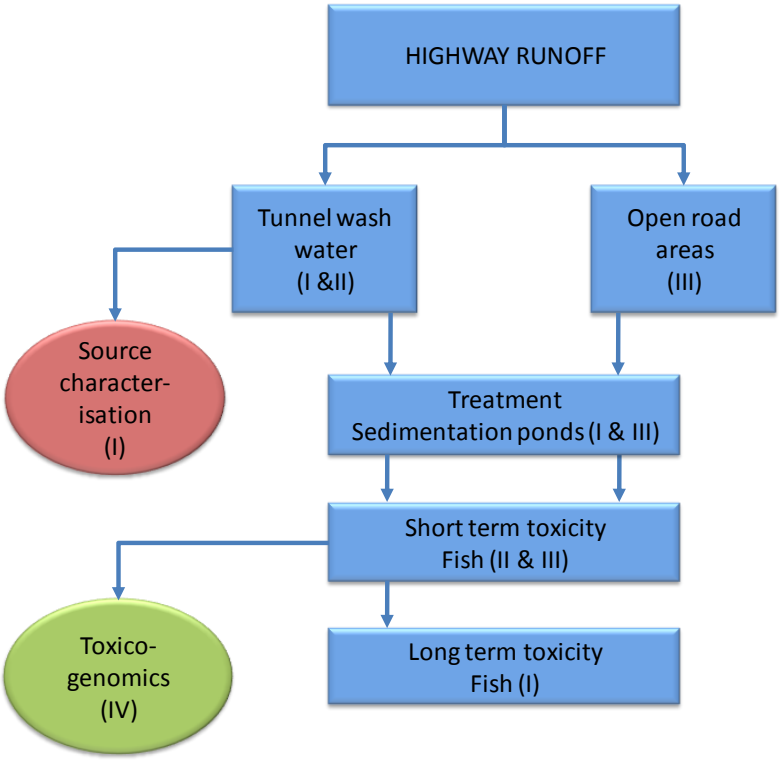

Figure 1. Outline of the four papers (I – IV) included in this thesis.

2 Background for the thesis

2.1 Runoff processes

The hydrological runoff patterns from impervious areas such as paved roads and highways are distinctly different from those normally occurring in natural vegetated areas. One major hydrological feature in impervious areas is the so-called "first flush", a phenomenon which is assigned to the rapid and high increase in the pollutant concentrations and/or masses found in the initial phase of a runoff episode with a subsequent rapid decline in concentrations and/or masses (Sansalone & Buchberger 1997). In addition, Kayhanian et al. (2008) demonstrated that highway runoff was generally most toxic in the initial phase of a storm event. However, a united definition of the first flush term seems still missing (Han et al. 2006; Sansalone & Buchberger 1997; Sansalone & Cristina 2004). Nevertheless, several authors have reported the occurrence of first flush events in highway runoffs (e.g. Han et al. 2006; Sansalone & Buchberger 1997; Sansalone & Glenn 2000; Shinya et al. 2000), while others have not (e.g. Farm 2002; Waara & Farm 2008).

Highway runoff shows a great deal of variation in terms of concentration and masses of various contaminants. Figure 2 gives a brief and general introduction of the transport mechanisms involved.

Figure 2. Conceptual drawing over highway runoff transportation.

Factors which are likely to affect the concentration and mass fluxes in highway runoff are variation in weather and climate conditions (e.g. time between episodes, rain and snow fall patterns, seasonality such as summer vs. winter), variations in traffic parameters such as loads, driving speed, relationship between light- and heavy duty vehicles, amount of studded tires during winter etc. Also variation in runoff area characteristics such as size and percentage impervious area, type and age of the pavement (e.g. mineralogy of the aggregates in the pavement) are important. Finally, variations due to road maintenance activities such as road cleaning, de-icing, dust binding etc. As an example, the vehicular dust production as a function of studded tires and driving speed is shown in Figure 3. Hence, the stochastic nature of highway runoff makes it difficult to compare results from various studies.

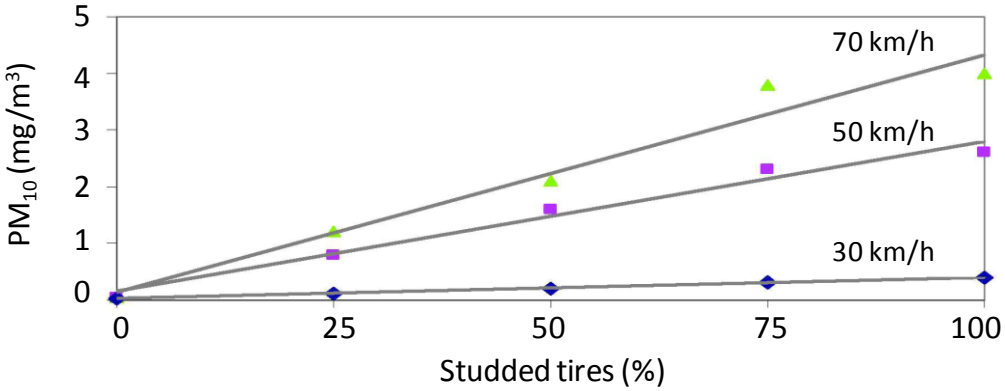

Figure 3. Air dust (PM_{10} = particles with aerodynamic diameter less than 10 µm) as a function of studded tires and driving speed. After Snilsberg (2008).

In contrast to the naturally occurring runoff processes in open road areas, manmade tunnel wash water runoff has received significantly less attention, although such runoffs have shown to be highly polluted (Andersen & Vethe 1994; Barbosa et al. 2006; Paruch & Roseth 2008a; Paruch & Roseth 2008b). A major difference between naturally occurring runoffs in open road areas and manmade tunnel wash water runoffs is the meteorological factor, i.e. the micro climate inside the tunnels are less affected by precipitation, wind, sunlight and large temperature fluctuations. Hence, contaminants in tunnels have been accumulated over time (months), and wash water runoffs are likely more concentrated than in runoffs from open road areas where precipitation frequently washes the pavement.

Washing and cleaning are a frequently conducted maintenance task in many tunnels, e.g. the washing frequency in major Norwegian tunnels ranges from 2 – 12 times per year

depending on traffic density and tunnel size. The cleaning and washing are performed typically by removing dust, debris and coarse material with a road sweeping machine prior to the detergent application and high pressure cleaning. Information provided by contractors indicates that the water volume utilised during a wash ranges between 60 – 100 L/m in a tunnel consisting of two tubes and two driving lanes in each tube. The amount of detergents sums up to roughly 0.5 – 1% of the total water consumption. Hence, up to 100 m^3 of polluted wash water, containing up to 1 m^3 detergents can potentially be discharged during cleaning of a 1 km long tunnel, a process lasting 5 – 6 h.

2.2 Chemical contaminants in highway and tunnel wash water runoff

Highway runoff typically contains a broad range of contaminants, both organic and inorganic compounds. In addition, the contaminants originate from multiple sources. For example, some contaminants (e.g. antimony (Sb) and zinc (Zn)) have been assigned to originate from various vehicle components such as brakes and tires, while others originate mostly from combustion and road surface wear (e.g. naphthalene and iron (Fe)). Based on recent published material, Table 1 presents data on a range of traffic related contaminants included in this thesis. Their most likely sources are also displayed. A common approach to differentiate between vehicle and non-vehicle derived contaminants is to calculate enrichment factors (EF) and/or to use concentration ratios between compounds, a topic covered in Paper I by using tunnel wash water runoff. The use of tunnel wash water runoff in the EF calculations was preferred as the influence of contaminants from long-range transported air pollutants was then minimized.

Table 1. Information of highway runoff contaminants and their sources based on literature data. Contaminants in **bold** are included in the present thesis (not all contaminants are included in all papers).

Source		Contaminant[1]	References
Vehicle	Brakes	**Ba**, **Cu**, **Fe**, **Mo**, **Na**, **Ni**, **Pb**, **Sb**	Dongarra et al. 2009; McKenzie et al. 2009; Sternbeck et al. 2002; Thorpe & Harrison 2008
	Tires (incl. studded tires)	**Al, Zn**, **Ca**, **Cd**, **Co**, **Cu**, **Mn**, **Pb**, W, **hydrocarbons**, **PAH** (pyrene, fluoranthene, benzo(ghi)perylene)	Glaser et al. 2005; Karlsson & Viklander 2008; Lindgren 1998; McKenzie et al. 2009; Ravindra et al. 2008; Sternbeck et al. 2002; Thorpe & Harrison 2008
	Catalytic converters	Pt, Pd, Rh	Ek et al. 2004; Whiteley & Murray 2005
	Vehicle body	**Cr**, **Fe**, **Zn** (steel)	Taylor & Robertson 2009
	Combustion	Ag, **Ba**, **Cd**, **Cr**, **Co**, **Mo**, **Ni**, **V**, **Sb**, Sr, **Zn**, **PAH** (naphthalene), MTBE, BTEX	Brown & Peake 2006; Desta et al. 2007; Glaser et al. 2005; Lin et al. 2005; Marr et al. 1999; Ravindra et al. 2008; Wang et al. 2003; Weckwerth 2001
	Oil and petroleum spill, dripping, used lubricant oil	**PAH** (LMM)	Ravindra et al. 2008; Wang et al. 2000
Non-vehicle	Road surface (asphalt, bitumen)	**Al**, **Ca**, **Fe**, **K**, **Mg**, **Na**, **Pb**, **Si**, Sr, Ti, **PAH** (mix of HMM and petrogenic LMM, chrysene)	Brandt & de Groot 2001; Brown & Peake 2006; Sternbeck et al. 2002; Thorpe & Harrison 2008
	De-icing and dust suppression	**Ca**, **Mg**, **Na**, **Cl**, ferro-cyanide (anticaking agent)	Aldrin et al. 2008; Novotny et al. 2008; Ramakrishna & Viraraghavan 2005; Viklander et al. 2003
	Road equipment (e.g. crash barriers, traffic signs etc.)	**Zn** (galvanised steel)	Thorpe & Harrison 2008
	Detergents used in tunnel wash	**Tensides**	Paper II in the present thesis

[1]Abbrevations: Ag=silver, Al=aluminium, Ba=barium, BTEX= benzene, toluene, ethylbenzene and xylenes, Ca=calcium, Cd=cadmium, Cl=chloride, Co=cobalt, Cr=chromium, Cu=copper, HMM=high molecular mass species, K=potassium, LMM=low molecular mass species, , Mg=magnesium, Mn=manganese, Mo=molybdenum, MTBE=methyl tert-butyl ether, Na=sodium, Ni=nickel, Pb=lead, Pd=palladium, Pt=platinum, Rh=rhodium, Si=silicon, Sr=strontium, Ti=thallium, Zn=zinc

2.2.1 Metals

Trace metals are probably the most frequently reported group of contaminants in highway runoff studies, reflecting the concern of metal contamination of the aquatic environment due to their potentially toxicity towards aquatic organisms. Opposite to organic contaminants, they are neither created nor destroyed by biological or chemical processes, and in addition, some metals (e.g. Cu and Zn) are nutritionally essential at low levels (Fairbrother et al. 2007). An important feature of metals and metalloids are their appearance in a range of different physico-chemical forms (i.e. metal species). Hence, trace metal species are defined according to their physico-chemical properties such as nominal molecular mass, charge properties and valence, oxidation state, structure and morphology, density, degree of complexation etc. (Figure 4) (Salbu 2009).

Consequently, the mobility, bioavailability and toxicity of metals are strongly related to their speciation (Fairbrother et al. 2007). For example, there is a broad consensus in the scientific community that low molecular mass species (LMM) are considered mobile and potentially more bioavailable than high molecular mass species (HMM) such as colloids, polymers, pseudocolloids and particles. The aquatic system is dynamic and the partitioning between various species of a certain metal are largely influenced by water quality variables such as pH, ionic strength, redox potential, water temperature, suspended solids and inorganic and organic ligands such as carbonates and organic matter (Fairbrother et al. 2007; Salbu & Oughton 1995). This is of special interest when studying episodic events where nonequilibrium mixing zones may occur (e.g. Lydersen et al. 1994; Rosseland et al. 1992; Teien et al. 2008; Teien et al. 2004), involving molecular mass growth mechanisms (e.g. hydrolysis, complexation, polymerisation, colloid formation and aggregation) and/or mobilization processes (e.g. desorption, dissolution, dispersion) (Salbu 1987; Salbu 2009) (Figure 4). Currently, only a limited number of highway runoff studies exist where metal speciation has been conducted beyond the well established 0.45 μm filtration procedure to separate particles from dissolved species (Bechet et al. 2006; Durin et al. 2007; Flores-Rodriguez et al. 1994; Harrison & Wilson 1985; Karlsson et al. 2009; Tuccillo 2006).

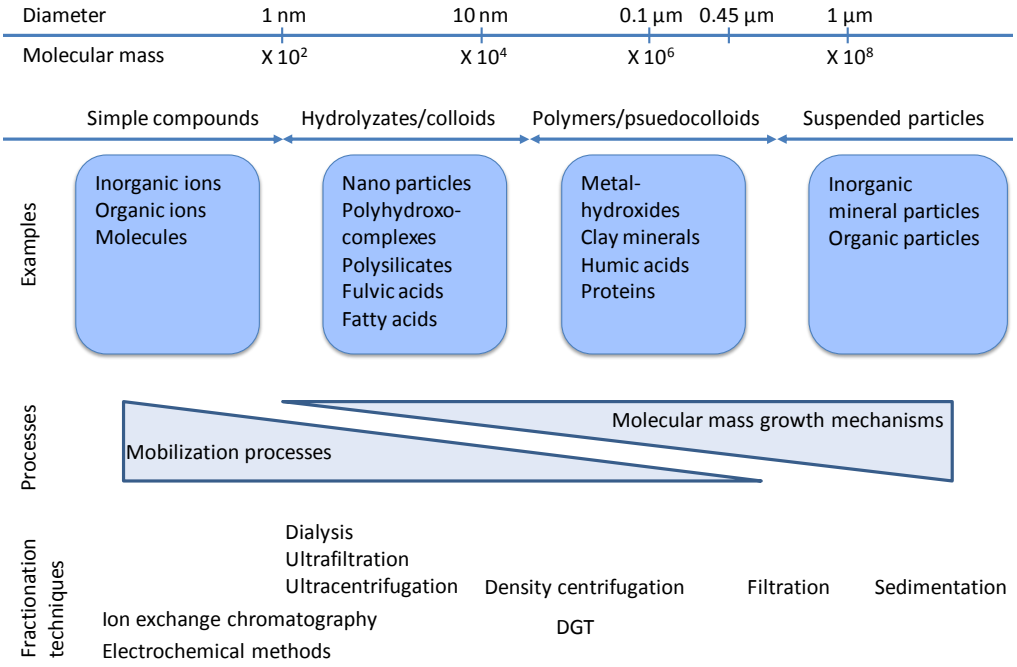

Figure 4. Association of metal and metalloid species with compounds in different size ranges. Molecular mass growth mechanisms include hydrolysis, complexation, polymerization, colloid formation and aggregation. Mobilisations mechanisms include desorption, dissolution and dispersion. Modified after Salbu (1987; 2009) and Salbu and Oughten (1995).

2.2.2 Polycyclic aromatic hydrocarbons (PAH)

PAHs are probably the organic contaminants having received greatest attention, and the release of automobile derived PAHs into the environment seems increasing (Beasley & Kneale 2002; Napier et al. 2008). PAH as a group consists of approximately 100 semivolatile compounds and all consist of two or more fused aromatic benzene rings (Baek et al. 1991; Gehle 2009; Srogi 2007). In addition, most of the PAHs can be photo-oxidized and degraded to simpler substances (Baek et al. 1991; Gehle 2009). PAHs differ in physical (e.g. vapour pressure, solubility, octanol-water partition coefficient (log K_{ow})) and chemical properties (e.g. resistance to oxidation and reduction), in part, due to differences in molecular masses (Baek et al. 1991; Logan 2007). For example, PAHs of five rings and more have low solubility and low vapour pressure and are therefore often predominantly associated with particles, whereas PAHs with two and three rings are more volatile (Baek et al. 1991; Srogi 2007) and can for example be found in high mountain areas after transportation by air (Grimalt et al. 2001; Vives et al. 2004).

PAHs are often divided into three or four classes based on their environmental origin: PAHs formed by combustion of organic matter (e.g. fossil fuels) are classified as pyrogenic, PAHs formed by geological processes in the earth (e.g. petroleum) are classified as petrogenic, and finally PAHs formed in peat lands and sediments from biogenic compounds (e.g. anaerobic processes) and in sediments directly by organisms are classified as diagenic and biogenic, respectively (Logan 2007). Their gross attention, both scientifically and regulatory, is due to their widespread distribution in air, soil and water, and due to their biological reactivity being a serious risk to all living organisms due to carcinogenic and mutagenic properties. As a rule of thumb, the carcinogenicity of PAHs increase, while the acute toxicity decrease, with increasing number of rings (increased molecular mass) (Ravindra et al. 2008). Presently, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene and dibenzo(ah)anthracene are known for their carcinogenic properties (Ravindra et al. 2008; Srogi 2007).

2.2.3 Road de-icing salt

Norway and other countries on the northern hemisphere use significant amount of de-icing chemicals to enhance good friction and thereby increasing the traffic safety on the roads during the winter period. The far most applied de-icing chemical in Norway is NaCl (99.5 %), and over the past few years the total use of NaCl on the major roads has substantially increased (NPRA 2007; NPRA 2009). In the winter season 2000/2001 70 000 tons of NaCl were used on Norwegian roads, while in 2008/2009 the consumption was nearly 200 000 tons, approximating 14 tons/km. Thus, the salt concentrations can be rather high during runoff episodes.

Several papers have addressed the concern of high salt concentrations found in highway runoff. These concerns may be categorized in different biological effects such as increased drift of lotic macroinvertebrates as a response to acute elevated salt concentrations (Crowther & Hynes 1977) and altered blood physiology in fish as demonstrated in a laboratory study by Vosyliene et al. (2006). In addition, elevated salt concentrations may cause chemical effects by mobilizing trace metals through ion exchange processes and by causing oxygen depletion in lakes and ponds (Amrhein et al. 1992; Bäckström et al. 2004; Lofgren 2001). Finally, high salt concentrations may cause physical effects such as altered circulation patterns in lakes and ponds due to increased density of the salt enriched bottom dwelling layer (Kjensmo 1997; Novotny et al. 2008).

2.3 Multiple stressors and biological responses

Environmental contaminants have the potential to harm and disturb organisms in a multiple way causing toxic effects in cells, tissues and vital organs. This may further impair growth, fitness, reproduction etc which in a long term may have a negative effect on populations and communities. In real life, pollutants often occur together and the

toxicity of such mixtures may act additively (e.g. 1 + 1 = 2), synergistically (e.g. 1 + 1 > 2) or antagonistically (e.g. 1 + 1 < 2) (Eggen et al. 2004; Salbu et al. 2005). Traditionally, regulatory authorities performing Ecological (environmental) Risk Assessment (ERA) are usually evaluating one stressor at time, or if mixed toxicity is considered, additive effects are often assumed.

The following review is not comprehensive but gives a brief introduction to some few important aspects that are essential in the present thesis, of water pollution and physiological effects on fish. In addition, the concept of biomarkers is introduced being a toolkit for bridging the gap between exposure and early responses, i.e. responses before the stage of more detrimental effects (e.g. community changes).

2.3.1 Bioavailability

As indicated in Chapter 2.2, the bioavailability of contaminants is influenced by several water quality variables. For example, the accumulation of metals in biota depends on the speciation and is strongly modified by the presence of ligands in water such as organic matter (TOC/DOC) and suspended solids being able to complex dissolved metal species in water. In addition, high levels of major metal cations (e.g. Ca) also modify the accumulation and uptake of metals as major cations are excellent competitors with high binding strength to biological membranes resulting in reduced bioavailability (e.g. fish gills) (Chapman 2008; Fairbrother et al. 2007; Grosell et al. 2006; Hollis et al. 1997; Niyogi & Wood 2004; Rosseland & Staurnes 1994). Finally, pH may be considered as the most important modifier due to its major influence on metal speciation (Chapman 2008; Fairbrother et al. 2007). Like for metals, the bioavailability of PAHs is reduced if the content of organic and inorganic ligands increases (Logan 2007).

The definition of *bioavailability* is not 100 % consistent in the scientific literature; Chapman (2008) defines it as the portion of a compound that is immediately available for uptake by an organism, Hare (1992) defines it as the portion of a compound that can potentially be taken up and finally Fairbrother et al. (2007) define it as the proportion of a compound that absorb onto, or into and across biological membranes of organisms. According to Erickson et al. (2008) bioavailability is a relative term and mirror the portion of a chemical species contributing, directly or indirectly, to absorption. Partly because of this existing discrepancy in the literature, they launched a more broad definition of the term bioavailability: "*the relative facility with which a chemical is transferred from the environment to a specific location in an organism of interest*". This definition was reasoned from four important factors important for further assessment of the term, and they can be briefly summarised as:

1. Chemical uptake, and thus bioavailability, depends on certain morphological, physiological and biochemical attributes of an organism.

2. Bioavailability must be referenced to a specific chemical concentration in the organism of interest, such as total chemical concentration in the entire organism or in a specific tissue/organ, or the chemical concentration associated with a specific molecular receptor.

3. Bioavailability must be referenced to a specific environmental concentration, e.g. total chemical concentration, with bioavailability being considered as an aggregate property of the combined chemical species.

4. Transfer pathways of interest must be specified, e.g. an assessment may consider a single or all routes of exposure.

In the experiments presented in Papers II and III the major pathway for contaminants is through the gills, and the term *gill reactivity* is there used to describe the accumulation of a chemical compound on the entire gill organ including mucus, blood soft- and bone tissue.

In terms of metal bioavailability and ERA, several models have been developed to bridge the gap between the aquatic chemistry representing the exposure situation and the physiology representing the biological/ecological impairment (Paquin et al. 2002). Examples of such models are the Free Ion Activity Model (FIAM), the Gill Surface Interaction Model (GSIM), and perhaps the currently most popular model, the Biotic Ligand Model (BLM) (Niyogi & Wood 2004; Paquin et al. 2002). The latter combines the influences of speciation (e.g. DOC complexation) and cation competition on metal toxicity in e.g. fish. Although recent progress has been made (Kamo & Nagai 2008), most of these models are originally built for single compounds during more or less acute water borne exposure situations and do not incorporate the more realistic real life situation, where an orchestra of contaminants may play together episodically and/or chronically and interact, in terms of toxicity, additively, synergistically and/or antagonistically (Eggen et al. 2004; Fairbrother et al. 2007; Salbu et al. 2005). Highway and tunnel wash water runoffs, contain a vast number of contaminants, typically representing a real life situation, being able to cause multiple biological effects through series of interactions with multiple target sites. For example, elevated levels of Cd can inhibit Zn uptake from the ambient water, but Zn seems not to exclude Cd uptake (Bentley 1991; Bentley 1992). Another example of the unpredictable outcome of multiple stressors was demonstrated by Fleeger et al. (2007), where the joint exposure effects of binary metal-PAH mixtures (e.g. Cd and phenanthrene) on benthic copepods were synergistic while multiple metal-PAH mixtures (i.e. Cd, Hg, Pb, fluoranthene and phenanthrene) seemed to moderate the effects. Hence, the prediction of mixed toxicity is still a major challenge in the ERA context, and although *in situ* experiments were performed during the present work, they were not exclusively designed with the aim of addressing this challenge.

2.3.2 Gill toxicity; ion regulatory and respiratory dysfunctions

In freshwater fish, there is a constantly osmotic and ionic gradient across the branchial epithelia due to difference in osmolarity between the ambient water and the blood plasma (Evans et al. 1999; Rosseland & Staurnes 1994). The diffusional loss of Na and Cl ions and the osmotic influx of water into cells are counteracted and controlled by active uptake of the very same ions, and by producing dilute urine excreting the water in excess (Wendelaar Bonga & Lock 1992). These processes occur mainly in cells with high metabolic activity termed mitochondrion-rich cells (MRC, previously and often still termed chloride cells) which contain various transport enzymes and apical channels, e.g. Na/K-ATPase and Na-Cl co-transporters, respectively (Evans et al. 1999; Evans 2008; Perry & Gilmour 2006). Any damage to the gills, caused by chemical or physical perturbations, will have immediate effects on this well tuned osmoregulatory system (Wendelaar Bonga & Lock 1992).

As fish does not drink freshwater, there is a broad consensus in the scientific community that the main site of action regarding toxicity from water borne contaminants takes place in the gills, which is the case both for inorganic as well as organic compounds (Evans 1987; Niyogi & Wood 2004; Paquin et al. 2002; Rosseland & Staurnes 1994; Wendelaar Bonga & Lock 1992). One reason for that is the gill anatomy having a large surface area with thin epithelial membranes working in a countercurrent water blood system, which facilities a delicate site for uptake of contaminants from the water and further transfer to the blood (Evans et al. 1999; Wendelaar Bonga & Lock 1992). For example, in a trout weighing 250 g, 48 L/h of water passes the gills during normal metabolic conditions (Reid & McDonald 1991).

Toxic compounds may cause structural damages on the gill tissue such as uplifting of the lamellar epithelium from the underlying tissue, necrosis of MRC and pavement cells (PVC), epithelial swelling by intercellular spaces, rupture of the epithelium and lamellar fusion, are accompanied with loss of Na^+ and Cl^- ions from the blood plasma (Evans 1987; Peuranen et al. 1994; Wendelaar Bonga & Lock 1992; Wilson & Taylor 1993). There are mainly two causes leading to a reduction in plasma ions. Firstly an increased passive efflux of ions across the gills due to increased membrane permeability or disruption of the membrane, and secondly an inhibition of active ion uptake by the MRCs (e.g. inhibited carbonic anhydrase and inactivated Na/K-ATPase) (Lauren & McDonald 1985; Lauren & McDonald 1987; Rogers et al. 2005; Rosseland & Staurnes 1994; Wendelaar Bonga & Lock 1992). In addition, cell necrosis and apoptosis are also observed in acute metal exposed fish (Li et al. 1998; Rosseland & Staurnes 1994; Wendelaar Bonga 1997). The increased membrane permeability is related to some metals (e.g. Cu and Al) ability to displace Ca^{2+} from the anionic sites of the intercellular cement allowing Na^+ and Cl^- to diffuse through the tight junctions within the gill epithelium (Evans 1987; Wendelaar Bonga & Lock 1992). Opposite to metals, there seem to be no specific effect of organic contaminants on the ion-transporting mechanisms but loss of

plasma ions are rather caused by a nonspecific damage to the gill epithelium (Wendelaar Bonga & Lock 2008).

A third, and possibly more indirect reason for ion loss, is the induction of a stress response which is rapidly followed by a release of catecholamine hormones (e.g. adrenaline and noradrenalin) into the blood and a subsequent raise in blood pressure in the gills causing for example lamellar perfusion and secondly influx of water (Wendelaar Bonga & Lock 1992; Wendelaar Bonga 1997; Wendelaar Bonga & Lock 2008; Wilson & Taylor 1993). Presently there are indications that, at least some metals can stimulate the proliferation of new MRCs (hyperplasia) in the epithelium to compensate the loss of plasma ions or to replace necrotic and/or apoptotic MRCs (Wendelaar Bonga & Lock 1992). An increased density of MRC which might be beneficial for the ionic regulation, will, however, increase the blood to water diffusion barrier which in the end may disturb normal respiration by impairing the gas transfer (O_2 and CO_2) and indirectly the acid – base metabolism (CO_2, H^+ and HCO_3^-) (Laurent & Dunel 1980; Perry et al. 1992; Perry 1998).

Presently, it seems like trace metals such as Ag, Cd, Cu, Fe, Pb appear mainly as ion regulatory toxicants (McGeer et al. 2000; Peuranen et al. 1994; Pilgaard et al. 1994; Rogers et al. 2003), and according to Pilgaard et al. (1994) many metals have higher threshold for impairing respiration compared to impairment of the osmoregulatory system. This is in contrast to Ni which acts as a respiratory toxicant by damaging the respiratory epithelium, including swelling (hypertrophy) of the secondary lamellae, and decreased lamellar height together with an increased lamellar width reducing the area for gas diffusion (Pane et al. 2003; Pane et al. 2004). Typically, respiratory effects are manifested by low arterial O_2 tension (pO_2), high arterial CO_2 tension (pCO_2) and blood acidosis (Wood et al. 1988). Finally, Zn and Al (pH dependent) seems to have dual effects, and may potentially impair ion regulation as well as respiration (Gensemer & Playle 1999; Rosseland & Staurnes 1994; Spry & Wood 1985).

2.3.3 Biotransformation and bioactivation of PAH

The accumulation of PAHs in fish, which mostly is driven by passive diffusion, is mainly through the gills, gastrointestinal tract and to some extent through the skin (Logan 2007). Once absorbed, PAHs can, due to their lipid soluble properties, readily cross biological membrane (e.g. the gill surface and the intestinal tract) by diffusion into the blood. The uptake rate is largely dependent on the hydrophobic properties of the specific PAH compound, i.e. the log octanol-water partition coefficient (log K_{ow}). Higher log K_{ow} values indicate more lipophilic character and therefore more easily transported across biological membranes. However, this is only true up to a certain threshold because the molecule size will finally limit the uptake rate (Kleinow et al. 2008). Within the blood, the PAHs are further transported to tissues by diffusion. PAHs are compared to many other more persistent organic contaminants (e.g. polychlorinated biphenyls (PCBs)) efficiently

metabolized and detoxified by the cells, and do in general not bioaccumulate or biomagnify (Jonsson et al. 2006; van der Oost et al. 2003). Hence, measuring PAH concentrations in tissues is not recommended for field monitoring purposes (van der Oost et al. 2003).

The detoxification of PAH occur to some degree in all major organs, although the liver is considered most important in that respect, and the exposure to PAH is therefore in vertebrates measured through their breakdown products (metabolites) in bile (Logan 2007; Pollak 1998; van der Oost et al. 2003). The PAH metabolism is bioactivated by interacting with the intracellular aryl hydrocarbon receptor (AhR) which sets off the phase I, in a two phased biotransformation process. These multiple step processes are catalytic driven and make the PAHs normally less toxic, more water soluble and thereby more readily excreted from the body (van der Oost et al. 2003).

In brief, during phase I the PAHs are oxidized by membrane bound mixed functioning oxidase enzymes, known as cytochrome P450 (e.g. CYP1A), by adding a hydroxyl or epoxide group to the molecule. This is subsequently facilitated by conjugation with larger endogenous molecules in the phase II such as UDP-glucoronosyl transferase (UDPGT) and glutathione S-transferase (GST). Finally, the PAH metabolites are then excreted mainly from the gills or by secretion of bile (Kleinow et al. 2008; Reynaud & Deschaux 2006). However, during phase I, several reactive intermediates are produced being often more toxic than the parent compound (Pollak 1998; van der Oost et al. 2003; Xue & Warshawsky 2005). This may be exemplified with the PAH benzo(a)pyrene which is oxidized by the CYP1A to carcinogenic and mutagenic intermediates such as benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide, quinones and subsequently generation of reactive oxygen species (ROS) due to redox cycling, radical banzo(a)pyrene cations and benzylic carbenium ions (Flowers et al. 1997; van der Oost et al. 2003; Xue & Warshawsky 2005). All are more or less capable to interact with the DNA, causing DNA and protein adducts and DNA fragmentation which may be detrimental for organisms (Flowers et al. 1997; Xue & Warshawsky 2005). For example, observations of tumour epizootics in wild fish populations linked to PAH contamination were already reported in early 1980s (Baumann 1998), and according to Logan (2007) and to Reynaud and Deschaux (2006) detrimental PAH related effects on fish include e.g. mortality in all life stages, decrease in growth, reduced condition factor, edema, cardiac dysfunction, deformities, cataracts, immune system dysfunctions and estrogenic effects.

2.3.4 Free radicals and oxidative stress

Aerobic organisms cannot live without sufficiently amount of oxygen (O_2) as it is fundamental for an efficient energy production by electron transport chains that ultimately donate electrons to O_2 (Halliwell & Gutteridge 2007). However, oxygen in its atomic form (O) is a free radical which during metabolic processes can generate several reactive intermediates (also termed reactive oxygen species (ROS)). Reactive nitrogen

species (RNS), reactive chlorine species (RCS) and reactive bromine species (RBS) are also free radicals). A simple definition of a free radical, launched by Halliwell and Gutteridge (2007), is: "*a free radical is any species capable of independent existence (hence the term "free") that contains one or more unpaired electrons*". Examples of intermediates that may cause deleterious effects in cells are superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (·OH) (Martinez-Alvarez et al. 2005). For example, 1 – 3 % of the O_2 reduced in the mitochondrial electron transport chain may form $O_2^{\cdot-}$ (Halliwell & Gutteridge 2007). Hence, organisms totally dependent on oxygen are inherently at risk due to oxidative stress (Davies 2000), and is populistic phrased as the "oxygen paradox".

Oxidative stress is broadly defined as an imbalance between ROS and the antioxidant defence system which includes a battery of enzymes and repair mechanisms. This imbalance is manifested either as an excess of ROS and/or as a deficit of antioxidants which may cause cell damages such as oxidations of membrane lipids, proteins and DNA or even cell death (Felton & Summers 1995; van der Oost et al. 2003). A brief outline of the mechanisms and pathways involved when cells are exposed to oxidants and free radicals is depicted in Figure 5.

In addition to normal aerobic homeostasis, ROS and oxidative stress can originate from various inorganic as well as organic contaminants, e.g. transition metals such as Cu and Fe, and planar aromatic hydrocarbons, e.g. PAH (Figure 6). Increased ROS generation by transition metals appear through a pathway including Fenton chemistry where ·OH is formed via the catalytic presence of for example Cu and Fe (Halliwell & Gutteridge 2007):

$$Cu^{+}/Fe^{2+} + H_2O_2 \rightarrow \text{intermediate oxidizing species} \rightarrow Cu^{2+}/Fe(III) + {}^{\cdot}OH + OH^{-}$$

The CYP1A metabolic activation pathway where PAH quinones are generated is also believed to be a significant donor to ROS, and Fenton chemistry is involved (Flowers et al. 1997; Xue & Warshawsky 2005). During redox cycling, an initial enzymatic (DT diaphorase) one-electron oxidation of the parent compound produces a semiquinone anion radical and hydrogen peroxide (H_2O_2) which is subsequently followed by a second one-electron oxidation resulting in a fully oxidized quinone and a $O_2^{\cdot-}$ (Xue & Warshawsky 2005). Once formed, quinones can undergo multiple redox cycling and thus generate ROS several times (Flowers et al. 1997).

In addition to the PAH quinone pathway, ROS may be produced by occasionally uncoupling of the electron transfer and O_2 reduction during the CYP1A biotransformation cycle with $O_2^{\cdot-}$ and H_2O_2 as the outcome (Di Giulio & Meyer 2008). However, this seems to be only valid for contaminants such as planar halogenated aromatic hydrocarbons (PHAHs) and PCBs and not for PAHs (Schlezinger et al. 1999; Schlezinger et al. 2006).

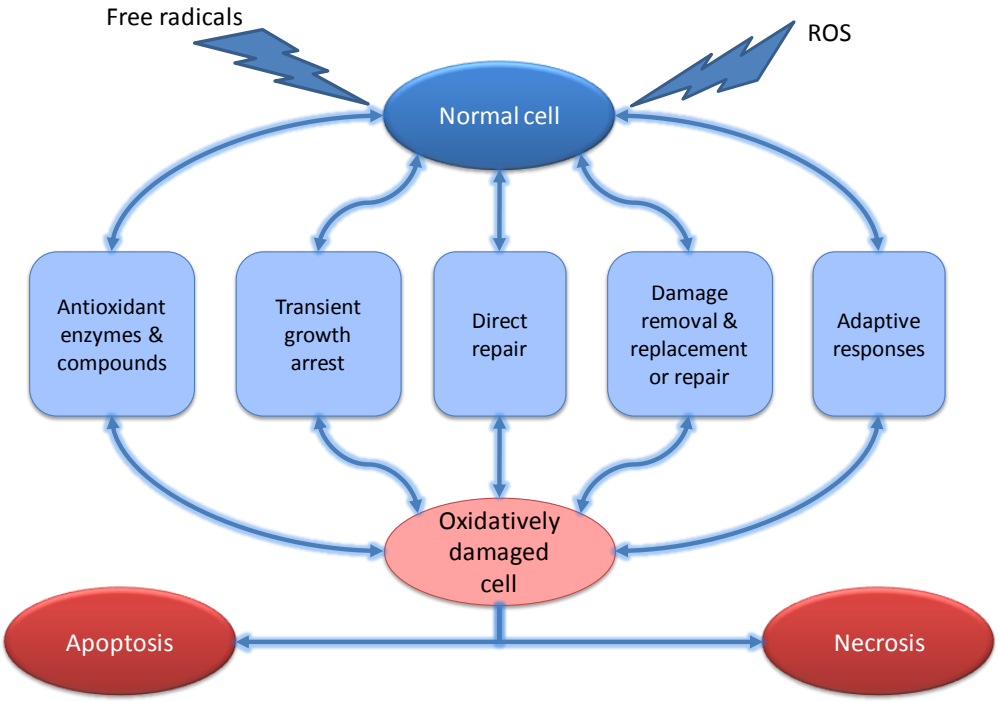

Figure 5. A conceptual model depicting oxidative stress and antioxidant defense system, modified after Davies (2000). A normal mitotic eukaryotic cell converted into an oxidatively damaged cell after ROS exposure, which then dies by either apoptosis or necrosis. The antioxidant defense system acting against cell damaging includes antioxidant enzymes and compounds (primary defenses) and the facility of mitotic cells to enter a protective transient growth arrested state. If insufficient, damaged proteins, lipids and DNA will undergo direct repair or they will be partially or completely degraded and then repaired or replaced. Meanwhile, a series of temporary adaptive responses will take place as programmed cell death (apoptosis) or direct cell death (necrosis).

Along the evolutionary road, organisms became adapted to utilize the highly reactive O_2 in the energy production. Hence, all aerobic organisms needed to synchronically develop defence systems against metabolically produced ROS to prevent oxidative stress (Figure 5 and Figure 6). This system is collectively known as the "antioxidant defence system" and includes important antioxidant compounds acting as scavengers such as vitamin A, E and C, uric acid, glutathione (GSH), ferritin (Fe regulatory), ceruplasmin (Cu regulatory) and metallothionein. In addition, the defence system consists of numerous

antioxidant enzymes working directly or indirectly on ROS, e.g. superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GSH reductase), GST, γ-glutamylcysteine synthetase (GCS), thioredoxin (TRX) and DT diaphorase (quinone reductase) (Halliwell & Gutteridge 2007). Finally, cellular processes like transient growth arrest, various directly and indirectly repair mechanisms and adaptive responses are all crucial aspects in the antioxidant defence system (Davies 2000). Many of the above mentioned enzymes and proteins are now frequently used as biomarkers for oxidative stress in environmental science, including the present thesis (Papers II, III and IV).

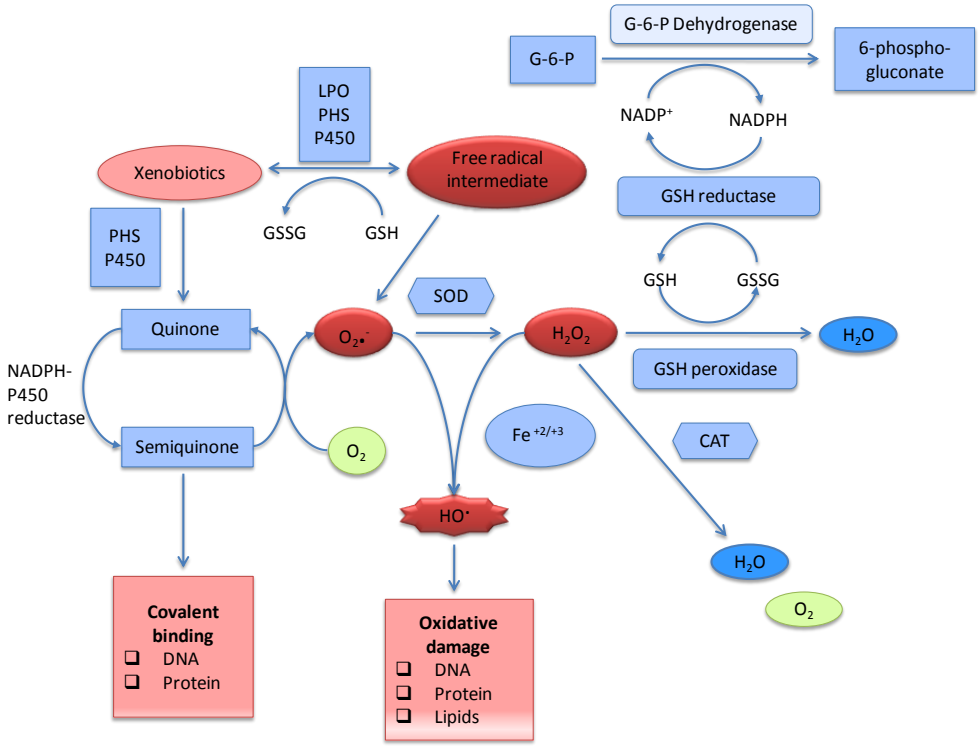

Figure 6. Overview of contaminant induced oxidative stress, antioxidant enzymes and catalytic reactions involved in the antioxidant defense system. For abbreviations, see the abbreviation list, page 9. Adopted from Sigma-Aldrich (www.sigmaaldrich.com/catalog).

2.3.5 Energy metabolism

It is obvious that chemical perturbations impairing normal cellular metabolism in various organs may escalate and be expressed at a higher biological level (e.g. individual), which may be exemplified by reduced growth, decreased immune defence and condition

(fitness). Reduced growth and fitness in fish have been reported and linked to pollution exposure (Brotheridge et al. 1998; Luckenbach et al. 2001; Luckenbach et al. 2003). For example, chemical stressors at sub-lethal concentrations may impair the overall behaviour (e.g. foraging behaviour) and social interactions (e.g. hierarchal dominance) within a fish population by affecting the sensory system (e.g. olfaction), and thereby reducing the energy uptake (Atchison et al. 1987; Heath 1995; Kasumyan 2001; Kazlauskiene et al. 2008; Kuzmina & Ushakova 2008; Sloman 2007). It is also well known that energy reallocation may occur, i.e. metabolic tradeoffs between growth and reproduction on one side and repair and detoxification processes on the other side (e.g. resisting oxidative stress) (Chapman 2008; Hall et al. 1992; Scott & Sloman 2004; Sloman 2007). Finally and more indirectly, reduced food quality and prey availability due to chemical contamination may also reduce the energy uptake (Coghlan & Ringler 2005).

2.3.6 Monitoring biological effects

As described in the previous chapters, toxicity in fish involves a cascade of physical, chemical and biological processes at various levels. This is known as biological complexity (Figure 7). The complexity increases from left to right, e.g. damages on a cellular level typically appear before any damages on population level. Hence, the biological complexity involves both spatial and temporal variability. This concept is of great significance when studying the effects of pollution on fish, and it is important to state that no level of organisation is more important than another, and studies at any level may give valuable insight in the mechanisms involved.

The sequential order of responses to pollution as depicted in Figure 7 has generated a lot of effort developing the "biomarker concept", providing early-warning signals at an early level which reflects the possibility to cause detrimental effects on the biota caused by anthropogenic perturbations (Hyne & Maher 2003; van der Oost et al. 2003). Several definitions of a *biomarker* have been launched, and in its broad sense it may be defined as: "*any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction*" (WHO 1993). In the review by van der Oost et al. (2003), a biomarker refers to all biological indicators (i.e. biochemical, physiological, histological, morphological, behavioural, etc.) measured inside an organism or its product.

The measurements of biomarkers offer a great opportunity to gain knowledge needed in ERA. However, limitations and obstacles do exist, e.g. confounding factors that are not related to the pollution may perturb the interpretation. Another example, often used in an ERA context, is the assessment of effects by extrapolating toxicity data from one organism to another, from acute to chronic, from laboratory to field, from low to high biological levels etc. Such exercises can be very challenging and may be associated with great deal of uncertainties because the mechanistic understanding of how processes at

each level are functionally integrated is still incomplete (Eggen et al. 2004; Forbes et al. 2006; Hyne & Maher 2003; Salbu et al. 2005; Schlenk et al. 2008). Typically, these uncertainties are often compensated by introducing safety factors, e.g. 100, 10 and 1 for acute, chronic and field data, respectively (Salbu et al. 2005). However, although still in its youth, microarray technology is in that respect very promising, allowing rapid measurements of the up- or down regulation of thousands of genes and their expression simultaneously. Hence, complex pathways and strategies that an exposed organism applies in response to environmental stressors may be revealed (Steinberg et al. 2008), and thereby in an ERA contexts being able to predict toxicant responses across different phylogenetic groups present in the aquatic ecosystem and estimating how changes at one level of biological organisation will affect another (Snape et al. 2004). For example, the U.S. Environmental Protection Agency (US EPA) accepts toxicogenomics data as part of a weight-of-evidence approach for establishing mechanisms of toxicity for regulated substances (Van Aggelen et al. 2010). Microarray technology also provides leads for identification of novel biomarkers of exposure and detrimental effects (Van Aggelen et al. 2010).

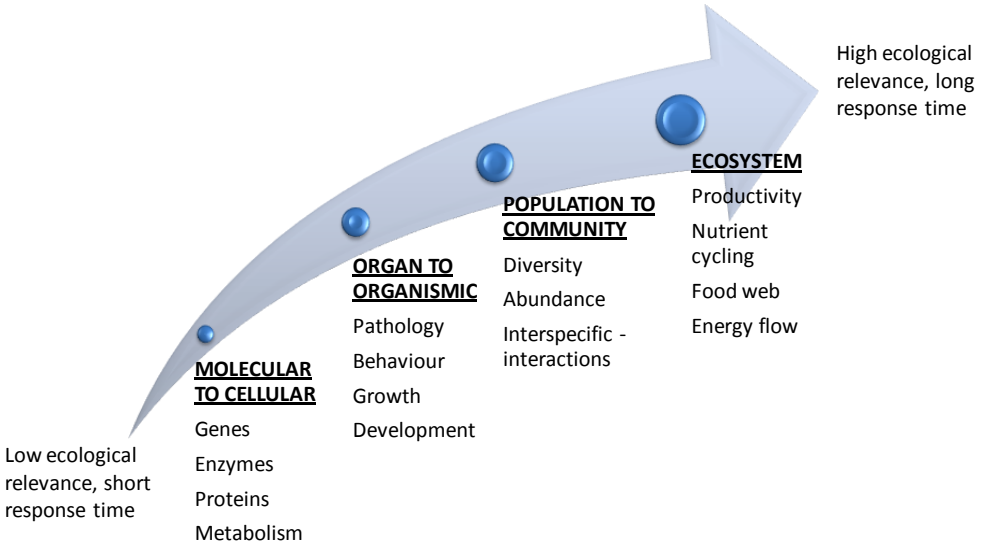

Figure 7. A conceptual model over biological complexity showing the relationship of response time, response sensitivity and ecological relevance with typical response parameters associated with selected biological levels. Modified after (Schlenk et al. 2008).

In the present thesis, we have utilised several chemical and biological endpoints which may be considered as biomarkers indicating exposure and effects at various levels. Haematological variables (Papers II and III), catalytic enzyme activity (Paper III), protein

induction (Paper III) and protein mRNA transcription (Papers II and IV) represent the molecular and cellular level, while the liver somatic index (Paper III), condition factor (Paper III) and growth (Paper I) represent the organ and individual level. In addition, the chemical accumulation of metals in gills (Papers II and III) and liver (Papers III and IV) has been important linking chemical exposure of gill reactive or transient species in the water together with uptake and biological responses.

3 Experimental outline and analytical methods

All exposure studies involving fish (Papers II, III and IV) were planned and conducted within the concept of the 3 Rs, reduce, refine and replace (Russell & Burch 1959), which implies the reduction of experimental animals, refinement of experimental methods and the replacement of experimental animals with substitutes, respectively. In addition, animal welfare was essential throughout the present work which implies good exposure conditions in terms of maintaining sufficiently oxygenated water, avoidance of high animal densities circumventing problems with elevated CO_2, and ammonia / nitrite concentrations. All experiments have been approved by the Norwegian Animal Research Authority (Forsøksdyrutvalget, FDU)

3.1 Study sites

The results presented in this thesis were obtained from field work conducted at two different sites along one of the major highways (E6) outside the City of Oslo; the Skullerud junction (Figure 8) representing the natural runoff events from open roads (Paper III), and the Vassum junction (Figure 9), representing the manmade tunnel wash water runoff (Papers I, II and IV). The latter, includes three adjacent tunnels, the Nordby-, Vassum- and the Smiehagen tunnel, being 3.84, 0.95 and 0.85 km long, respectively.

Figure 8. Map showing the Skullerud junction. The localization of the sedimentation pond is denoted by the red arrow.

Figure 9. Map showing the Vassum junction. The localization of the sedimentation pond is denoted by the red arrow.

3.1.1 Vassum – tunnel wash water runoff

Two field studies were conducted during washing and cleaning of the Nordby tunnel which consists of two separate tubes and four driving lanes. The traffic density, measured as annual average daily traffic (AADT), is currently around 25 000 vehicles (11 % is heavy duty vehicles) and the average driving speed is currently 89 km/h. The Nordby tunnel is frequently cleaned, normally 4 to 6 times per year including a full wash after the winter season which means washing of the road surface, walls, roof and technical infrastructure (e.g. traffic signs, lights etc.). From year 2000, the drainage system in the Nordby tunnel was divided in two separate systems preventing mixing of clean drainage water from the surrounding rock and polluted wash water runoff. A significant fraction of coarse material and debris are removed from the runoff as it flows through several gully pots. Finally, the wash water is pumped to a sedimentation pond outside the tunnel before being discharged into the stream Årungselva (Figure 10). The sedimentation pond receives, in addition, wash water runoff from the two other nearby tunnels and some runoff from the open road junctions in between (17 000 m^2).

Figure 10. The Vassum junction with the Nordby tunnel to the right hand side and the sedimentation pond to the left hand side. The pond inlet is in front of the picture, while the pond outlet is in the back.

The first field study in the Nordby tunnel was conducted in the end of April 2006 (Paper I), while the second study was conducted in mid December 2008 (Papers II and IV). The main purpose of the first study was to characterize and quantify the discharged tunnel wash water, in terms of inorganic and organic contaminants, before reaching the recipient stream Årungselva. Hence, water samples were therefore obtained from the pond outlet by using *in situ* size and charge fractionation techniques. The second study was focusing on the effects of untreated tunnel wash water on exposed brown trout by using untreated wash water prior to the sedimentation pond.

3.1.2 Skullerud – highway runoff

The study presented in Paper III was performed in May 2007 by simulating four consecutive runoff episodes from a sedimentation pond (Figure 11). The pond is located at the Skullerud junction, receiving runoff from a four lane motorway having an AADT around 45 000 vehicles (13 % heavy duty vehicles), and an average driving speed of 85 km/h. The drainage area is approximately 34 000 m^2 (65 % paved) and the pond retention time is estimated to be minimum 72 h. The pond is constructed with two basins with a total wet volume of 800 m^3; the first being a small closed pre-sedimentation basin and the second being the main pond. In the last few years, dense aquatic vegetation has emerged from the midpoint of the pond and towards the outlet. The runoff water is finally discharged from the pond and into the stream Ljanselva.

Figure 11. Picture showing the bridge on E6 crossing the Skullerud sedimentation pond. The picture is taken from the pond outlet towards the pond inlet. The blue steel container in back of the picture was used as a field laboratory during the experiment presented in Paper III.

3.2 Water quality assessment

As the bioavailability of contaminants, especially metals, was of major interest throughout the present work, *in situ* fractionation techniques were applied to obtain information on metal speciation. Historically, numerous fractionation techniques have been utilised in the laboratories for metal speciation purposes, although *in situ* fractionation techniques should be applied to avoid storage effects affecting the species distribution. Utilizing size exclusion techniques such as filtration and ultrafiltration, LMM species can be differentiated from colloids and particles (see Figure 4). Combining size exclusion with charge separation techniques such as ion chromatography (i.e. cation and/or anion resins), information on LMM species being positively or negatively charged or neutral, can also be attained (e.g. Salbu & Oughton 1995; Salbu 2009; Teien et al. 2004).

In the present work (Paper I), ultrafiltration was performed *in situ* using an Amicon H1P1-20 hollow-fibre cartridge with a nominal molecular cut-off mass of 10 kDa. This nominal cut-off has successfully been adopted and utilised in several studies to quantify the concentrations of LMM metal species, including environmental water quality monitoring (e.g. Heier et al. 2010), toxicity studies (e.g. Rosseland et al. 1992; Salbu et al. 2008; Teien et al. 2006) and highway runoff studies (e.g. Tuccillo 2006). The advantages of using hollow fibre can be summarised: sharp and distinct cut-off level, low retention

of metal species and minimal impairments of clogging due to high tangential flow and low cross filter flow (Salbu 2009). Finally, sorption is insignificant when conditioning of the system with a sample aliquot is performed (Salbu & Oughton 1995).

In addition to size exclusion, cation and anion exchange chromatography was included to gain information regarding charge properties of the dissolved metal species (metal species < 0.45 μm). The concentrations of positively (Papers I and II) and negatively charged (Paper I) metal species were determined by using Chelex-100 (Biorad) resin (Na-form) and AG1-X8 (Biorad), respectively. Neutral species and neutral/negative species were estimated by subtraction in Papers I and II, respectively. The fraction retained by chromatography techniques adopted in the present work, is influenced by the stability constants and the flow rate (i.e. equilibrium time). Hence, the retained fraction will include charged species in solution and species dissociated from weak complexes or desorbed from solid surfaces (Salbu 2009; Teien et al. 2004).

All metal concentrations were obtained by analysing the water samples with ICP-MS (Perkin Elmer ELAN 6000) and ICP-OES (Perkin Elmer Optima 5300DV). Internal standards (indium (In) and thallium (Tl)), several blanks and certified reference material (SRM 1643e, National Institute of Standards and Technology) were included in the analyses for quality control purposes.

3.3 Fish samples

Although the number of published papers regarding highway runoff toxicity has increased in the past few years (Bækken 1994; Grapentine et al. 2008; Karlsson et al. 2010; Kayhanian et al. 2008; Maltby et al. 1995b; Sriyaraj & Shutes 2001; Waara & Farm 2008), hardly any have utilised an holistic approach, i.e. including series of biomarker responses in fish at different biological levels (see Figure 7). Fish, represented by brown trout (*Salmo trutta*), were chosen as the preferred organism in the present work for many reasons. First of all, brown trout is very abundant in Norwegian water courses and is considered sensitive and probably the most sensitive fish species in Norway after Atlantic salmon (*Salmo salar*). In addition, the biology and physiology of salmonids are extensively studied and they are therefore often applied in the field of ecotoxicological science (e.g. Kroglund et al. 2008; Rosseland & Staurnes 1994). Therefore, fish is generally considered to be one of the most feasible organism for pollution monitoring in aquatic systems (van der Oost et al. 2003), and in addition fish have gained the most attention on the subject of gene expression profiling under chemical stress (Steinberg et al. 2008).

In all fish experiments, sampling of biological material was done following the EMERGE protocol standard (Rosseland et al. 2001), which is currently an adopted international standard used in several EU projects (MOLAR, EMERGE, EUROLIMPACS) and in UNECE ICP).

3.3.1 Trace metal accumulation in biological tissue

The accumulation of water borne contaminants in brown trout organs such as gills and liver has been reported in field studies (e.g. Brotheridge et al. 1998; Heier et al. 2009; Rosseland et al. 2007), and may represent an estimate on the gill reactive and bioavailable fractions, respectively. In the current work (Papers I, II and III) only trace metal concentrations were measured and quantified in biological samples, and no PAH concentrations were measured. PAHs are rather rapidly metabolized during biotransformation both in gills and liver (Jonsson et al. 2006; Levine & Oris 1999) and the use of concentration data is therefore debated because they do not tend to accumulate in quantities that reflect the exposure (van der Oost et al. 2003).

The trace metal concentrations in gills (i.e. metals within the gill organ and metals precipitated on the gill surface) (Papers II and III) and liver (Papers III and IV) were determined by freeze drying the samples and subsequently digesting the samples by using an ultraclave before analysing the digested with an ICP-MS instrument. Internal standard (In), blanks and certified reference material (DORM-2 and DOLT-3, National Research Council Canada) were included in the analyses for quality control purposes.

3.3.2 Haematological measurements

Haematological variables in fish blood were analyzed *in situ* by utilizing a portable clinical blood analyzer, I-STAT (Abbot, USA) (Papers II and III). Depending on analytical cassettes, various parameters can simultaneously be measured in a small drop of blood (approximately 10 μL blood is sufficiently) and results are obtained within few minutes. All fish blood analyses in the conducted experiments were performed with an EC8+ cassette, including parameters such as glucose, plasma ions (Na, K and Cl), blood partial pressure of CO_2 and hematocrit.

3.3.3 Enzyme and protein measurements

The enzymatic activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) presented in Paper III was measured in liver samples by using commercial available kits. In brief, liver samples were homogenized in buffer before centrifugation. The SOD and CAT analyses were performed according to the kit instructions on the withdrawn supernatants by measuring the absorbance on a Multiscan Ascent 96 well plate reader. A more detailed description of the assays is presented in Paper III. The results were normalised by the protein content which was obtained from each sample by using the Bradford method (Bradford 1976).

The Cd/Zn metallothionein assay (MT) used in Paper III was originally developed by (Bartsch et al. 1990) and later modified by a research group at the Norwegian University of Science and Technology (Olsvik et al. 2001) and from whom we kindly received the

assay manual. This assay is based on a radiochemical method using ^{109}Cd as a tracer, and where the concentration of Cd bound MT is calculated by measuring the ^{109}Cd activity in the samples and by using the assumption that the MT molecular mass is 7 kDa with a molecular ratio of 7 g atoms of Cd per mole protein.

3.3.4 Gene expression

Gene expression is a sensitive indicator of toxicant exposure and cellular metabolism and in the present thesis two different techniques were applied, the quantitative real-time polymerase chain reaction (qrtPCR) technique (Papers II and IV) and the DNA microarray technique (Paper IV). The analyses were performed by the Norwegian Institute for Water Research (NIVA). Hence, only a brief introduction of these two techniques will be given here, while a short description of weaknesses and strengths are presented in Chapter 4.2.3 due to the novelty of these technologies in ecotoxicology.

QrtPCR is a well established and the preferred method for quantifying mRNA in biological samples (Wang et al. 2006). PCR was first invented and developed by the Nobel Prize awarded American scientist Kary B. Mullis in 1983, and the novelty of the technology laid in its simplicity being able to copy pieces of DNA in an exponential rate in only few hours by using readily available laboratory reagents (Mullis 1990). In brief, messenger RNA (mRNA) is purified from the sample (tissue/cells) before two DNA primers, matching the genes of interest, reverse transcriptase, DNA polymerase and the four deoxynucleoside triphosphates (dNTPs) needed for DNA synthesis are added. The first round of synthesis is the reverse transcription of the mRNA into complementary DNA (cDNA) using one of the primers which then are followed by a series of heating and cooling cycles which finally amplify the DNA strand (Alberts et al. 2008). Hence, by using fluorescent dyes (e.g. Sybr Green) in a qrtPCR it is possible to determine whether a DNA sequence is present in the sample and the number of copies (gene expression). Exact description of the applied method including primers can be found in Paper II.

Recently, DNA microarray technology has been increasingly utilised in environmental science, and by adopting this technology it is possible to identify the effects of stressors (e.g. chemicals) on a large number of genes (thousands) in a single experiment (Lettieri 2006). Generally, a DNA microarray is normally a glass slide on which one deposits single stranded DNAs (probe) with various sequences coding for genes in interest. These probes hybridize with labelled single stranded DNAs (targets) obtained from the experimental samples. The target reflects the amount of isolated mRNA in the samples. The amount of emitted fluorescence from each spot on the glass slide is proportional with the amount of mRNA produced from the gene having the corresponding DNA sequence. After scanning the microarray, the fluorescence signals are converted to numerical values (raw data) which finally are statistically analysed (Drăghici 2003). In the present thesis (Paper IV), microarray analysis was performed with an Atlantic salmon custom array from Agilent (Agilent technologies, Santa Clara, California, USA), after re-

annotation by the cGRASP consortium (http://web.uvic.ca/grasp/) and selective in-house re-annotation by Blast2Go (http://www.blast2go.org/) using default parameters with minor modifications. The experimental design was employed whereby exposed fish (treatment group) were compared with their control counterparts.

3.4 Multivariate statistics

The multivariate statistics with supplementary ordination plots (Papers I and III) and response curves (Paper II) were conducted by using the software CANOCO 4.55 and CanoDraw 4.14, respectively.

Three different multivariate statistical models have been utilized in the present thesis, namely principal component analysis (PCA), redundancy analysis (RDA) and principal response curve (PRC). They are only briefly described in the following text.

3.4.1 Principal component analysis (PCA)

PCA is an unconstrained linear method (indirect gradient analysis) which seeks to describe the explanatory variable by ordination axes that best explains the measured variability. The axes can thus be interpreted as the best obtained theoretical explanatory variables. Hence, PCA reduces the multidimensionality down to few gradients (i.e. ordination axis). PCA is probably one of the most applied multivariate techniques and is commonly included in statistical packages. Its popularity might be attributed to its ease of calculation and its strength against deviations from multivariate normality in data.

3.4.2 Redundancy analysis (RDA)

The linear method redundancy analysis (RDA) is a constrained form (direct gradient analysis) of the principal component analysis (PCA). The advantage of RDA is the possibility to combine a set of response variables with a set of explanatory variables, which finally can be tested for significance by Monte Carlo permutation tests. The ordination axes are thus weighted sums of explanatory variables, i.e. the constrained ordination axes correspond to the directions of the greatest data set variability that can be explained by the explanatory variables. The final results of a RDA analysis are normally displayed in ordination diagrams. The interpretation of such diagrams is presented in Papers I and III.

3.4.3 Principal response curve (PRC)

In Paper II, PRC was conducted on three groups of endpoints (response variables), i.e. trace metal accumulation, blood physiology and biomarkers. This was done to better display the spatial pattern in the data and based on the knowledge that trace metal accumulation and alteration of blood compounds like glucose and ions is rather quick

responses, while transcription of different mRNA coding for various proteins, enzymes etc is a more delayed response.

Principal response curve (PRC) is based on a partial redundancy analysis (pRDA) and is a very applicable method for time dependent multivariate data (Borcard et al. 1992; Van den Brink & Ter Braak 1999). In brief, time is used as categorical covariables and the interaction between time and treatment is used as response variables. Hence, the focus is put on the deviation of the selected endpoints (y-axis) along a time gradient (x-axis) in the exposed group from that in the control group. The significance is validated by utilizing Monte Carlo permutation test of the treatments, i.e. permuting whole time series. The interpretation and the calculation of explained variance related to time, exposure and the amount of captured explained variance of the PRC axis are described in Paper II.

4 Methodological considerations

4.1 Experimental design

In experimental animal studies there will always be a trade off between the number of animals needed to obtain the scientific objectives on one side, and ethical and economical aspects on the other side. For rather obvious reasons such as pain and suffering, too many experimental animals are unethical. Similar, too few animals would also be unethical, due to the fact that animals would have been sacrificed for "nothing" (Festing 2002), and in addition, the scientific objectives would stay unsolved. This issue is indeed essential in one of the Rs in the frame work of the 3 Rs, namely *Reduction* (Russell & Burch 1959).

Several different endpoints from different organs in the fish (gill, blood, liver and kidney) were measure throughout the experiments (Papers II, III and IV). These endpoints have varying degree of variance, which mostly were unknown prior the experiments, and it was therefore difficult to statistically estimate (e.g. power analysis) an optimal sample size for the various endpoints. However, based on years of experience at our laboratory with Al accumulation on fish gills and blood variables such as Na and Cl (e.g. Kroglund et al. 2008), we decided to have a sample size of 6 fish (i.e. endpoints obtained from 6 fish in each group and repeat).

4.2 Analytical considerations

4.2.1 Water quality assessment

Water samples were obtained by a closed *in situ* sampling and sequentially fractionating with respect to size and charge. The in situ sampling technique will be more representative than collecting samples in containers at certain intervals, as the water quality shows temporal variability. Uncertainty is, however, introduced as the partitioning calculations are based on subtracting metal concentrations in the different fractions. This approach was preferred as it would be less affected by changes in the speciation of unstable metal species, and to avoid contamination during sampling, as the system was closed. In addition, the equipment was pre conditioned with sample water before each sampling (i.e. hoses, sample container, filters, hollow fiber and ion exchange material).

Regarding ion exchange chromatography, the interpretation of the analytical results is not necessarily straight forward as the chemical equilibrium may shift during the fractionation because of its slow procedure. Column size, bed size and flow rate are essential in this respect. In addition, the resins may act as filters retaining for example colloids or particles which may also impair the interpretation of the analytical results, i.e. the risk of overestimating the results (Apte & Batley 1995; Salbu & Oughton 1995; Salbu

2009). This is however, minimised when applying chromatography after size filtration and especially after ultrafiltration where particles and colloids are excluded.

Highway runoff typically contains high concentrations of salts, which can lead to methodological effects and instrumental influences of the quantification of trace elements by ICP-MS (and ICP-OES) such as interference (e.g. overlap of signals from elements having masses close to each other). Hence, water samples with high salt concentrations (Na and Cl > 100 mg/L) were diluted to reduce potentially problems regarding interference. In addition, internal standard (In), blanks and reference material were included in the analytical work.

4.2.2 Enzyme and protein assays

The SOD, CAT and protein assays performed on liver tissue sampled from the Skullerud experiment (Paper III) were done according to the protocols provided by the manufacturers. However, all assays involve multiple steps throughout the experimental work and errors and variations may occur. Such variability was minimized by measuring the samples in triplicates. The CAT analyses also included a positive control. Although SOD and CAT are important antioxidant enzymes, their use as biomarkers for ROS are debated. According to van der Oost et al. (2003) they are less responsive to pollutants compared with phase I and II enzymes (e.g. CYP1A and GST). In addition, numerous of SOD/CAT assays and methods currently exists which make comparisons with other comparable studies difficult (e.g. units differ among studies). However, as a control group was included in the fish experiment, the results were internally comparable.

4.2.3 Hepatic gene expression - qrtPCR and DNA microarray

The major advantage with the qrtPCR technology is its high sensitivity (e.g. only minute amount of DNA is needed), large dynamic range, high throughput and precise reproducible quantification. However, obstacles do exist and appear in the context of specificity, efficiency and normalisation (Hansen 2006; Wang et al. 2006). For example, unreliable data are expected if the amplified products are not correct, e.g. the selected primer should be specific for gene of interest in the studied organism. In addition, care must be taken to avoid nonspecific product formation and primer dimers. Regarding efficiency, the optimum is to have a two-fold increase in product formation during each PCR cycle. Finally, normalisation of the expression data has gained much attention to reduce the variability and uncertainties introduced in the experimental work. Various normalisation methods exist (e.g. housekeeping gene) but in Paper II we normalised the results with the amount of RNA in each sample, a suitable approach when working with mixed exposures (e.g. highway runoff) (Finne 2008).

The obvious advantage of DNA microarray technology is the rather rapid performance and the high output of results, i.e. the expression of thousand of genes. However, obstacles and challenges do exist, and examples given by (Drăghici 2003) are:

1. Noise: microarrays tend to be noisy due to the many steps involved such as mRNA preparation, transcription, labelling, surface chemistry, target volume, hybridization processes, artefacts (e.g. dust), scanning and quantification.

2. Normalization which aims to account for systematic differences across data sets (e.g. quantity of mRNA) and to eliminate artefacts.

3. Experimental design.

4. Large number of genes which introduces the classical problem with the p-value when conducting multiple comparisons, i.e. the increased probability of gaining false positives.

5. Significance: revealing the significance between expression profiles from the experimental groups cannot be directly answered by classical statistical techniques such as chi-square tests because the number of variables (genes) is much greater than the number of experiments. Hence, novel techniques need to be applied,

6. Biological factors: the amount of mRNA is not always directly proportional to the amount of protein and even if they were proportional, proteins require a number of post-translational modifications in order to become active and fulfil their role in cells. In addition, translating the up- and down regulation of genes into biological knowledge, e.g. their interactions and regulatory pathways are more important than which particular genes are regulated.

7. Array quality assessment which is linked to quality measures allowing the discarding the data coming from below standard arrays as well as the identification of possible causes of failure in the microarray process.

All this issues are related to methodological uncertainties. However, of equally importance is the challenging biological interpretation of up- and down regulation of a vast number of genes, i.e. the complex relationship between genetic responses and ecological outcome limits its use in ERA and in regulatory decision making (Van Aggelen et al. 2010). However, in Paper IV the focus was on functional pathways rather than single gene expression. Several studies have advocated the use of functional pathways because individual genes do not operate alone in the cell, but in a sophisticated network of interactions (Al-Shahrour et al. 2006; Luebke et al. 2006). In addition, when the transcriptional changes are minimal or moderate, e.g. pollution episodes of modest

character (Olsvik et al. 2008), single gene expression may be confounded by temporal variation in responses.

4.3 Evaluation of the multivariate statistical methods

The experiments presented in this thesis, focusing on multiple stressors and multiple effects faced the challenge of interpreting huge and complex data sets. Hence, to better interpret and understand the toxicity of highway runoff some simplification was needed. Multivariate statistics has in this context proved to be a promising tool as it can reveal pattern of responses and inter-individual relationships between endpoints that otherwise might be undetected by univariate statistics (e.g. Astley et al. 1999; Galloway et al. 2006; Rodríguez-Ortega et al. 2009; Rognerud et al. 2002; van der Oost et al. 1997). Another major advantage with multivariate statistics is its possibility to overcome the problem with multiple statistical testing, i.e. the increased probability of committing "type I error (false positive)" and the risk of misinterpret random noise (Shaw 2003).

Limitations and drawbacks with multivariate methods do, however, exist. One major drawback compared to univariate methods, is the challenging interpretation of abstract results presented in various ordination plots (Van den Brink & Ter Braak 1999). However, the PRC overcomes some of this problem at least in time dependent studies or experiments (Van den Brink & Ter Braak 1999; van den Brink et al. 2009). Another weakness is that details might unfortunately be missed when adopting multivariate methods in the search of global patterns (Van den Brink & Ter Braak 1999). In addition, multivariate approaches are sensitive to outliers (that would of cause be true both for erroneous recorded values as well as extreme values). However, transformation of the data typically reduces the significance of extreme values.

When working with large datasets, missing values for reasons such as instrument error, values below detection limit etc. is almost inevitable. Unfortunately, most multivariate methods cannot include missing values in their algorithms. Hence, variables or observations including missing values must be removed, or alternatively, missing values must be substituted with an estimated value typically based on replacement methods, regression methods or maximum likelihood methods (Tsanis et al. 1994). One common approach in water quality studies is to replace concentration data below the limit of detection with values equal to half the detection limit. This falls under the method of replacement (Tsanis et al. 1994).

5 Summary of the scientific papers

5.1 Paper I – tunnel wash water runoff

Chemical and ecological effects of contaminated tunnel wash water runoff to a small Norwegian stream

In the present study, traffic related contaminants were quantified in tunnel wash water (the Nordby tunnel, Norway) discharged from a sedimentation pond to a nearby small stream, Årungselva. High concentrations of metals and PAHs were measured in the discharged tunnel wash water, and several exceeded their corresponding environmental quality standard (e.g. Cu, Pb, Zn, fluoranthene, pyrene). The contaminants PAHs, Al, Cd, Cr, Cu, Fe and Pb were associated with particles and colloids, while As, Ca, K, Mg, Mo, Ni, Sb and Zn were more associated with low molecular mass species (< 10 kDa). Calculated enrichment factors revealed that many of the metals were derived from anthropogenic sources, originating most likely from wear of tires (Zn), brakes (Cu, Sb), and from road salt (Na, Cl). The enrichment factors for Al, Ba, Ca, Cr, Fe, K, Mg and Ni were low, suggesting a crustal origin, e.g. asphalt wear. Based on calculated PAH ratios, PAH seemed to originate from a mixture of sources such as wear from tires, asphalt and combustion. Finally, historical fish length measurement data indicates that the fish population in the receiving stream Årungselva may have been influenced by the chemical perturbations in runoffs originating from the nearby roads and tunnels during the years, as the growth in summer old sea trout (*Salmo trutta* L.) in downstream sections of the stream is 21 % reduced compared with the upstream sections.

5.2 Paper II – tunnel wash water runoff

Exposure of brown trout (*Salmo trutta* L.) to tunnel wash water runoff – chemical characterisation and physiological impact

The present experiment was conducted to evaluate the short term toxicity (4 h exposure) of a tunnel wash episode by measuring several endpoints and biomarkers in brown trout, including metal accumulation in gills, haematological variables and hepatic gene expression. Our findings showed that the runoff water was highly polluted, but most of the contaminants were associated with particles which are normally considered biological inert. In addition, high concentrations of calcium (Ca) and dissolved organic carbon (DOC) were identified in the wash water, thus reducing metal toxicity. However, compared to the control fish, a rapid accumulation of trace metals in gills was observed. This was immediately followed by a modest change in blood ions and glucose in exposed fish shortly after the exposure start. However, after 38-86 h post wash, gill metal concentrations, plasma ions and glucose levels recovered to control levels. In contrast, the mRNA transcription of the CYP1A and the oxidative stress related biomarkers TRX

and GCS did not increase until 14 h after the exposure start and this increase was still apparent when the experiment was terminated 86 h after the beginning of the tunnel wash. The triggering of the defence systems seemed to have successfully restored homeostasis of the physiological variables measured, but the fish still used energy for detoxification four days after the episode, measured as increased biomarker synthesis.

5.3 Paper III – highway runoff

Ecotoxicological impact of highway runoff using brown trout (*Salmo trutta* L.) as an indicator model

The current experiment had three objectives: 1) quantify chemical concentrations of contaminants in runoff episodes; 2) characterize the associated physiological responses in exposed brown trout; 3) assess whether treatment ponds based on sedimentation are able to reduce the toxicity of highway runoff. The pollution levels, apart from road salt, were rather low during the first two consecutive runoff episodes (each episode lasted 24 h). The pollution levels increased in the two last consecutive episodes due to a heavy rainfall. In addition, lowered oxygen concentrations lead to hypoxic conditions. Overall the fish exposed to highway runoff had, compared to the control fish, higher concentrations of trace metals in gills and liver, increased activity of the antioxidant defence system represented by superoxide dismutase, catalase and metallothionein, problems with the regulation of plasma Cl and Na, as well as increased levels of blood glucose and pCO_2. Finally, this seemed to affect the metabolism of the fish expressed by reduced condition factor. The observed effects were likely caused by multiple stressors and not by a single contaminant. The sedimentation pond clearly reduced the toxicity of the highway runoff. But even in the least polluted exposure tank (pond outlet + stream water) signs of physiological disturbances were evident.

5.4 Paper IV – Genomics

Hepatic gene expression profile in brown trout (*Salmo trutta*) exposed to traffic related contaminants

The present paper presents results from a DNA microarray screening analysis performed on a subset of liver samples obtained from the exposure study presented in Paper II. The aim was to increase the mechanistic understanding of the effects of traffic related contaminants, common in highway- as well as in tunnel wash water runoff, on salmonids. In addition, the focus was on biological and molecular pathways by the means of gene ontology (GO) assessment rather than studying single gene expression. At time 38 h (i.e. 34 h after the exposure), several gene ontology terms (GO) were significantly suppressed in exposed fish including GOs within the immune system. In addition, several enzymes involved in the biosynthesis of cholesterol were apparently strongly

inhibited. Readily bioavailable polycyclic aromatic hydrocarbons (PAHs) or other traffic related organic pollutants may have caused these alterations in exposed fish. The initial responses were subsequently followed by up-regulation of xenobiotic biotransformation and antioxidant defense system at time 86 h (i.e. 82 h after the exposure), including classical biomarkers such as cytochrome P450 (CYP1A1 and CYP1B1), cytosolic sulfotransferase 3 (SULT) and glutathione peroxidase 1 (GPX). Of special interest was the apparently up-regulation of the paraoxonase (PON) enzyme, indicating the presence of organophosphorus compounds (OPs) in the runoff water. In addition, the apparently up-regulation of the arsenite methyltransferase (AMT) may indicate that metalloids such as arsenic (As) and antimony (Sb) were readily bioavailable despite that no liver accumulation of these metalloids (or other metals) was observed.

6 Discussion

6.1 Highway and tunnel wash water runoff - water quality

Apart from the road salt concentrations, metals and PAHs appeared at higher concentrations in the tunnel wash water studies at the Nordby tunnel (Papers I and II) than in the open road area study at Skullerud (Paper III) (Figure 12), despite that the traffic density at Skullerud is almost twice the density in the Nordby tunnel. In addition, the runoff area at the two sites is quite similar taking into account that untreated wash water was sampled during washing of one tunnel tube. The relatively large differences between these two cases, in terms of contaminant concentrations, can therefore be attributed to meteorological patterns. For example, contaminants accumulated over time inside the tunnel are sheltered from being frequently flushed out by precipitation, and typically less affected by wind or/and dispersed by wind. This demonstrates the significance of tunnel wash water runoff as a major pollution source, being potentially detrimental for receiving waters. However, these results are somewhat contradictory to a Portuguese study which concluded that the accumulation of contaminants outside the tunnel was higher than inside the tunnel due to high wind velocities inside the tunnel (both due to mechanical ventilation and unidirectional traffic) (Barbosa et al. 2006). The very different climate conditions in Norway and Portugal may, however, explain this controversy. For example, compared to Norway Portugal has a dry climate (semi-arid) with little precipitation, and the use of studded tires in Portugal is probably non-existing.

Based on calculated enrichment factors (EF), many of the metals seemed to originate from the vehicle (Paper I). Especially the high EF for Zn and high EFs for Cu and Sb indicated wear from tires and brakes as sources, respectively. According to the review by Wik and Dave (2009), tire wear is found in all environmental compartments and is potentially detrimental for aquatic organisms. In contrast to Zn, Cu and Sb, the metals Ba, Al, Ni, Cr and Fe had low EFs. This demonstrates their association with crustal material, e.g. originating from asphalt wear. The PAH ratios indicated contribution from combustion, asphalt- and tire wear. In addition, the dominance of pyrene and fluoranthene, both in the tunnel wash water and in the open road area runoff, is in accordance with other studies (Boxall & Maltby 1997; Shinya et al. 2000).

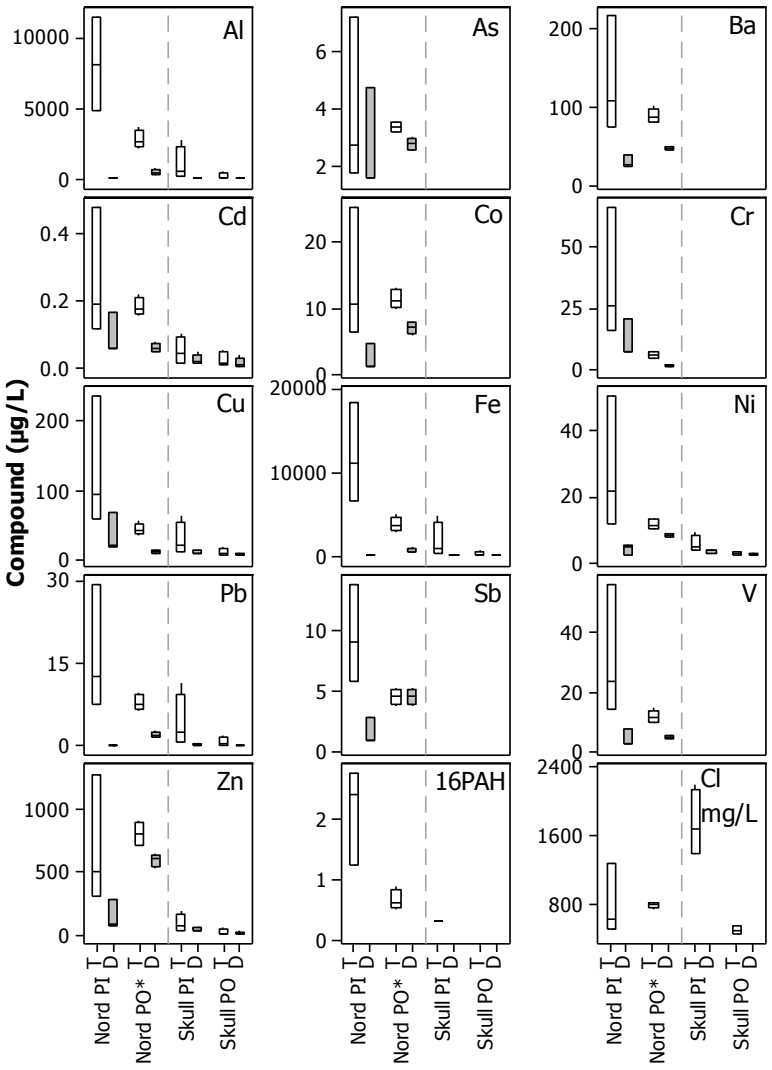

Figure 12. Box plots presenting the total (T) concentrations of metals (and dissolved (D)), chloride and PAH obtained from the various studies (n = 3 – 4). The rectangular box for each group represents the interquartile range of the data including the median value displayed as a horizontal line, while the whiskers extending from the boxes represents the upper and lower 25 % of the distribution. Nord PI = untreated pond inlet water from the Nordby tunnel (Paper II), Nord PO = treated pound outlet water from the Nordby tunnel (*10kDa) (Paper I), Skull PI and Skull PO = untreated pond inlet water and treated pond outlet water from Skullerud, respectively (both Paper III). As, Ba, Co, Cr, Sb and V were not measured in the Skullerud experiment.

In an EQS context, and in line with other studies (e.g. Kalainesan et al. 2009; Karlsson & Viklander 2008; Kayhanian et al. 2008; Lundberg et al. 1999; Sansalone & Buchberger 1997; Semadeni-Davies 2006; Shinya et al. 2000), Al, Cu, Fe, Pb, Zn and several of the PAHs (e.g. pyrene and fluoranthene) were the compounds of most concern. These contaminants exceeded benchmarks utilized in Canadian, American and European EQSs (Table 2). Being quite different from the EU and North American approach, the Scandinavian EQS system is stricter in its assessment as the concentrations are scaled and classified according to the deviation from natural or background concentrations (Table 3). Overall, the tunnel wash water was classified as being more polluted than the open road runoff, having an average score of 4.0 compared to 3.1. In addition, the pond inlet water in both cases was more polluted than the discharged outlet water, i.e. 3.5 vs. 2.7 in open road runoff and 4.3 vs. 3.6 in the tunnel wash water.

Data regarding Al and Fe concentrations are less often reported in highway runoff studies compared to many other metals, such as Cd, Cu, Ni, Pb and Zn. The reason for this is not obvious, but might be due to the fact that Al and Fe are considered as elements of crustal origin. However, the high concentrations of Al and Fe found in the present work underlines that more attention should be paid also to these two metals. Especially because, toxic transient Fe and Al species may be formed under certain conditions due to their chemical properties. Examples are conditions with low pH, sea salt episodes and mixing zones where different water qualities meet (e.g. Teien et al. 2008; Teien et al. 2005).

Apart from As, Ni and Zn, most of the contaminants were highly associated with particles, and in terms of total concentrations, the untreated pond inlet water was generally more polluted than the pond outlet water (Figure 12). The removal rates in the Skullerud sedimentation pond obtained from the present work (Paper III), although based on a limited number of samples, are consistent with previous measurements in Skullerud. For example, the annual average removal rate in 2003 - 2004 of Pb, Zn, Cd and Cu was on average estimated to be 66 % (Åstebøl 2004; Vollertsen et al. 2006), while our value of the same metals was 70 % (72 % including all measured metals). This is also comparable with other published studies (e.g. Farm 2002; Semadeni-Davies 2006). It also demonstrates that particles are important transporting agents for traffic related contaminants, and that they are removed by sedimentation along their passage from inlet to outlet of the pond. However, remobilization of particle associated metals in sediments may occur over time due to redox, complexation (with organics) and ion exchange (salt effect) processes and contaminated sediments can act as a point source in the future. The removal of dissolved contaminants were considerable less efficient than the removal of particles, i.e. dissolved metal concentrations (< 0.45 μm) were reduced on average 42 % when comparing the inlet and outlet samples.

Table 2. Classification of highway (Paper III) and tunnel wash water runoff (Papers I and II) in terms of existing environmental quality standards (EQS) for fresh water obtained from Canada (CA) (CCME 2007), USA (US) (USEPA 2009) and EU (EC 2006). The classification is based on the maximum measured concentration in the various experiments.

	Skullerud – Highway runoff						Nordby – Tunnel wash water					
	In			Out			In			Out[e]		
Compound	CA[a]	US[b]	EU[c]	CA[a]	USA[b]	EU[c]	CA[a]	US[b]	EU[c]	CA[a]	US[b]	EU[c]
Al	X	CMC		X	CCC		X	CMC		X	CMC	
As							X					
Cd	X			X			X		AA[d]	X		
Cr							X					
Cu	X	CMC		X	CCC		X	CMC		X	CMC	
Fe	X			X			X			X	CCC	
Ni												
Pb	X			X			X			X	CCC	
Zn	X			X			X	CMC		X	CMC	
Anthracene							X			X		
Benzo(a)anthracene							X			X		
Benzo(a)pyrene							X		MAC	X		
Fluoranthene	X						X			X		
Phenanthrene												
Pyrene	X						X			X		
Benzo(b)fluoranthene Benzo(k)fluoranthene								}	AA		}	AA
Benzo(ghi)perylene Indeno(1,2,3-cd)pyrene		}	AA					}	AA		}	AA

[a] The EQS for Cu, Pb and Ni are hardness dependent.

[b] CMC = Criteria Maximum Concentration, CCC = Criterion Continuously Concentration. All metals beside Al (total) are expressed as dissolved (0.45 µm) and with hardness 100 mg/L $CaCO_3$.

[c] MAC = maximum allowable concentration, AA = annual average value. In the case of metals EQS refers to the dissolved concentration (0.45 µm). In the case of PAH, one of the EQS represent the sum of benzo(b)fluoranthene and benzo(k)fluoranthene, and one represent the sum of benzo(ghi)perylene and indeno(1,2,3-cd)pyrene.

[d] Cd is based on hardness class 3.

[e] The ultrafiltrated metal concentrations (< 10 kDa) represent the dissolved concentrations in the EQS from USA and EU.

Table 3. Classification of highway (Paper III) and tunnel wash water runoff (Papers I and II) in terms of existing environmental quality standards (EQS) for fresh water obtained from Norway (NOR) (Andersen et al. 1997) and Sweden (SWE) (SWEPA 2000). A Scandinavian classification system for salmonids (SAL) is also included (Lydersen et al. 2002). The classification is based on the maximum measured concentration in the various experiments.

	Skullerud – Highway runoff						Nordby – Tunnel wash water					
	In			Out			In			Out		
Metal	NOR[a]	SWE[b]	SAL[c]	NOR[a]	SWE[b]	SAL[c]	NOR[a]	SWE[b]	SAL[c]	NOR[a]	SWE[b]	SAL[c]
As								3			2	
Cd	2	3	1	2	2	1	5	4	3	4	3	2
Cr							5	4	4	3	3	2
Cu	5	5	4	5	4	3	5	5	4	5	5	4
Ni	4	2	1	3	2	1	5	4	3	5	3	2
Pb	5	4	3	3	3	2	5	5	4	5	4	3
Zn	5	4	4	4	3	2	5	5	4	5	5	4

[a] Class 1 – 5 = very low concentration – very high concentration
[b] Class 1 – 5 = very low concentration – very high concentration
[c] Class 1 – 4 = very low concentration – high concentration

Unlike the Skullerud data, the inlet (Paper II) and outlet (Paper I) concentrations measured in the tunnel wash water were obtained from two different studies being performed over two years apart. In addition, the inlet concentrations represent the pollutant loadings from a "half wash" while the outlet concentrations is obtained from a "full wash". Hence, calculation of removal rates was therefore not applicable in this case. Nevertheless, the total concentration of most contaminants was on average higher in the untreated pond inlet water compared to the pond outlet water (Figure 12). In terms of the dissolved concentrations, however, this pattern was in fact opposite, at least for Al, Ba, Co, Fe, Ni, Pb, Sb and Zn. Although associated with large uncertainty, this discrepancy between the pond inlet and outlet in terms of total and dissolved metal concentrations may indicate that the pond performance is not optimal. In a biological context, this is of concern as dissolved contaminants are assumed more bioavailable than those attached to particles. In fact, 25 % of the measured metals were discharged as LMM species (< 10 kDa) during the full wash of the Nordby tunnel presented in Paper II. These LMM species are considered mobile and potentially bioavailable

On a general basis, low and negative removal rates reported in the literature have been linked to ion exchange processes, chloride complexation and oxygen depletion effectuated by high road salt concentrations (Revitt et al. 2004), and due to the release of metals bound to organic matter decomposed from aquatic plants (Shutes et al. 2001). As high concentrations of salt, organic matter, as well as oxygen depletion were observed in the present studies, it was interesting to evaluate the significance of these variables on the dissolved metal concentrations found in Papers I, II and III. This was done by running a

partial RDA using Cl, DOC and dissolved oxygen (DO) as predictors and dissolved metal concentrations as response variables. Overall, Cl, DOC and DO significantly explained 46.5 % (p = 0.008) of the observed variation (Figure 13). However, their individual contribution to the observed variation was 20.6 %, 12.7 % and 7.6 %, for DOC, DO and Cl, respectively. However, only DOC was significant.

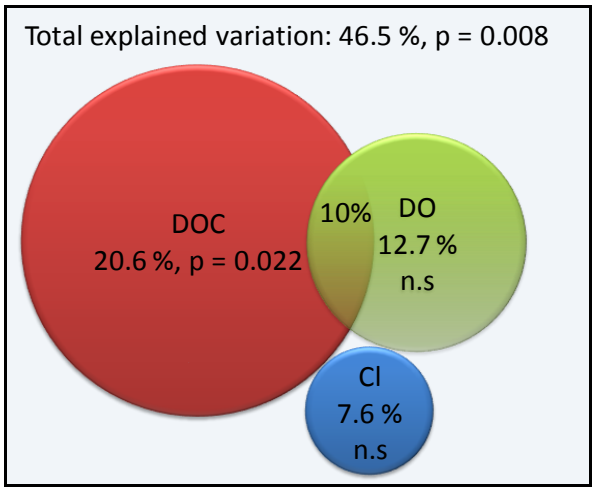

Figure 13. A Venn diagram showing the results from the partial RDA, including Monte Carlo permutation tests statistics. The circle sizes are proportional to their corresponding explained variability of the dissolved metal concentrations obtained from the Skullerud pond receiving highway runoff and the Vassum pond receiving tunnel wash water runoff. Overlapping circles denotes shared explained variability which cannot be separated. DOC = dissolved organic carbon, DO = dissolved oxygen and Cl = Chloride.

The interpretation of this statistical exercise is that despite their joint effect on the dissolved metal concentrations was significant, only DOC alone could significantly explain parts of the variability. Hence, the elevated road salt concentrations seem not to have any major impact on the dissolved metal concentrations in the present work. DOC is an important ligand in the aquatic system, being able to complex metal ions as well as other dissolved contaminants. Both ponds have during their lifespan become vegetated with both macrophytes and periphyton. Biodegradation of dead plant material may therefore explain the relatively high DOC concentrations in the ponds, and partly some of the reduced oxygen levels. Hence, dissolved metals associated with DOC may during runoff episodes or washing events be readily flushed out from the ponds. The dominance of neutrally charged LMM metal species and the high concentrations of LMM organic matter (e.g. fulvic acids) in the Vassum pond outlet (Paper I) may in fact

support the presence of transport mechanisms described above. This is also coherent with results published by Shutes et al. (2001) which showed that improper pond maintenance, in terms of not removing dead plant material, was a source of organically bound Cu readily discharged to the recipient.

6.2 Biological impairments and toxicity assessment

The concern about highway runoff being potentially detrimental for aquatic organisms is not new and has sporadically been addressed in published papers at least since late 1970s (e.g. Crowther & Hynes 1977; Gjessing et al. 1984). Common approaches are small-scale toxicity tests performed under controlled laboratory conditions involving small aquatic organisms such as bacteria, algae and crustacean (Christensen et al. 2006; Grapentine et al. 2008; Karlsson et al. 2010; Kayhanian et al. 2008; Marsalek et al. 1999; Waara & Farm 2008; Wik et al. 2008), and perhaps more ecological relevant, *in situ* macroinvertebrate surveys (Bækken 1994; Blasius & Merritt 2002; Grapentine et al. 2008; Maltby et al. 1995b; Perdikaki & Mason 1999; Sriyaraj & Shutes 2001; Wik et al. 2008; Woodcock & Huryn 2008). However, both approaches may be too insensitive as they rely on endpoints such as growth, lethality and community changes. Biological effects may therefore stay undetected, especially when the pollution levels are moderate. For example, Farm (2002) and Waara and Farm (2008) observed no toxicity when using a battery of small-scale toxicity test in 65 untreated highway runoff samples (AADT ~ 20 000), although the pollution load was comparable with the concentrations presented in this work. Through the present work (Papers II, III and IV) we have utilised brown trout, a well studied, native and sensitive species, to address an alternative, and hopefully a more complementary approach for studying highway and tunnel wash water toxicity. The experiments in the present thesis were therefore performed *in situ* and included several biological endpoints being able to study sub-lethal effects and thereby increasing the novelty of our approach.

As previously discussed, the highway runoff and tunnel wash water runoff were highly contaminated, and metals did accumulate on gill tissue, indicating their gill reactivity. The overall metal concentrations in gills were on average within the same range in the two experiments at Skullerud and the Nordby tunnel, respectively (Papers II and III). In the Skullerud experiment, the accumulation of Cu and Fe on gills was highest in the fish exposed to untreated runoff water (Pond inlet, PI). Unfortunately, Al and Pb were not included in the multivariate analysis due to a high number of values below the limit of detection. However, values obtained from the first simulated episode published by Meland et al. (2010) clearly showed accumulation of Al and Pb in gills. These four metals were together with Sb and Co also those who appeared at highest concentrations in gills sampled from the tunnel wash water exposed fish. Hence, the accumulation of several metals on gills indicated co-precipitation, a process likely to occur in exposures containing multiple contaminants.

Contrary to the previous mentioned metals, Zn seemed not to be gill reactive despite high dissolved concentrations (<0.45 μm) in both experiments. This harmonizes with other mixed exposure studies (Hansen et al. 2007; Heier et al. 2009; Wepener et al. 2001). According to the review of the biotic ligand model (BLM) by Niyogi and Wood (2004) Zn has a lower gill binding affinity than metals like Cu and Pb, and according to Wepener et al. (2001) also lower binding affinity than Fe which makes Zn less competitive for available binding sites at the gill surface. In fact, Wepener et al. (2001) observed a decrease in Zn concentrations in gill tissue during a mixed exposure study with Cu, Fe and Zn. This was attributed to the displacement of Zn from gill binding sites by the more competitive metals Cu and Fe. A similar decrease was observed in our experiments (Papers II and III), supporting the conclusions by Wepener et al. (2001) that metal mixtures may compete in an antagonistic fashion. Another interesting aspect of the presumed lack of Zn toxicity in the present work is that these results are very contradicting to two other recent published studies investigating toxicity of highway runoff (Camponelli et al. 2009; Kayhanian et al. 2008). However, they have not used brown trout but other organisms such as algae, water flea, sea urchin, Microtox™, frog eggs and larvae and one fish species (fathead minnow, *Pimephales promelas*). Hence, the different conclusions may therefore be ascribed to for example other uptake mechanisms, e.g. diffusion of contaminants through the body which is presumed more important in small animals.

The gill metal analysis included both adsorbed metals, metals retained in mucus and within the gill tissue, and it is therefore impossible to know the exact quantity of metal species crossing the gill membrane. Mucus secretion is believed to be a defense mechanism against uptake of toxic compounds, and has been demonstrated in exposure studies (Hansen et al. 2007; Mallatt 1985; Tao et al. 2006). For example, Al and Pb which were almost entirely associated with mineral particles in the tunnel wash water (Paper II) were most likely retained in the mucus as particles and subsequently removed by coughing off mucus. However, there are evidences that particle bound metals (Cd, Pb and Cu) retained in the gill mucus may to a certain extent become bioavailable due to pH changes in the gill microenvironment which may alter the metal speciation and/or the solubility (Playle 1998; Tao et al. 1999a; Tao et al. 1999b; Tao et al. 2000a; Tao et al. 2000b). This process is believed to be of higher significance in poorly buffered water systems, as the pH deviance between ambient water and gill microenvironment is expected to be greatest under such conditions (Playle 1998). In both our experiments the water had high concentrations of Ca, indicating good buffer capacity and, in addition, indicating that these processes probably played only a minor role.

Impaired blood chemistry was evident in exposed fish from both experiments (Papers II and III) demonstrating that waterborne contaminants to some degree were bioavailable. The most evident pattern was a rapid stress response manifested by increased plasma glucose together with a concurrent decrease in plasma ions (Cl and Na), indicating impairment of the ion regulatory system. Although alterations in blood chemistry were

evident, they were most likely of modest character, i.e. sub-lethal. Even so, the impairment of the ion regulatory system was evident and probably caused by several contaminants. For example, many of the gill reactive metals in the present thesis (e.g. Al, Cu, Fe and Pb) are known ion regulatory toxicants (McGeer et al. 2000; Peuranen et al. 1994; Rogers et al. 2003; Rosseland & Staurnes 1994).

Two different methods were used in the current thesis to study the cellular effects in liver from fish exposed to highway and tunnel wash water runoff. In Paper III effects were investigated by measuring protein (MT) and enzymatic activity (SOD and CAT), while in Papers II and IV we adopted mRNA transcription technology to study the regulation of genes (qrtPCR and DNA microarray, respectively). It is worth mentioning that although up-regulation (or down-regulation) of the mRNA for a specific protein after an exposure episode does not necessarily imply a coherent increase in concentration or activity for that specific protein, as demonstrated by Hansen et al. (2006). However, the overall pattern in our experiments on single biomarkers (Papers II and III) was that the antioxidant defense system was triggered in exposed fish, probably reflecting the presence of ROS. This was manifested by a slight but notable increase in SOD and CAT activity together with increased level of MT in exposed fish from the Skullerud experiment (Paper III), and a slight but notable up-regulation of TRX and GCS in exposed fish from the tunnel wash water experiment (Paper II). In contrast to the more modest up-regulation of the genetic antioxidant biomarkers, the up-regulation of CYP1A mRNA, coding for the important phase I enzyme involved in the PAH biotransformation process, was more distinct (Paper II). An appealing thought could therefore be; that the high correlation between CYP1A and the antioxidant biomarkers in the tunnel wash water experiment, may in fact indicate that ROS production was elevated as a result of biotransformation processes of PAH or other organic contaminants, rather than being induced by transition metals like Fe and Cu. As the mRNA transcription of the metal binding protein MT-A was more or less unchanged, this hypothesis is strengthened.

The microarray analysis in Paper IV, performed on a subset of liver samples from the tunnel wash experiment presented in Paper II, confirmed the overall responses discussed in the previous paragraph. The novelty of this technology being able to conduct genome-wide screening provided, in addition, new and even more detailed mechanistic understanding of the effects of traffic related contaminants. The most evident effects were the initial inhibition of several immunological processes together with an apparent inhibition of several enzymes involved in the cholesterol biosynthesis. Although Cu has proved to inhibit the cholesterol pathway (Santos et al. 2010), the lack of MT-A transcription and accumulation of Cu in liver of the exposed fish support the conclusions from the previous section, emphasizing the presence of PAHs and/or other organic contaminants as the main toxins in this respect. In fact, the apparent up-regulation of the main organophosphorus (OP) detoxifying enzyme paraoxonase (PON) in exposed fish indicated the presence of OPs in the exposure water. OPs have

previously been documented in highway runoff due to its presence in e.g. lubricant oil and hydraulic fluids (Marklund et al. 2005; Regnery & Puttmann 2010), and additionally, OPs have been documented to have inhibitory effects on the cholesterol biosynthesis (Elhalwagy & Zaki 2009). Hence, OPs could, similar to PAHs, have contributed to the observed responses in the present work. Finally, the apparent up-regulation of the arsenite methyltransferase (AMT) indicated the presence of bioreactive metalloids. This is interesting as neither hepatic accumulation of metalloids (As and Sb) nor other trace metals were observed in exposed fish.

As highlighted in Chapter 2.3.6, assessing effects at a higher biological level (see also Figure 7) by extrapolating effects from a lower level are challenging. However, the alterations of molecular and cellular endpoints presented in Papers II, III and IV and the reduced growth (21 %) in summer old sea trout (0+) downstream E6 (Paper I) may reflect an escalation of effects from an early warning signal at a low biological level to an effect on an individual level. Hence, this may be considered as a step on the road towards effects at even higher biological levels, e.g. population or community alterations, and strengthening the evidence that runoffs from highways and maintenance tasks such as tunnel wash may in fact be of ecological significance.

6.3 Regulatory considerations

In an environmental regulatory context, the results obtained from the present work, both chemically and biologically suggest that treating highway and tunnel wash water runoff by the means of sedimentation ponds may be insufficient. Partly because of poor removal efficiencies of contaminants associated to colloids and LMM species, which are believed to be more bioavailable than contaminants associated with particles. In addition, a maintenance strategy of these ponds, in terms of removing contaminated sediment and/or plant material, seems presently to be absent. Although not documented in this work, remobilization of trapped contaminants may therefore occur during large runoff events due to chemically processes and/or due to physical disturbance of the bottom sediment. Finally, high salt concentrations, organic matter and oxygen depletion may also affect the overall treatment performance. The significance of these factors has also been pinpointed in other studies (Kalainesan et al. 2009; Kamalakkannan et al. 2004; Lundberg et al. 1999; Marsalek et al. 1999; Starzec et al. 2005).

As demonstrated in this work, high concentrations of road salt, LMM metal species and PAHs are being discharged from the ponds. In addition, the overall performance in terms of reducing ecotoxicological effects both in a short term and a long term scale can be questioned. Hence, it might be advisable to look for new solutions or new strategies on how to mitigate the risk of causing negative environmental effects. For example, in Denmark there is an ongoing, and promising, project studying the contaminant removal performance of a sedimentation pond designed and constructed with an additional sand filter compartment and a fixed media sorption filter at the pond outlet (Vollertsen et al.

2009). Another reflection, and perhaps a more sustainable solution, is to let the runoff water filter along the road ditches into the ground instead of leading the runoff from a large area into a small pond before it is discharged into a recipient, as concentrated contaminated discharges from one single point may increase the environmental hazard. A recent study from Switzerland showed that diffuse infiltration of road contaminants in constructed infiltration slopes may be a suitable alternative for road runoff mitigation (Piquet et al. 2008). However, it should be stressed that this study were conducted at low traffic (AADT = 2 500) and the performance in high traffic areas is still unclear. In addition, the treatment performance during winter may also be a challenge as such facilities are less appropriate when covered by ice and snow.

Opposite to naturally occurring runoffs in open road areas, tunnel wash events can be more easily controlled. Hence, washing routines and strategies (BMPs) should be designed and implemented in a way that minimizes the risk of contaminating the receiving waters. Three factors that should be considered in that respect are for example: 1) washing frequency, 2) time of year and 3) water volumes. 1) A frequent washing regime is beneficial in terms of preventing the build-up of large contaminated dust depots which are readily washed out. This might be more important during the winter season when a significant part of the vehicles utilise studded tires. 2) Washing of tunnels polluting rivers or streams with anadromous species should be avoided during periods when aquatic animals are most vulnerable, e.g. pollution episodes during the swim-up stages of fish larvae and during smoltification of salmonids in the spring. In fact, all tunnels surrounding the Vassum junction have their full wash the week after eastern, which in a biological context may be undesirable. 3) Increasing the water volumes during washing would dilute the concentrations and thereby reducing the risk of short term biological effects. However, the total pollutant loadings in terms of masses would be the same and accumulation of contaminants in the sediment may become a threat to aquatic organisms in a long term perspective. For example, a British study revealed that the macroinvertebrate community was changed in a highway runoff impacted small stream due to sediment associated contaminants (Maltby et al. 1995a; Maltby et al. 1995b). They also concluded that the observed toxicity was mainly due to the PAHs pyrene, fluoranthene and phenanthrene (Boxall & Maltby 1997). Except, phenanthrene, these are the very same PAHs that dominated in the present work (Papers I and II). Too much water during the washing could also increase the risk of flushing out already trapped contaminants in the sediment (i.e. if the tunnel wash water is discharged into a sedimentation pond or other treatment facility).

A final aspect worth considering during planning and constructing of new tunnels is the use of alternative covering materials in walls and roof of the tunnels. For example, the current practise is to use concrete which causes a rough surface, while an alternative could be to use tiles or other smooth material being easier to clean. The use of detergents could then be reduced or even stopped. This strategy will be launched at the Bjørvika tunnel in Oslo, which will be partly opened in 2010.

At the moment, the NPRA are launching a project, based on the results published by Paruch and Roseth (2008a) and Paruch and Roseth (2008b), where wash water from the 3.7 km long Nøstvedt (opened 2009) will be treated in a two step process; first sedimentation and secondly filtration. Hopefully, this will contribute to reducing the concentrations of contaminant found in tunnel wash water and thereby reducing the risk of causing environmental harm.

7 Conclusions and future perspectives

The results presented in this thesis showed that runoff water caused by both precipitation and by tunnel wash were polluted, and several contaminants exceeded various EQS standards (e.g. Al, Cu, Fe, Pb, Zn and various PAHs). In addition, the total pollutant levels were generally higher in pond inlet water than in pond outlet water. However, poor removal of dissolved metal species and road salt in the Skullerud sedimentation pond (highway runoff) and relatively high discharges of LMM metal species from the Vassum sedimentation pond (tunnel wash water runoff) were observed. The source characterization of tunnel wash water runoff revealed that metals such as Zn, Cu and Sb originated mostly from vehicles, while e.g. Fe and Al seemed to be associated with pavement wear. Finally, PAHs originated most likely from combustion, tire wear and asphalt. The results also indicate that tunnel wash water, in terms of chemical concentrations, may be of most concern.

The present thesis also show that the biological short term effects in brown trout was sub-lethal in terms of causing general stress, impairing the ion regulatory system and triggering the antioxidant defense system, as well as activating the PAH biotransformation system. In addition, microarray analysis revealed molecular effects on immunological functions and on the cholesterol pathway. It should be stressed that the fish exposure studies were conducted in undiluted or in slightly diluted runoff water (e.g. 50:50 in Skullerud pond outlet water) and that the dilution factor would in most cases be higher in many recipients. Nevertheless, the reduced growth in the summer old sea trout population downstream the Vassum sedimentation pond, receiving tunnel wash water runoff, indicates in fact a long term biological effect.

The obtained results both in respect of chemical and biological measurements indicate that the treatment performance of the sedimentation ponds may be questioned. For example, high concentrations of organic matter, road salt and oxygen depletion may have a negative influence on the overall treatment performance and efficiency. Hence, there is a need for more research on new and/or alternative mitigation strategies incorporating chemical speciation as well as biological endpoints in the assessment of treatment performance. The establishment of BMPs for existing pond systems is also warranted.

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Chemical and ecological effects of contaminated tunnel wash water runoff to a small Norwegian stream

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ABSTRACT

Cleaning and washing of road tunnels are routinely performed and large volumes of contaminated wash water are often discharged into nearby recipients. In the present study, traffic related contaminants were quantified in tunnel wash water (the Nordby tunnel, Norway) discharged from a sedimentation pond to a nearby small stream, Årungselva. *In situ* size and charge fractionation techniques were applied to quantify traffic related metal species, while PAHs were quantified in total samples. All metals and several PAHs appeared at elevated concentrations in the discharged wash water compared with concentrations measured in Årungselva upstream the pond outlet, and to concentrations measured in the pond outlet before the tunnel wash event. In addition, several contaminants (e.g. Cu, Pb, Zn, fluoranthene, pyrene) exceeded their corresponding EQS. PAH and metals like Al, Cd, Cr, Cu, Fe and Pb were associated with particles and colloids, while As, Ca, K, Mg, Mo, Ni, Sb and Zn were more associated with low molecular mass species (<10 kDa). Calculated enrichment factors revealed that many of the metals were derived from anthropogenic sources, originating most likely from wear of tires (Zn), brakes (Cu and Sb), and from road salt (Na and Cl). The enrichment factors for Al, Ba, Ca, Cr, Fe, K, Mg and Ni were low, suggesting a crustal origin, e.g. asphalt wear. Based on calculated PAH ratios, PAH seemed to originate from a mixture of sources such as wear from tires, asphalt and combustion. Finally, historical fish length measurement data indicates that the fish population in the receiving stream Årungselva may have been adversely influenced by the chemical perturbations in runoffs originating from the nearby roads and tunnels during the years, as the growth in summer old sea trout (*Salmo trutta L.*) in downstream sections of the stream is significantly reduced compared to the upstream sections.

1. Introduction

In Norway, more than 1000 road tunnels having a combined length close to 800 km have been built because of the challenging landscape with fjords and mountains, and additionally, in order to protect citizens in urban areas from noise and air pollution.

Although the discharge of highly polluted wash water during cleaning of tunnels has recently gained increased awareness (Paruch and Roseth, 2008a, b), the environmental concern related to road tunnels has traditionally been on air pollution (e.g. Sternbeck et al., 2002).

Many Norwegian tunnels are frequently washed (2–12 times/year) to prevent dusty conditions and poor visibility, and additionally, to increase their life span. In brief, a washing event includes removal of dust, debris and coarse material with a road sweeping machine before detergent is applied on the tunnel surfaces and other technical infrastructure. This is followed by high pressure cleaning before a road sweeping machine makes a last run removing both dirt and un-drained wash water. According to the contractors, 60–100 L/m of wash water (0.5–1% detergent) is utilised in a two tube tunnel with four driving lanes.

Metals are often found in elevated concentrations in highway runoff as well as in tunnel wash water (e.g. Sansalone and Buchberger, 1997; Paruch and Roseth, 2008b), and can be present in different physico-chemical forms (i.e. metal species), varying in size (nominal molecular mass), charge properties and valence, oxidation state, structure and morphology, density, ligands etc. (Salbu and Oughton, 1995). Compared with high molecular mass (HMM) species such as

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colloids, polymers, pseudocolloids and particles, low molecular mass (LMM) species are believed to be more mobile and potentially more bioavailable. The aquatic system is highly dynamic and alterations in water quality variables such as pH, ionic strength, redox potential, temperature, suspended solids and the availability of inorganic and organic ligands (e.g. carbonates and organic matter) may change the speciation of metals and metalloids (Salbu and Oughton, 1995; Teien et al., 2004). A series of fractionation techniques have been utilised in the laboratories for metal speciation purposes, although *in situ* fractionation techniques should be applied to avoid storage effects affecting the species distribution. Utilising size exclusion techniques such as filtration and ultrafiltration, LMM species can be differentiated from colloids and particles. Combining size exclusion with charge separation techniques such as ion chromatography, information on positively-, negatively- and neutrally-charged LMM species can be attained (Teien et al., 2004). Therefore, the application of *in situ* size and charge fractionation techniques, as applied in the present work, is judged to be the most useful technique for characterizing fluctuating tunnel wash runoff, as well as the water quality which aquatic organisms are exposed to.

Metal toxicity towards aquatic organisms is well documented, and effects have been observed at different biotic levels represented by effects at a molecular and cellular level (e.g. protein damages, lipid peroxidation, chemosensory impairments and osmoregulatory failures) (e.g. Golovanova, 2008), effects at an individual level (e.g. changed behavior, reduced growth and condition factor) (e.g. Scott and Sloman, 2004; Golovanova, 2008), and finally, effects at a population level (e.g. alterations of the social hierarchies among fish) (e.g. Scott and Sloman, 2004). Hence, in the context of environmental management it is crucial to obtain data concerning metal speciation to better understand and predict the fate of metal contamination, as well as for the implementation of treatment by best management practices (BMPs).

Along with several metals, polycyclic aromatic hydrocarbons (PAHs) often appear at elevated concentrations in highway runoff, and their main sources are combustion, wear from tires, bitumen from the asphalt and leakage and spill of petroleum products (Kose et al., 2008; Napier et al., 2008). According to a recent review by Napier et al. (2008), the release of automobile derived PAHs in the environment shows an upward trend. This is of major concern since PAHs can cause serious harm to aquatic organisms, like mortality in all life stages, decrease in growth, reduced condition factor, edema, cardiac dysfunction, deformities, lesions and tumors, cataracts, immune system dysfunctions and estrogenic effects (Logan, 2007).

In Norway, harsh winter conditions requires the use of significant amounts of de-icing chemicals (normally NaCl) on the roads to enhance good friction and traffic safety for the road users. Road pavements inside tunnels are normally not de-iced, but deposition of salt may occur as a consequence of the vehicles passing through the tunnels. In addition, $MgCl_2$ is occasionally applied as a dust suppressor. High salt concentrations in highway runoff are frequently reported to affect freshwater ecosystems and may cause increased drift of lotic macroinvertebrates (Crowther and Hynes, 1977), alteration in fish blood physiology (Vosyliene et al., 2006) and alterations on amphibian community structures by excluding salt intolerant species (Collins and Russell, 2009). In addition, application of road salt may mobilize trace metals through ion exchange processes, thus potentially increasing their bioavailability (Amrhein et al., 1992).

The objective of the present study was to *in situ* characterize and quantify traffic related contaminants in runoff from a tunnel wash event discharged from a sedimentation pond into the stream Årungselva, and identify if any impact from these discharges could be identified for the downstream fish population, such as long-term changes in growth and density of juvenile sea trout (*Salmo trutta L.*).

2. Materials and methods

2.1. Study site

The water sampling survey was conducted April 20–21, 2006 during the last night of washing of the Nordby tunnel on the motorway E6. The 3.84 km long tunnel, with two separate tubes (each tube 10 m wide with two lanes) is situated 30 km south east of the City of Oslo, Norway (Fig. 1). The walls and the roof of the tunnel are covered with concrete. It was opened for traffic in 1993 and the annual average daily traffic (AADT) is approximately 25 000 vehicles (11% heavy duty vehicles >3500 kg) with an average vehicle speed of 89 km/h.

The tunnel is cleaned typically 4 to 6 times per year, one being a full wash. Before year 2000 the tunnel wash waters were diluted and discharged together with clean drainage water from the surrounding rock, while after year 2000 they were discharged separately. A significant fraction of coarse material and debris are removed from the runoff as it flows through several gully pots. Finally, the runoff is pumped to a sedimentation pond outside the tunnel before it is discharged into the stream Årungselva (Fig. 1). The sedimentation pond was established in spring 2000 as part of a larger extension of the motorway E6, which also included the construction of two additional new tunnels, the Smiehagen tunnel (0.95 km) and the Vassum tunnel (0.85 km). Hence, the sedimentation pond also receives runoff from these two tunnels and from 17 000 m^2 (1.7 ha) of open road areas between these tunnels.

Årungselva has a length of 2.5 km, from the eutrophic Lake Årungen to the Oslofjord. The discharge from the Lake Årungen, which mainly occurs during the spring and autumn floods, varies between nearly 0 m^3/s to at least 25 m^3/s. However, during the summer drought, long sections of the stream may be dry, except in the lower part where groundwater supply gives a wetted area even in such periods (Borgstrøm and Heggenes, 1988).

2.2. Water quality assessment

The tunnel wash water volume was estimated by recording the run time (hours) of the two drainage pumps and by multiplying the run time with their corresponding pump capacity (m^3/h). An automatic data logger (CR200 Campbell Scientific Ltd., recording every 10 min) was installed in a manhole in the outlet of the sedimentation pond and equipped with a multi water quality sensor (HORIBA W21SDI: temperature, pH, conductivity, dissolved oxygen and redox potential) and a turbidity sensor (WQ710 Global Water). All wash water samples from four sampling rounds during the washing event were collected from the manhole by using a peristaltic pump. In addition, one sampling round in the manhole and one upstream the pond outlet in Årungselva were performed prior to the washing event, and used as references in the data interpretation.

PAHs were analysed at the Bioforsk laboratory according to the USEPA analytic technique (EPA 8100). The limits of quantification (LOQ) were 0.01 µg/L for acenaphthylene, anthracene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene, 0.05 µg/L for fluorene, phenanthrene, fluoranthene, pyrene and chrycene and 0.1 µg/L for naphthalene and acenaphthene.

2.2.1. Metal partitioning by in situ fractionation of water

Ultrafiltration and ion exchange chromatography (IEC) were applied *in situ* to characterize the water with respect to size and charge distribution of the elements, as described by Salbu and Oughton (1995) and Teien et al. (2004). Ultrafiltration was performed using an Amicon H1P1-20 hollow-fibre cartridge with a nominal molecular cut-off mass of 10 kDa. IEC was performed on ultrafiltered water by cation exchange chromatography (Chelex-100 resin, Biorad)

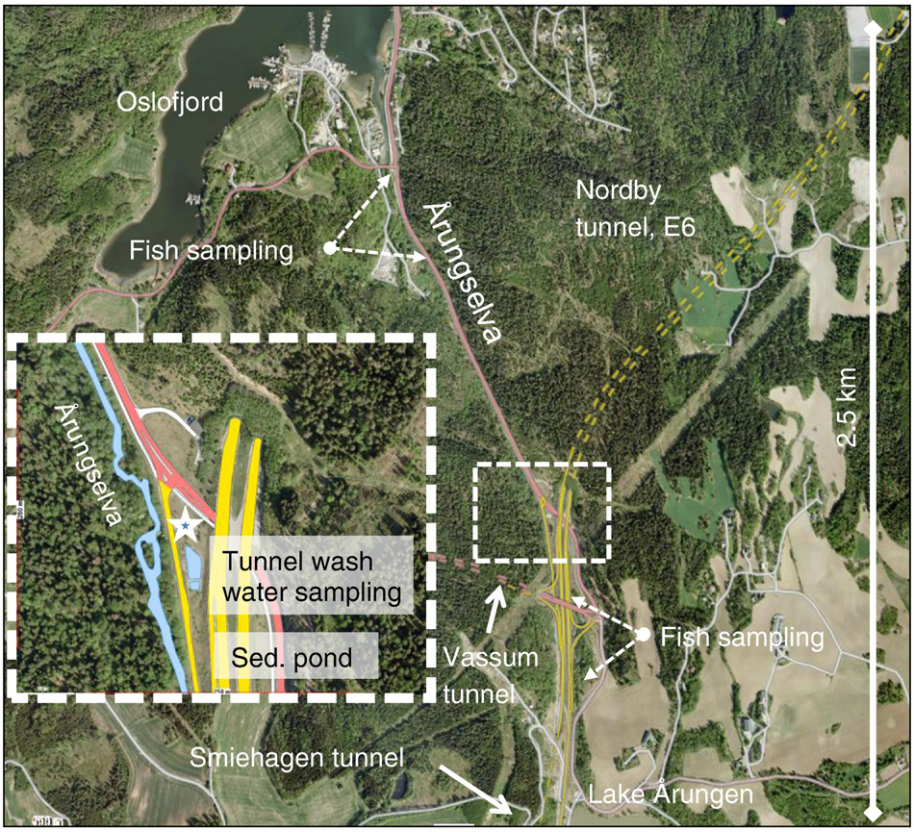

Fig. 1. Overview of the investigated area, including roads, tunnels, sedimentation pond and the recipient stream, Årungselva. The fish samplings were conducted within the sections marked with dashed arrows. The small dashed square indicates the magnified area displayed in the enlarged dashed square to better display the pond. The discharged tunnel wash water was sampled from the pond outlet indicated by a star.

and anion exchange chromatography (AG1-X8, Biorad), using approximately 15 mL resin material in a column with a water flow rate of 30 mL/min. Thus, operationally defined, the following fractions were obtained:

- Total (unfiltered sample);
- Particulate and HMM (high molecular mass)/colloidal species = concentration in total sample subtracted concentration in ultrafiltered sample;
- LMM species (low molecular mass) = concentration in ultrafiltered sample (<10 kDa);
- LMM + species, positively charged = concentration retained on Chelex-100, derived by subtracting total LMM concentration by the cation exchange eluat concentration;
- LMM − species, negatively charged = concentration retained on AG1-X8, derived by subtracting total LMM concentration by the anion exchange eluat concentration; and
- LMM 0 species, neutral = total LMM species subtracted LMM positively charged and negatively charged.

All samples were acidified in 1% suprapure concentrated HNO_3 immediately after sampling and stored cold and dark until instrumental analysis. The concentration of aluminum (Al), calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), potassium (K), sodium (Na) and silicon (Si) were determined using ICP-OES (Perkin Elmer Optima 5300DV), while the concentrations of arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), cupper (Cu), molybdenum (Mo), nickel (Ni), lead (Pb), antimony (Sb), vanadium (V) and zinc (Zn) were determined using ICP-MS (Perkin Elmer ELAN 6000). Internal standards, blanks and reference material (SRM 1643e, National Institute of Standards and Technology) were used as quality control. The detection limits (LOD) for the metals expressed in µg/L were 0.2 (Al), 0.01 (As), 0.003 (Ba), 50.0 (Ca), 0.002 (Cd), 0.001 (Co), 0.03 (Cr), 0.02 (Cu), 50.0 (K), 10.0 (Mg) 1.0 (Fe), 0.004 (Mo), 3.0 (Mn), 200 (Na), 0.8 (Ni), 0.02 (Pb), 0.002 (Sb), 30.0 (Si), 0.04 (V), and 0.6 (Zn). The measurements of metals in the reference material were all within 5% of certified value.

2.2.2. Mass balance and enrichment factor

The total mass of the various contaminants was estimated from their total concentrations and the calculated discharged water volume from the pump house to the pond. These estimates are rough approximations as the calculations are based on the average concentrations obtained from four sampling rounds during the last night of washing. In addition, the water volume is based on wash water entering the pond and not actual water flow from the outlet.

To discriminate between contaminants with an anthropogenic source (vehicles) from those having a crustal origin (road surface/tunnel walls) the data was normalized with respect to silicon (Si) and an enrichment factor (EF) was calculated for all contaminants. The use of EF is based on the assumption that contaminants linked to an anthropogenic source will have an EF>1, while those having an EF close to 1 are likely to have a crustal origin (Balasubramanian and Qian, 2004). Enrichment factors have commonly been utilised in metal contaminated sediments and most of them have utilised aluminium (Al) or iron (Fe) as a normalisation factor due to their crustal abundance (e.g. Sutherland, 2000). However, both Al and Fe are significant elements in car manufacturing. Si was therefore selected for normalisation of the data, as this metal is abundant in soil and bedrock in this area (i.e. quartz and feldspar) but, as far as we know, is of minor importance in car manufacturing. Hence, the EF was obtained by calculating the ratio between the concentrations of each contaminant measured in the tunnel wash water and in the stream water (background value) after normalizing with the Si concentrations. Hence, the EF was obtained by the formulae $(C\ X_{wash\ water}/C\ Si_{wash\ water})/(C\ X_{SW}/C\ Si_{SW})$, where

C $X_{wash\ water}$ = concentration of contaminant X in the wash water, C $Si_{wash\ water}$ = concentration of Si in wash water, C $X_{stream\ water}$ = concentration of contaminant X in stream water, C $Si_{stream\ water}$ = concentration of Si in stream water.

Different papers operate with different threshold values, however, in the present paper the following values, set by Sutherland (2000), are used: EF<2 no/minimal anthropogenic signal, 2–5 moderate, 5–20 significant, 20–40 very strong and EF>40 extreme anthropogenic signal. The concentration of total PAH in the stream water was below limit of quantification (0.1 µg/L) and an EF for PAH was therefore not calculated.

2.3. Fish sampling

The stream Årungselva is a spawning locality for sea trout. In the autumn, the population in the stream consists of parr, mainly 0+ and 1+, and older fish which have returned from the Oslo fjord. Fish have been collected by electrofishing, using a direct current back pack shocker (Geomega, Trondheim) with current output 600 V at 1000 Ω, pulse length 1.8 ms at 70 Hz (Borgstrøm and Skaala, 1993). The anode was a wand-mounted ring, ca. 15 cm in diameter and covered by fine-meshed net to increase the catch probability. In addition, a hand net was used for capturing stunned fish. The cathode was a wire placed on the stream bottom behind the operator. The battery was used at a voltage between 12 and 13 V. Total length of the fish was measured to the nearest mm, and otoliths were collected from subsamples to control the age. The sampling has been carried out on two stream sections, one in the lowermost part near the fjord, and one upstream the pond outlet (Fig. 1). Fishing was performed over the whole stream bed in both sections. However, as this fish sampling has not been part of any monitoring program related to highway runoff, far more data exists from the downstream section (1995, 1996, and 1998–2009) compared with the upstream section (1995, 1998, 1999, 2006, and 2009).

2.4. Statistics

The univariate and multivariate statistics were conducted by using Minitab 15 software and CANOCO 4.55, respectively. Results with $p<0.05$ were assigned statistically significant.

One-sample *t*-test was utilised to statistically evaluate the EF values against the threshold values. The data, based on the four consecutive water samples during the tunnel wash, was checked for normality prior the one-sample *t*-test.

The assumptions of normality and equal variance were met after a square root transformation of the fish length data, while no transformation was necessary for the density data. One-way ANOVA followed by Tukey Simultaneous Tests were applied both to test for any differences between fish lengths sampled upstream and downstream the sedimentation pond outlet prior and after the pond was established, and to test for any differences in density.

A multivariate redundancy analysis (RDA) was applied to evaluate the variation in the water quality data set. The metal concentrations were used as response variables, while a set of dummy variables (1 and 0) coding for the various size and charge fractions were used as explanatory variables (i.e. particles and colloids, LMM +, LMM − and LMM 0). Only samples from the discharged tunnel wash water, sampled from the outlet, were included in the model. Hence, the data set consisted of 16 samples (4 fractions x 4 sampling rounds) and 15 variables summing up to a total of 240 measurements.

The data were log $(x+1)$ transformed and we used a forward selection procedure combined with Monte Carlo permutation tests (499 permutations) to identify which of the categorical explanatory variables contributed significantly to the observed variation.

The results of the RDA are displayed in ordination diagrams. Both response variables and explanatory variables are displayed in the diagram as arrows pointing in the direction of steepest increase of their corresponding values. Arrows going in the same direction are positively correlated, while arrows going in opposite direction are negatively correlated. Arrows forming an angle close to 90 degrees are considered uncorrelated. Also, the variables with the longest arrows have the greatest variance in the data material.

More detailed information about the RDA is given by Lepš and Šmilauer (2003).

3. Results and discussion

3.1. Water quality and total concentrations of contaminants

Approximately 300 m^3 of wash water was pumped to the sedimentation pond during the three nights of washing, of which 65 m^3 (22%) was discharged during the last night (Fig. 2). Based on earlier information from the contractors, the water consumption was slightly increased compared to earlier maintenance practice.

Opposite to pH, which remained circumneutral, temperature, turbidity, conductivity, dissolved oxygen and redox potential increased rather rapidly due to the incoming tunnel wash water (Fig. 3). The high increase in turbidity clearly demonstrated the significance of particles in highway runoff as well as in tunnel wash water runoff. Although road salt is normally not applied inside the tunnel, the high conductivity, being approximately 0.95 S/m at its highest, indicated large deposition of road salt readily flushed out with the wash water. This is also confirmed by the high concentrations of sodium and chloride (Table 1) measured in the tunnel wash water, being 48 and 33 times the concentrations found in the stream water, respectively.

Organic matter is an important ligand for metals as well as for PAHs (Krein and Schorer, 2000). In the present study, the organic matter in the tunnel wash water, measured as TOC, was mainly found in the LMM fraction, indicating the presence of substances like fulvic acids, fatty acids, amino acids etc. (Thurman, 1985; Salbu and Oughton, 1995) probably originating from biodegraded plant material in the sedimentation pond (Table 1). Various LMM compounds from the applied detergent, oil etc. could also contribute to the organic matter loadings.

3.1.1. Concentrations and discharged loadings of trace metals and PAHs

The concentrations of the various metals in the pond outlet (Ref pond) were low prior to the tunnel wash and comparable with the concentrations measured upstream in the recipient stream, Årungselva (Fig. 4). In addition, the concentrations of PAHs were below quantification levels both in the stream and in the pond outlet prior to the tunnel wash event. According to Amundsen and Roseth (2004), tunnel wash water runoff is normally more contaminated than runoff from open road areas, and as expected, the concentrations

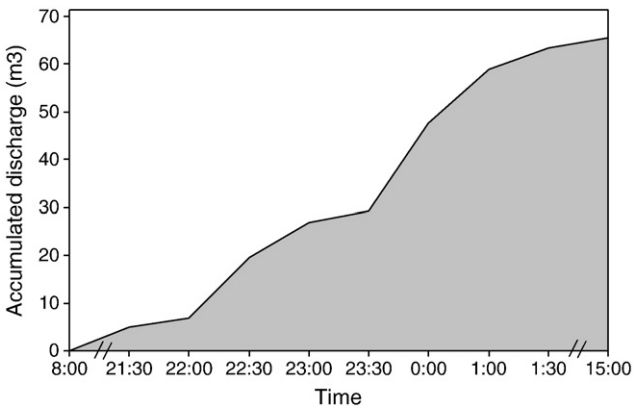

Fig. 2. Accumulated tunnel wash water entering the pond during the studied tunnel wash event.

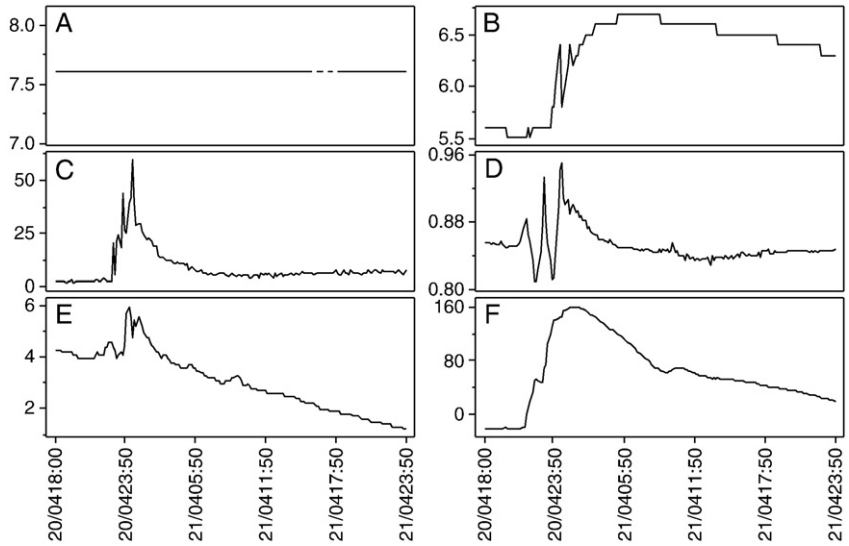

Fig. 3. Variation of logged water quality data from the pond outlet during the tunnel wash event. A = pH, B = temperature (°C), C = turbidity (NTU), D = conductivity (S/m), E = dissolved oxygen (mg/L) and F = redox potential (mV).

of metals and PAHs increased substantially in the pond outlet during the tunnel wash. In contrast to what is often observed in open road areas (e.g. Sansalone and Buchberger, 1997), and despite that the concentrations were slightly higher in the initial phase of the wash event for most of the contaminants, a distinct "first-flush" effect was not apparent in the present study (Figs. 4 and 5). This might be due to the hydrological difference between precipitations, which immediately remove the contaminants over a large area, and successively cleaning by high pressure working from one end of the tunnel to another.

Twelve of the 16 PAHs in the tunnel wash water had concentrations above LOQ (Fig. 5), and a majority of these compounds are classified as carcinogenic and mutagenic. The quantification of PAHs in the LMM fraction was conducted in only one sample (01:00 h). All the measured compounds were, however, below LOQ. Hence, the PAHs seemed to be associated with the particulate and colloidal fraction which is in line with other reported studies (e.g. Karlsson and Viklander, 2008).

Table 1
Variables measured in water samples collected from the stream Årungselva (SW, $n = 1$) and from the outlet of the sedimentation pond prior (Ref pond, $n = 1$) and during the tunnel wash event. The tunnel wash water data ($n = 3$–4) are presented as mean ± standard error of mean (SEM), minimum (Min.) and maximum (Max.).

Variable	Unit	SW	Ref pond	Tunnel wash[a]			
				Mean	SEM	Min.	Max.
pH	–	7.0	7.5	–	–	–	–
Conductivity	mS/m	22.8	66.1	–	–	–	–
Temperature	°C	–	3.1	–	–	–	–
Hardness	mg $CaCO_3$/L	72	105	160	3	154	165
TOC	mg/L	6.7	3.1	10.6	0.1	10.3	10.8
TOC LMM	mg/L	4.2	2.3	11.4	0.8	9.8	13.3
Ca	mg/L	20.7	35.4	47	0.5	46	48
K	mg/L	3.8	3.3	12.6	0.4	11.7	13.3
Mg	mg/L	4.9	4.0	10.3	0.4	9.4	11.4
Mn	mg/L	0.04	0.02	0.18	0.01	0.15	0.21
Na	mg/L	13.5	86.3	645	21	592	692
Si	mg/L	4.3	2.5	7.5	0.6	6.5	9.0
Chloride	mg/L	23.6	98.6	788	18	736	820
Sulphate	mg/L	13.3	41.4	59.6	0.7	58.1	61.4
Nitrate	mg/L	3.2	0.9	2.3	0.4	1.9	3.4
Fluoride	mg/L	0.3	0.2	<LOD	<LOD	<LOD	<LOD

[a] pH, conductivity and temperature in the discharged tunnel wash water are displayed in Fig. 3.

The total amount of discharged PAHs, trace metals and road salt to the stream Årungselva during the entire washing event was 0.8, 2.3 and 435 kg, respectively (Table 2). Twenty four percent of the trace metal loadings were discharged as LMM species.

3.2. Source characterisations

The calculated enrichment factors (EF), based on total concentrations normalized by Si concentrations, are displayed in Fig. 6. Mn, V, As and Cd were significantly higher than EF 2, indicating a moderate enrichment. Pb, Mo, Cu, Co, Cl and Sb were significantly higher than EF 5, corresponding to a significant enrichment. Finally, Na and Zn had an EF significantly higher than 20 and 40, indicating very strong and extreme enrichment, respectively.

3.2.1. Sources of metal contamination

Based on present knowledge, most of the enriched metals can be associated with different sources from vehicle components and combustion. For example, Sb has been suggested as an applicable fingerprint for brake wear, while Zn and Cu are highly abundant both in tires and brakes (Sternbeck et al., 2002; Thorpe and Harrison, 2008; McKenzie et al., 2009). To identify the presence of wear from brakes in the environment, a Cu:Sb ratio around 5:1 has been proposed (Sternbeck et al., 2002). Based on total concentrations and LMM concentrations, the Cu:Sb ratios in the present study were 9.8 ± 1.6 and 2.5 ± 0.5, respectively. In comparison, the Cu:Sb ratio in the stream water was 22.9 and 13.0, respectively. Dongarra et al. (2009) showed that the Cu:Sb ratio varied among different environmental matrices (e.g. particulate matter vs. plant material). Hence, differences in matrices may explain why the ratio, in the present study, is not completely comparable with the suggested 5:1. The extreme enrichment of Zn may indicate that there were other contributing sources, e.g. Weckwerth (2001) argued that diesel soot is an important Zn source. Finally, zinc galvanized steel and iron abundant in equipment inside the tunnel, might have enhanced the Zn contribution observed in the present study.

A possible source for Co emissions in highway environment is diesel combustion (Wang et al., 2003), while diesel combustion together with brake wear are important for Mo (Weckwerth, 2001; Wang et al., 2003; Dongarra et al., 2009).

Pb has been a major concern, in highway runoff as well as in highway air pollution, due to its high abundance in gasoline. However, Pb as an antiknock agent in gasoline has been banned in EU since 2000

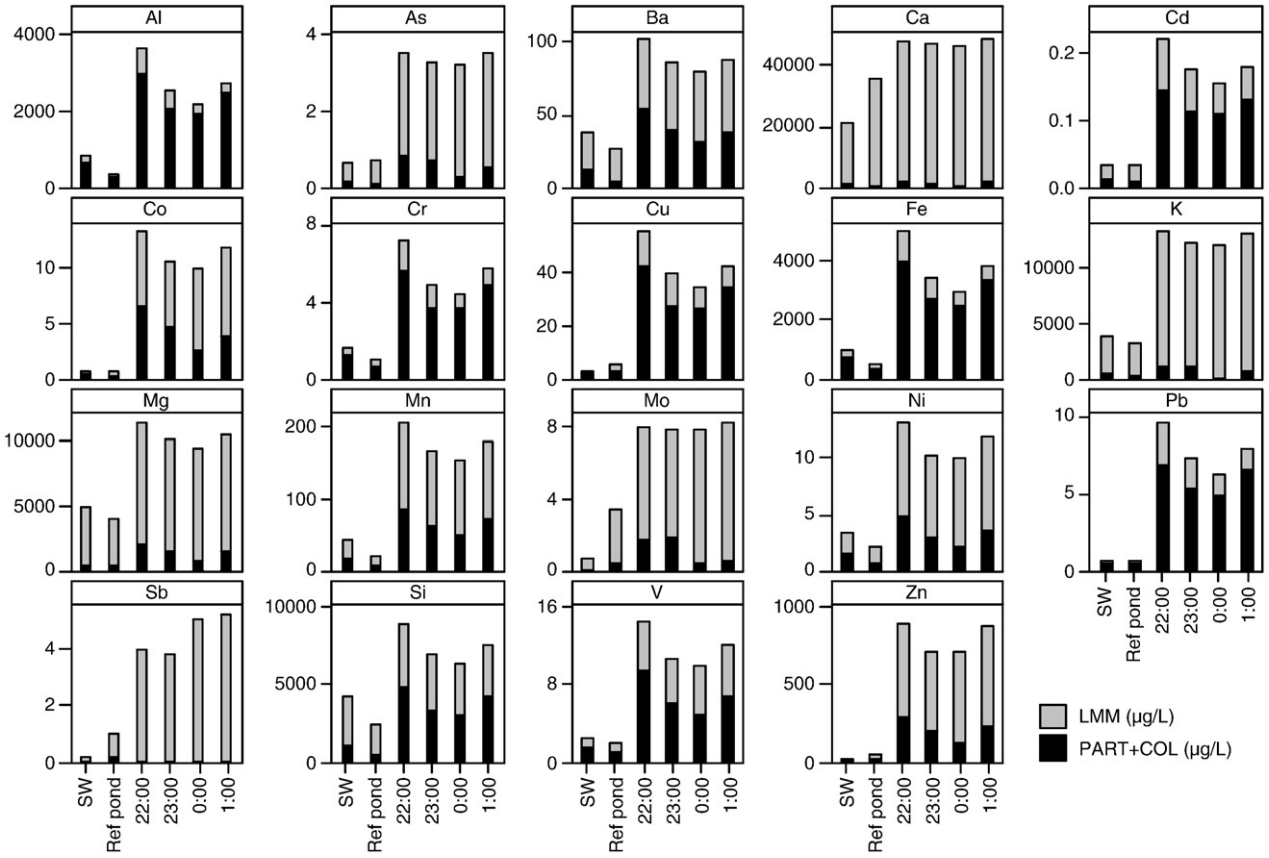

Fig. 4. Concentrations of metals in discharged tunnel wash water compared to the concentrations in water sampled upstream the recipient Årungselva (SW) and to the concentrations in water sampled from the pond outlet before the tunnel wash event (Ref pond). In addition, the partitioning between LMM species and the particle and colloidal fraction is displayed in the bars with grey and black colors, respectively.

(Hjortenkrans et al., 2006). Thus, other sources gave rise to the observed anthropogenic signal. According to Thorpe and Harrison (2008), Pb is highly present in brake linings, tires and asphalt.

Mn, V, As and Cd were only slightly enriched in the tunnel wash water, indicating that these elements have a mixed source, more or less equally portioned between vehicle wear and road surface wear (crustal origin). Brake wear and fuel emissions may have contributed to the enrichment of Mn, and additionally, fuel might be a source for V as well (Sansalone and Buchberger, 1997; Lin et al., 2005). Brake and tire abrasion could be a source for Cd (Sansalone and Buchberger, 1997; McKenzie et al., 2009), while the origin of As seems to be poorly evaluated in the scientific literature regarding highway pollution.

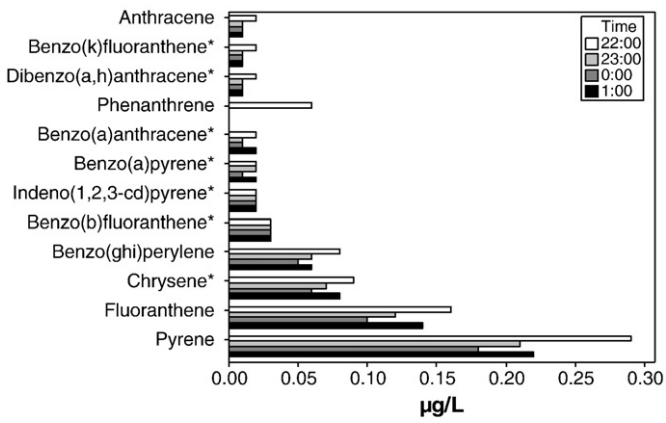

Fig. 5. The concentrations of various PAH compounds in the discharged tunnel wash water. Carcinogenic PAHs are indicated with *.

The high EF observed for Na and Cl clearly demonstrates the contribution of road salt. It appeared that Na was more enriched than Cl in the discharged tunnel wash water. Hence, this might indicate that there were other sources, in addition to road salt, that enhanced the EF for Na, for example brakes, as suggested by McKenzie et al. (2009).

Table 2
Estimated loadings discharged from the sedimentation pond during the tunnel wash. The loadings are estimated by multiplying the discharge from the pump house with the average concentration of each contaminant ($n=4$). In addition, the % partitioning between the particulate and colloidal fraction (Part + Col) and the low molecular mass species (LMM) are displayed.

Contaminant	Total loadings (g)		% Partitioning	
	3rd night	Whole wash	% Part + Col	% LMM
Fe	246	1143	83	17
Al	181	841	85	15
Zn	53	245	26	74
Mn	12	54	38	62
Ba	6	27	47	53
Cu	3	13	75	25
V	0.8	3.6	58	42
Ni	0.7	3.4	29	71
Co	0.7	3.4	39	61
Mo	0.5	2.4	14	86
Pb	0.5	2.4	76	24
Cr	0.4	1.7	80	20
Sb	0.3	1.4	0	100
As	0.2	1.0	18	82
Cd	0.01	0.1	68	32
∑metals	505	2343	76	24
Road salt (∑Na + Cl)	93 874	435 671	–	–
16 PAH	0.18	0.82	100	–

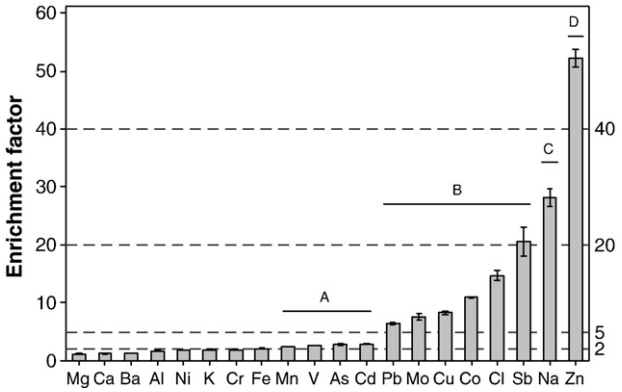

Fig. 6. Enrichment factors of contaminants measured in the discharged tunnel wash water (EF, mean ± SEM). Various threshold values (2 = moderate, 5 = significant, 20 = very strong and 40 = extreme) are displayed as horizontal dotted lines. A = significant higher than 2, B = significant higher than 5, C = significant higher than 20 and D = significant higher than 40.

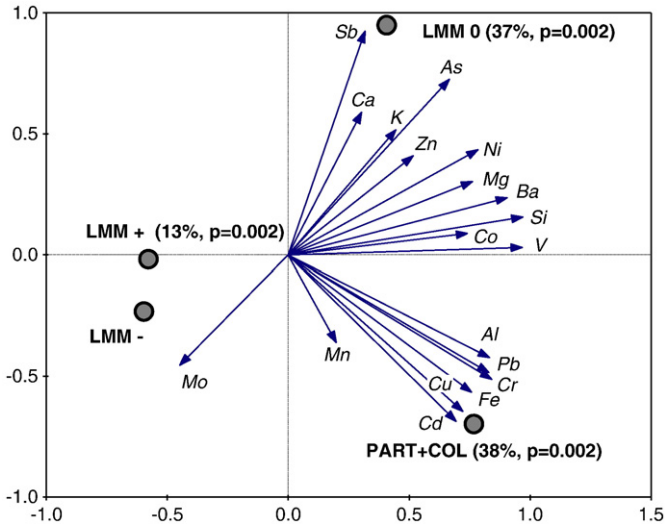

Fig. 7. RDA-plot obtained from the RDA analysis showing the variation explained by the three significant fractions from the Monte Carlo forward selection procedure: particle and colloidal fraction, LMM 0 and LMM +. Percentage explained variation of each fraction is indicated next to its corresponding centroide. The metals are displayed as arrows.

The major elements Mg, Ca, K, Al and Fe are typically crustal elements, indicating wear from the road surface as well as input from the concrete inside the tunnel (Thorpe and Harrison, 2008). Ba, Ni and Cr are trace metals that have been related to brake dust (Ba) (Sternbeck et al., 2002) and diesel emissions (Ni and Cr) (Weckwerth, 2001; Wang et al., 2003). In the present study they were, however, not anthropogenically enriched.

3.2.2. Sources of PAH contamination

A common approach to characterize possible emission sources of PAHs is to utilize different ratios between single PAHs or groups of PAHs. In the present study, there was a dominance of HMM PAHs (>4 rings) compared to LMM (2–3 rings) giving the LMM:HMM concentration ratio 0.08. A LMM:HMM ratio less than one indicates a pyrogenic origin (e.g. incomplete combustion) (e.g. Karlsson and Viklander, 2008). Alternative approaches utilize the formula anthracene/anthracene + phenanthrene or fluoranthene/fluoranthene + pyrene to determine the PAH origin, where values greater than 0.1 and 0.5 indicate pyrogenic origin, respectively (e.g. Karlsson and Viklander, 2008). In our study, these corresponding values were 0.3 and 0.4, indicating pyrogenic and a mix of pyrogenic and petrogenic sources, respectively. Examples of petrogenic sources are bitumen in asphalt and tires. The high content of pyrene and chrysene in the wash water supports the latter (Brandt and de Groot, 2001; Karlsson and Viklander, 2008), and a Japanese study concluded that tire wear was the most important source of PAH in road dust, followed by asphalt as the second largest contributor (Kose et al., 2008). Hence, the PAH profile in our study indicates, based on the various approaches, a mix of sources including wear from tires and road surface and combustion, coherent with results published by Karlsson and Viklander (2008).

Most of these EFs and ratios are derived from studies on airborne particles, road deposited sediments, stormwater sediments etc. and might not be fully applicable for source characterization of water and runoff samples as in the present study. Further investigations on this topic are needed to better understand the dynamic, transfer and fate of pollutant from highway runoff as well as from tunnel wash water discharges to the aquatic environment. More research on tunnel wash water is, in this respect, very useful because it only reflects traffic derived pollutants and not other anthropogenic sources, e.g. deposition of long-range air pollutants. This would also be beneficial from an environmental management perspective.

3.3. Metal speciation

Based on the size fractionating technique and the following RDA (Figs. 4 and 7), it was evident that Al, Cd, Cr, Cu, Fe, Pb and to some extent Mn were strongly correlated with particles and colloids, while As, Ca, K, Mg, Mo, Ni, Sb and Zn were more associated with LMM species. According to the RDA, 38% of the total variation was significantly explained by the particle and colloidal fraction. A further 50% could be significantly assigned to the LMM fraction, which is of great concern as LMM species are believed to be more bioavailable than particle associated contaminants (Steinnes and Salbu, 1995). Ba, Co, Si and V were more or less equally partitioned between the two size fractions. These results are comparable with other studies, although different speciation techniques have been applied, e.g. the nominal cut off used to separate different size classes differ (Harrison and Wilson, 1985; Bechet et al., 2006; Tuccillo, 2006).

As the major part of TOC was found in the LMM fraction, we may conclude that the metals found in the particulate and colloidal fractions originated from road surface wear and precipitates of e.g. oxides and hydroxides. The high concentrations of Al, Fe and Mn support the latter processes, as oxyhydroxides of these metals have the ability to scavenge other trace metals efficiently (Bechet et al., 2006; Tuccillo, 2006). This is also supported by the correlation between Al and Fe and Cd, Cr, Cu and Pb (Fig. 7).

The metals associated to LMM species varied with respect to charge (Figs. 7 and 8). As much as 37% of the total variation, revealed in the RDA, was attributed to the neutrally charged LMM fraction. This fraction was associated with As, Ca, K, Mg, Ni, Sb and Zn. The high abundance of neutrally charged metal species could indicate binding to various complexes, or the formation of neutrally charged ion pairs by electrostatic interactions between cations and anions, which is likely to occur in water with high ionic strength (Simkiss and Taylor, 1995). In contrast, Mo was mostly present as negatively charged species. Mo together with the metalloids As and Sb normally occur in surface water as anionic species. However, both As and Sb were present mainly as neutrally charged species.

3.4. Environmental criteria assessment

Different countries have diverging approaches for benchmarking environmental quality standards (EQS). For example, USA, Canada and EU use hardness dependent standards derived mainly from toxicity data, while the Nordic countries Norway and Sweden have classifying systems scaled according to the deviation from background or natural concentrations (Table 3). EQS for metals are sometimes

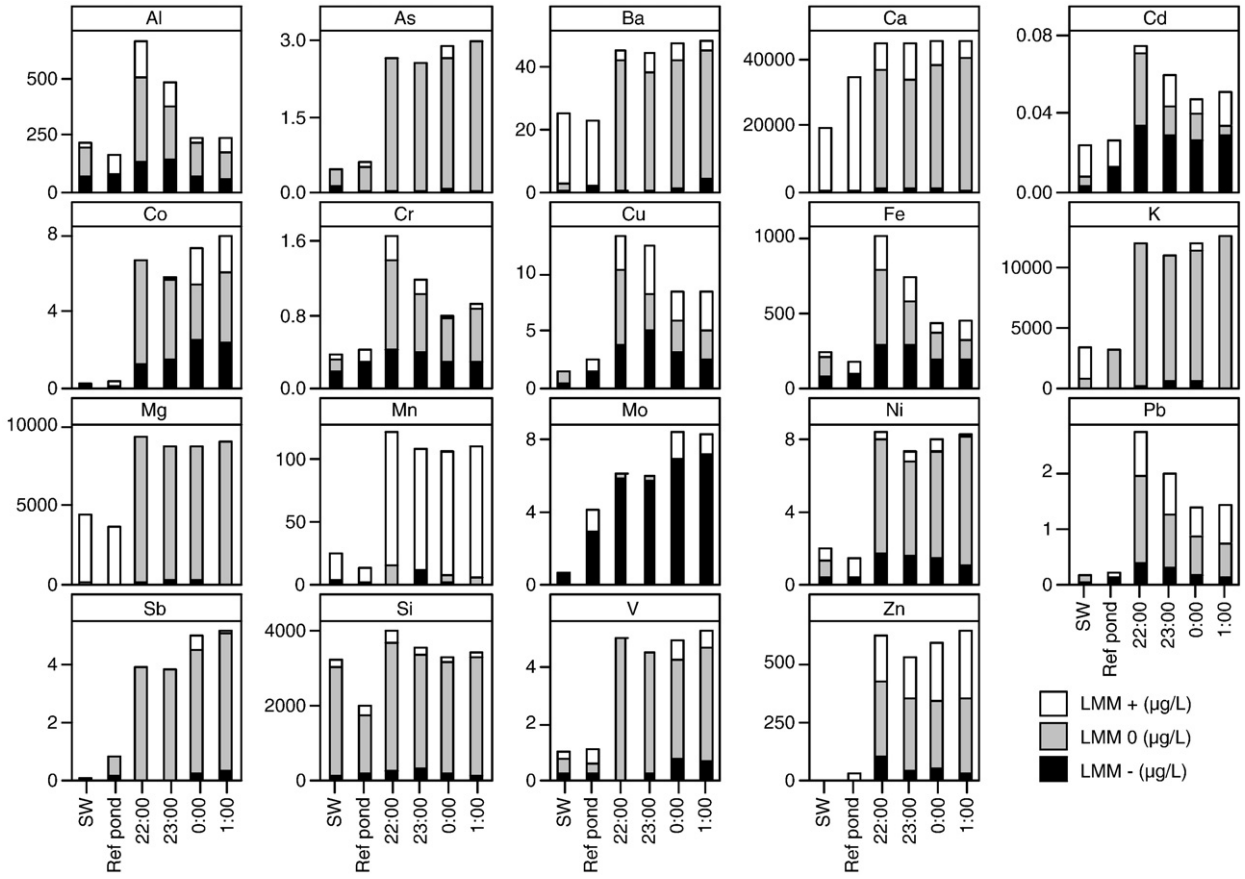

Fig. 8. Concentrations of LMM metals in discharged tunnel wash water compared to the concentrations of LMM metals in water sampled upstream the recipient Årungselva (SW) and to the concentrations of LMM metals in water sampled from the pond outlet prior the tunnel wash event. In addition, the partitioning between positively charged species (LMM +), neutral species (LMM 0) and negatively charged species (LMM −) are displayed in the bars with white, grey and black colors, respectively.

based on the dissolved fraction operationally defined by 0.45 µm filtered water samples, despite the fact that this cut-off will include, in addition to simple ions, hydrolysis products, complexes, polymers and colloids (Salbu and Oughton, 1995). In the present study we utilised ultrafiltration which excludes the colloidal fraction. Hence, the environmental toxicity assessment for trace metals using EQS obtained from US EPA and EU, which is based on 0.45 µm filtered water samples, might be somewhat underestimated. However, it is worth mentioning that Tuccillo (2006) concluded that trace metal contaminants associated to colloids (10 kDa–0.45 µm) were of minor importance compared to the LMM fraction (<10 kDa) and the particulate fraction (especially particles >5 µm) in discharged highway runoff.

Only phenanthrene and As, Cr and Ni did not exceed any of the benchmarks listed in Table 3. In contrast, Al, Zn and Cu exceeded the benchmarks given for chronic exposures in USA and Canada, and additionally, the former two also exceeded the USA benchmarks related to acute exposure (i.e. CMC is the highest level for a 1 h average exposure not to be exceeded more than once every 3 years). Fluoranthene and pyrene were the PAHs that most clearly exceeded the Canadian guidelines. The EU has, beside benchmarking the single compound benzo(a)pyrene, given two threshold values based on the sum of benzo(b)fluoranthene and benzo(k)fluoranthene, and the sum of benzo(g,h,i)perylene and indeno(1,2,3-cd)pyrene, respectively. These two values were exceeded in the tunnel wash water, while the benzo(a)pyrene threshold was not.

Cu, Pb and Zn concentrations in discharged tunnel wash water were all ranked in class 4 or 5 in the Norwegian and the Swedish classification system (Table 3). According to the Swedish system, class 4 indicates a growing risk of biological effects, and class 5 indicates reduced survival of organisms even during short term exposures. Although different approaches, this classification is consistent with the benchmark values obtained from Canada, USA and EU. The same three metals were also those with the highest rank in the Norwegian classification system related to salmonids in surface water (Table 3) (Lydersen et al., 2002). The Cu and Zn concentrations were ranked in class 4 “no salmonids and serious effects also on many other species,” while the Pb concentration was ranked in class 3 “effects on salmonids, reduced number of species with a dominance of tolerant species.”

Although the wash water runoff is treated by the means of a sedimentation pond, the observed concentrations of Al, Cu, Pb, Zn and several of the PAHs are, according to the EQS, quite worrying. It should be stressed that these concentrations were measured in the discharged wash water, and not in the receiving recipient where dilution would lower the concentrations rapidly. However, several have highlighted these pollutants in highway runoff as a major concern (e.g. Sansalone and Buchberger, 1997; Karlsson and Viklander, 2008). To put this into a broader perspective, several Norwegian tunnels have no kinds of pre-treatment measures of discharged tunnel wash water other than gully pots. In addition, tunnels with higher traffic loadings than the Nordby tunnel (AADT>25 000) and tunnels of greater size/length are likely to contribute to the input of even higher pollutant loadings to the aquatic environment. Hence, contaminants released during washing of tunnels should be removed at least by sedimentation before entering water bodies. The present study also demonstrates that a significant part of the pollution is released as dissolved species. Consequently, implementation of some kind of organic sorbent material in order to reduce the contribution from dissolved

Table 3
The concentration of waterborne contaminants measured in tunnel wash water runoff ($n=4$) compared to %nvironmental quality standards (EQS) for fresh waters obtained from EU (EC, 2006), USA (USEPA, 2009), Canada (CCME, 2007), Sweden (SWEPA, 2000) and Norway (Andersen et al., 1997; Lydersen et al., 2002). The dissolved concentrations in the present study are based on ultrafiltration (LMM species) and not directly comparable with the dissolved concentrations used in the EQS benchmarks which are based on 0.45 µm filtration.

Contaminant (µg/L)	Tunnel wash water runoff		Environmental quality standards (EQS)							
	Total (µg/L)	Dissolved (<10 kDa, µg/L)	EU AA[a]	EU MAC [b]	USA CCC[c]	USA CMC[d]	Canada[e]	Sweden class 1–5[f]	Norway class 1–5[g]	Norway fish class 1–4[h]
Al	2766 ± 314	404 ± 104			87	750	100			
As	3.4 ± 0.1	2.8 ± 0.1			150	340	5	2		
Cd	0.18 ± 0.01	0.06 ± 0.01	0.09[i]	0.6[i]	0.25	2.0	0.017	3	3	1
Cr (III)	5.6 ± 0.6	1.1 ± 0.2			74	570	8.9	3	3	2
Cu	43.3 ± 4.5	10.7 ± 1.3			9	13	3	4	5	4
Fe	3759 ± 444	652 ± 138			1000		300			
Ni	11.3 ± 0.7	8.0 ± 0.2	20	20	52	470	110	2	5	2
Pb	7.8 ± 0.7	1.9 ± 0.3	7.2	7.2	2.5	65	4	4	5	3
Zn	808 ± 51	599 ± 25			120	120	30	5	5	4
Anthracene	0.013 ± 0.003						0.012			
Benzo(a)anthracene	0.015 ± 0.003						0.018			
Benzo(a)pyrene	0.018 ± 0.003		0.05	0.1			0.015			
Fluoranthene	0.13 ± 0.013						0.04			
Phenanthrene	0.034 ± 0.009						0.4			
Pyrene	0.225 ± 0.023						0.025			
Benzo(b)fluoranthene Benzo(k)fluoranthene	∑ = 0.043 ± 0.003		∑ = 0.03	∑ = 0.03						
Benzo(g,h,i)perylene Indeno(1,2,3-cd)pyrene	∑ = 0.083 ± 0.006		∑ = 0.002	∑ = 0.002						

[a] AA = annual average value. In the case of metals EQS refers to the dissolved concentration (0.45 µm).
[b] MAC = maximum allowable concentration. In the case of metals EQS refers to the dissolved concentration (0.45 µm).
[c] CCC = Criterion Continuously Concentration. All metals beside Al (total) are expressed as dissolved (0.45 µm) and with hardness 100 mg/L $CaCO_3$.
[d] CMC = Criteria Maximum Concentration. All metals beside Al (total) are expressed as dissolved (0.45 µm) and with hardness 100 mg/L $CaCO_3$.
[e] The EQS for Cu, Pb and Ni are hardness dependent and are in the present table displayed with EQS corresponding to a hardness of 160 mg/L $CaCO_3$ (see Table 1).
[f] Class 1–5 = very low concentration–very high concentration.
[g] Class 1–5 = very low concentration–very high concentration.
[h] Class 1–4 = very low concentration–high concentration.
[i] Cd is based on hardness class 3 (EU).

contaminants should be considered. Paruch and Roseth (2008a; b) tested the removal efficiency of metals and PAHs in tunnel wash water with various commercial available sorbent materials. The removal efficiencies were fairly good, e.g. 67–97% for total PAH and above 90% for several metals.

3.5. Juvenile brown trout growth — long term effects?

We have documented that tunnel wash water runoff, although passing through a sedimentation pond, can be highly contaminated. Although based on a limited number of water samples, reduced growth (21% reduction, $p=0.000$) in summer old sea trout (0+) downstream the pond outlet compared to 0+ caught upstream (Fig. 9), might be linked to the polluted runoff discharged from the three tunnels and the nearby open road areas, as no growth difference was apparent in the years prior to the establishment of the new tunnels and the sedimentation pond. No significant change in number of captured 0+ per sampling was observed between the sites before and after year 2000 ($p=0.53$, not shown), indicating that the reduced growth rate cannot be explained by density dependent effects, as have been observed in other brown trout populations (e.g. Lobon-Cervia, 2007). Although the tunnel wash water from the Nordby tunnel was directly discharged into the Årungselva in the years before 2000, it was diluted with clean drainage water from the surrounding rock. Hence, after the year 2000, an average traffic increase of ca. 25% (based on 2002–2009 figures, (NPRA., 2010)) and extra pollutant loadings from the other tunnels as well as the roads in between have increased the total contaminant loads. In addition, trapped sediment has not been removed from the pond since the opening. In contrast to the upstream data, a significant time trend on growth reduction was evident in the downstream data (R^2 adjusted = 0.30, $p=0.024$) supporting the evidence of increased pollutant loadings downstream. There are no other anthropogenic inputs, other than traffic related contaminants between the investigated reaches, suggesting that the growth reduction may be linked to chemical perturbations originating from highway and tunnel wash water runoff.

The growth reduction of 0+ downstream the pond may imply that this fish has reduced energy uptake and/or energy reallocated from growth to other vital energy demanding processes. This would in varying degree be valid throughout all life stages, i.e. from the time of fertilization and embryonic development to the stage of swim-up and further development until smoltification. For example, toxicity is often more detrimental in early life stages compared with adults, and reduced growth in embryonic and juvenile stages has been

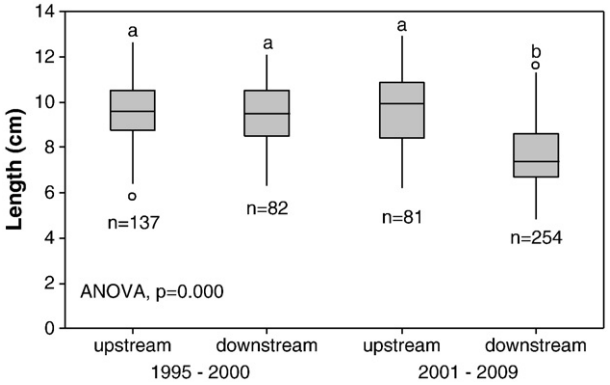

Fig. 9. Box plot presenting the length of brown trout sampled in the stream Årungselva from 1995 to 2009 ($n=81$–254). The data are grouped according to sampling site and time (i.e. before and after the extension of the E6 motorway and the establishing of the sedimentation pond). The rectangular box for each group represents the interquartile range of the data including the median value displayed as a horizontal line, while the whiskers extending from the boxes represents the upper and lower 25% of the distribution. The outliers are denoted by an open circle. The significance between the fish length in the various groups are indicated by different letters (lower-case).

demonstrated (e.g. Luckenbach et al., 2003; Coghlan and Ringler, 2005; Golovanova, 2008).

Reduced energy uptake at an individual level due to altered behavior (e.g. foraging, prey recognition) and changed social interactions caused by chemical stressors at sub-lethal concentrations affecting e.g. the chemosensory system (e.g. olfaction, gustatory and vision) has been suggested (Atchison et al., 1987; Kazlauskiene et al., 2008; Kuzmina and Ushakova, 2008). In addition, reduced food quality and prey availability due to pollution may reduce the prey availability and the energy uptake (Coghlan and Ringler, 2005). Finally, metabolic tradeoffs between repair and detoxification mechanisms and other processes are known (Hall et al., 1992; Scott and Sloman, 2004). Hence, energy reallocation from growth to repair and detoxifying may also have contributed to the reduced growth. In the stream Årungselva, the majority of sea trout juveniles previously smoltified after one winter had a mean length between 7.9 and 8.9 cm (Borgstrøm and Heggenes, 1988). A reduced growth rate during the last years may therefore imply a delay in age at smoltification, influencing both smolt production and competition between the different juvenile age-classes (Bohlin, 1977), and finally expanding the time of exposure to pollutants prior to smoltification.

Although the observed growth reduction in 0+ in the lower parts of the stream may be linked to highway and tunnel wash water runoff, more detailed monitoring of the stream is desirable, including longitudinal sampling for the analyses of water and sediment quality, supplementary biological and biomarker endpoints from the trout population and finally controlled *in situ* experiments.

4. Conclusions

The present study shows that washing of tunnels can produce relatively large volumes of highly contaminated water. Although the wash water from the Nordby tunnel are discharged into a sedimentation pond, several contaminants such as Cu, Pb, Zn, fluoranthene, pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(g,h,i) perylene and indeno(1,2,3-cd)pyrene measured in the pond outlet, exceeded benchmarks and gained high ranks in EQS obtained from EU, USA, Canada, Sweden and Norway. Although many of the contaminants were highly associated with particles and colloids, a large fraction appeared associated with the LMM fraction. For example, the RDA revealed that 50% of the variation of the metal concentrations could be assigned to the LMM fraction, which is of great concern as LMM species are believed to be more mobile and bioavailable than metals associated with particles.

The EFs revealed that many of the metals had an anthropogenic signal, indicating origins such as tires (Zn), brakes (Cu and Sb) and combustion (Co). In addition, the application of road salt caused high anthropogenic signal for Na and Cl. In contrast, Al, Ba, Ca, Cr, Fe, K, Mg and Ni had low EFs indicating that wear from the tunnel surfaces was their dominant source. The ratio between various PAH compounds indicated that they were of petrogenic and pyrogenic origin, e.g. tires and asphalt wear and combustion, respectively.

An evident growth reduction in sea trout (0+) in the lower parts of the stream Årungselva compared with sea trout in the upper parts may be attributed to contaminants discharged into the stream from the pond during the last 10 years. These indications demand a more detailed monitoring of the stream to reveal more knowledge about the connection between cause and effects. An additional treatment step after the pond outlet should also be considered to effectively reduce the outlet concentrations of contaminants entering the stream.

Acknowledgement

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Exposure of brown trout (*Salmo trutta* L.) to tunnel wash water runoff — Chemical characterisation and biological impact

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ABSTRACT

Washing and cleaning of road tunnels are a routinely performed maintenance task, which generate significant amount of polluted wash-water runoff that normally is discharged to the nearest recipient. The present study was designed to quantify chemical contaminants (trace metals, hydrocarbons, PAH and detergents) in such wash water and assess the short term impact on brown trout (*Salmo trutta* L.) based on in situ experiments. Selected endpoints were accumulation of trace metals in gills, haematological variables and hepatic mRNA transcription of five biomarkers reflecting defence against free radicals, trace metals, planar aromatic hydrocarbons and endocrine disruptions which were measured prior (−3 h), during (1 and 3 h) and after the tunnel wash (14, 38 and 86 h). Our findings showed that the runoff water was highly polluted, but most of the contaminants were associated with particles which are normally considered biologically inert. In addition, high concentrations of calcium and dissolved organic carbon were identified in the wash water, thus reducing metal toxicity. However, compared to the control fish, a rapid accumulation of trace metals in gills was observed. This was immediately followed by a modest change in blood ions and glucose in exposed fish shortly after the exposure start. However, after 38–86 h post wash, gill metal concentrations, plasma ions and glucose levels recovered back to control levels. In contrast, the mRNA transcription of the CYP1A and the oxidative stress related biomarkers TRX and GCS did not increase until 14 h after the exposure start and this increase was still apparent when the experiment was terminated 86 h after the beginning of the tunnel wash. The triggering of the defence systems seemed to have successfully restored homeostasis of the physiological variables measured, but the fish still used energy for detoxification four days after the episode, measured as increased biomarker synthesis.

1. Introduction

During the last decade the number of vehicles in Norway increased by 24%. The ever increasing traffic around major cities has led to a substantial use of tunnels to protect the citizens from noise and air pollution. Oslo, the capital of Norway, with surrounding municipalities has a network of approximately 40 tunnels which sums up to a distance close to 35 km. In addition, tunnels are on the national level often constructed due to the challenging Norwegian landscape with fjords, deep valleys and high mountains.

Washing and cleaning of tunnels are routinely performed mainly to remove dust and dirt and thereby increasing the lifespan of the tunnel and maintaining acceptable visibility for the road users. In addition, traffic safety is enhanced by removing oil, grease and particles from the road surface which otherwise may reduce the friction. The number of tunnel washes performed in a certain tunnel depends largely on its size and traffic load, in Norway typically 2–12 times per year. The majority of the washing events during a year are so-called “half wash”, while once a year the tunnels are completely washed (“full wash”). The former includes washing of the tunnel walls and the road surfaces while the latter also includes washing of the roof and other technical equipments and installations. Hence, a “full wash” would involve large water volumes that normally are more polluted than a “half wash”.

In brief, prior to the application of detergent a road sweeping machine removes dust, debris and other coarse material from the road surface. This is followed by application of some kind of detergent followed by high pressure cleaning. Finally the road sweeping machine makes a last run removing both dirt and un-drained washing

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water. Information provided by contractors indicates that the water volume utilised during a wash ranges between 60 and 100 L/m in a tunnel consisting of two tubes and two driving lanes in each tube. The amount of detergents sums up to roughly 0.5–1% of the total water consumption. Hence, around 60–100 m^3 of polluted wash water can potentially be discharged during cleaning of a 1 km long tunnel.

Normally, wash water runoff is discharged to the nearest water recipient and seldom treated other than through gully pots removing larger particles and coarse debris. In addition, some tunnels have oil separators. Tunnel wash water runoff normally appears with higher pollutant loadings compared to runoff from open roads (Amundsen and Roseth, 2004). This can be attributed to concentration processes of pollutants inside a tunnel (e.g. time span between washing events and reduced dispersion of air dust out of the tunnel compared to open areas). The pollutants include, however, the same contaminants such as trace metals, organic micro pollutants like PAHs, and road salt (Andersen and Vethe, 1994; Paruch and Roseth, 2008a,b). In addition, detergents used during washing will be part of the polluted wash water runoff. Some examples of concentrations for metals and PAHs measured in tunnel wash water are given in Table 1. According a recent review by Napier et al. (2008), the release of automobile derived contaminants like Cu, Zn and PAHs in the environment shows an upward trend. This is of great concern in a regulatory perspective as several metals and PAH compounds are on the list of priority substances under the European Water Framework Directive (EWFD). Several of these contaminants have been shown to give adverse effects in fish, both in laboratory experiment (e.g. Jonsson et al., 2006; Spry and Wood, 1985) and in field studies (e.g. Heier et al., 2009; Roberts et al., 2005). Trace metals are known to cause both osmoregulatory problems as well as respiratory dysfunctions, while PAHs can be carcinogenic and immunotoxic (e.g. Logan, 2007; Reynaud and Deschaux, 2006; Spry and Wood, 1985). The amount and concentrations of the various pollutants found in tunnel runoff will be a function of the tunnel construction (e.g. building material, length, width and slope), traffic (volume, vehicle type and driving speed) and the time lag between the washing events. The time of year will also play a part, at least in northern countries where road salt and studded tires are frequently used during the winter, increasing both the rate of corrosion and asphalt wear.

Eggen et al. (2004) listed five major future challenges in the field of ecotoxicology including “complex mixtures of pollutants”. Tunnel wash water runoff certainly fits into this category containing a cocktail of both organic and inorganic contaminants. Therefore, the discharges of potential toxic tunnel wash water into the aquatic environment represent a multiple stress exposure being able to cause multiple biological effects through series of interactions with multiple target sites (Eggen et al., 2004; Salbu et al., 2005). The complexity is further amplified by the fact that chemicals acting at the same target might set off antagonistic, additive or synergistic effects. Hence, a suitable toxicity assessment of tunnel wash water discharges should be based on measurements of the most important contaminants as well as performing in situ exposure experiments utilising abundant organisms like fish, as they on a general basis are considered to be one of the most feasible organism for pollution monitoring in aquatic systems (van der Oost et al., 2003). Endpoints such as survival and growth are frequently utilised, but do not necessarily provide a mode of action based assessment of toxicity and may therefore require complementation of endpoints reflecting uptake of pollutants and impairment of associated physiological and biological processes, e.g. uptake and accumulation of pollutants, haematological impairments like ion and glucose homeostasis, biotransformation processes including oxidative stress parameters.

Although chemical characterisations as well as biological effects of highway runoff is frequently reported (e.g. Gjessing et al., 1984; Grapentine et al., 2008; Klimaszewska et al., 2007; Maltby et al., 1995), knowledge about environmental effects due to tunnel wash water runoff are limited (Andersen and Vethe, 1994; Barbosa et al., 2006; Paruch and Roseth, 2008a,b). In addition to good chemical status in all surface waters within 2015, the requirement of biological and ecological protection is fundamental in the EWFD. Hence, our experiment faces this aspect and as far as we know no articles have been published where the evaluation of toxicity of polluted tunnel wash water has been based on in situ experiment.

The present study quantifies the concentrations of traffic related contaminants discharged during washing of the Nordby tunnel outside the City of Oslo, Norway. In addition, accumulation of trace metals in gills together with physiological responses in acutely exposed brown trout (*Salmo trutta* L.) is characterised. The physiological responses include measurements of blood physiology (glucose, Cl, K, Na, pCO_2 and hematocrit) and the hepatic mRNA transcription of 5 selected biomarker genes; metallothionein (MT-A), a cysteine-rich protein frequently used as a biomarker due to its capability to bind and regulate several trace metals (van der Oost et al., 2003); thioredoxin (TRX) and gamma glutamylcysteine synthetase (GCS) both being important in the antioxidant system (Finne et al., 2007; Kalinina et al., 2008); cytochrome *P450 1A* (CYP1A) a mixed function oxidase biotransformation enzyme utilised as biomarker for the exposure of planar aromatic hydrocarbons (van der Oost et al., 2003); and finally vitellogenin (VTG), which is a sensitive biomarker for exposure to estrogenic pollutants having endocrine disrupting properties (Matozzo et al., 2008; van der Oost et al., 2003). The multivariate technique principal response curve (PRC) was applied to statistically evaluate the overall toxicity of the tunnel wash water runoff.

Table 1
Concentration of various metals and PAHs measured in tunnel wash water from the Hanekleiv tunnel in Norway (Paruch and Roseth, 2008a, 2008b).

Metal/PAH	Min (μg/L)	Max (μg/L)	One sample (μg/L)
Aluminum	467	26 100	
Chromium	4.0	107.0	
Cupper	11.1	177.0	
Iron	2 590	26 800	
Molybdenum	15.4	21.0	
Nickel	<6.0	52.5	
Lead	<18.0	53.0	
Zinc	105.0	1560	
Acenaphthylene			0.06
Benzo(a)pyrene			0.36
Indeno(1,2,3-cd)pyrene			0.08
Fluorene			0.07
Naphthalene			1.15
Phenanthrene			0.17
Fluoranthene			0.37
Pyrene			0.61

2. Methods

2.1. Study site

This experiment was conducted in early December 2008 during washing of the Nordby tunnel, which is situated along the motorway E6, 30 km south east of the City of Oslo, Norway. The tunnel was washed in late June same year, i.e. pollutants had been accumulated for roughly 5 months. The tunnel, which opened in 1993, consists of two separate tubes with two driving lanes each. Each tunnel tube is 3840 m long and 10 m wide. The walls are covered with prefabricated concrete elements and the roof is covered with sprayed concrete. The annual average daily traffic (AADT) is around 25 000 vehicles, of that 11% is heavy duty vehicles above 3500 kg and the average vehicle speed is 89 km/h. The tunnel is regularly cleaned typically 4 to 6 times per year, one being a full wash. The drainage system is divided in two separate systems which prevent mixing of clean drainage water from the surrounding rock and the polluted runoff water. A significant

fraction of coarse material and debris are removed from the runoff as it flows through several gully pots. Finally, the runoff is pumped to a sedimentation pond outside the tunnel before it is discharged into the River Årungselva. In the present study, which was conducted during a "half wash", we used wash water prior to the final treatment in the sedimentation pond but after the gully pots. Hence, some reductions in the pollutant loadings were expected due to sedimentation.

2.2. Experimental setup

Animal welfare, which includes the three Rs (Replace, Reduce and Refine), was essential both during the planning and execution of the experiment. Brown trout (0+) were transported from the local hatchery (Oslo Fiskeadministrasjon, OFA) in plastic bags filled with cold (approximately 5 °C) oxygenated water. The transportation time was less than 1.5 h, and upon arrival the fish were immediately transferred randomly into 2 black circular exposure tanks (70 L) receiving Ås municipal tap water (5 L/min, 6.6 °C) in a flow through system installed inside a steel container (2.5 × 3.0 m). Both tanks were aerated and each tank contained approximately 40 fish. The fish in the control group had a mean weight of 46.9 ± 3.2 g, whereas the fish in the exposure group had a mean weight of 49.2 ± 2.6 g. Hence, the biomass in the control and the exposure tank were 24.1 g/L and 23.9 g/L, giving a specific water flow of 2.5 and 2.7 L/kg fish/min, respectively. This specific flow was nearly 6 times the mean flow used in aquaculture of Atlantic salmon (*Salmo salar* L.) ensuring a good water quality during reference conditions (Åtland et al., 2007). The trout were acclimated one week prior to the exposure. To reduce general stress in fish they were kept in darkness and not fed during the experiment.

The tunnel was cleaned during two nights of work, i.e. one tube each night. Probably due to a dry drainage system, no wash water runoff reached the pump house situated at the end of the drainage system the first night. Hence, the fish were only exposed to tunnel wash water during the second and last night of work.

Incoming polluted washing water was continuously pumped from the pump house into a 1000 L reservoir tank using a drainage pump. The fish were exposed by pumping the washing water from the reservoir tank to the exposure tanks using an aquarium pump (Eheim). The flow was set to 5 L/min. The tunnel wash was completed after 4 h and clean tap water was reintroduced as a water source.

2.3. Water quality assessment

2.3.1. Metals and anions

Water samples for metal analysis were sampled in situ using a peristaltic pump equipped with a 100 µm filter (Millipore) to prevent clogging of the system, and a 0.45 µm High Capacity In-Line Groundwater Sampling capsule (PALL). We also equipped the water sampling system with an ion exchange column to retain and quantify positively charged trace metal species from the water. The ion exchange column was filled with approximately 15 mL of chelex-100 resin (Biorad). A total of 3 samples were obtained from each sampling round; one total sample (unfiltered sample), a 0.45 µm filtered sample and a third sample being filtered (0.45 filtered) and subjected to cation exchanged chromatography, using an elution rate of 30 mL/min. Based on these three samples the following fractions were attained: 1) Particulate fraction (total–0.45 µm filtered sample) 2) Dissolved cations fraction (0.45 µm filtered sample–0.45 filtered cation exchanged sample) 3) Dissolved anions and neutral fraction (filtered sample–dissolved cation fraction). The water was sampled before and during the tunnel wash at 20:00 p.m., 01:30, 02:30 and 04:00 a.m., respectively. Each sampling round was completed within 30 min.

All samples were acidified with 1% suprapure concentrated HNO_3. The concentrations of arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), cupper (Cu), nickel (Ni), lead (Pb), antimony (Sb), vanadium (V) and zinc (Zn) were determined using ICP-MS (Perkin Elmer ELAN 6000), while the concentrations of aluminum (Al), iron (Fe), calcium (Ca), potassium (K), sodium (Na), magnesium (Mg) and manganese (Mn) were determined using ICP-OES (Perkin Elmer Optima 5300DV). The five latter metals were only determined in the total samples. Samples with high concentrations of Cl (road salt) were diluted to prevent interference on the measurements. Internal standards, several blanks and reference material (SRM 1643e, National Institute of Standards and Technology) were used as quality control. The detection limits (LOD) for the trace elements expressed in µg/L were 4.0 (Al), 0.01 (As), 0.02 (Ba), 0.002 (Cd), 0.001 (Co), 0.05 (Cr), 0.07 (Cu), 2.0 (Fe), 0.06 (Ni), 0.02 (Pb), 0.01 (Sb), 0.02 (V) and 0.55 (Zn). The measurement of the trace metals in the reference material were all within 5% of the certified value. The concentrations of chloride, sulphate and nitrate were determined by an Iachat IC5000 Ion Chromotograph.

2.3.2. Organic carbon, polycyclic aromatic hydrocarbons (PAH), hydrocarbons and detergents (tensides)

Total organic carbon (TOC, Shimadzu TOC cpn Total organic carbon analyzer) was measured in both unfiltered and 0.45 µm filtered samples, where the latter samples reflect the dissolved organic carbon (DOC) concentration.

The concentrations of 16PAH, total hydrocarbons (>C10–C40), non-ionic, cationic and anionic tensides were determined in unfiltered samples according to the methods EPA-8270-C, EN ISO 9377-2, DIN 38409-H23-2, DIN 38409-H20 and DIN 38409-H23-1, respectively. The analyses were performed by ALS Scandinavia. The limits of quantification (LOQ) for the various PAH compounds were 0.01 µg/L for acenaphthylene, acenaphthene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, indeno(1,2,3-cd)pyrene, dibenzo(ah)anthracene, benzo(ghi)perylene, 0.02 µg/L for fluorene, anthracene, benzo(a)pyrene, 0.03 µg/L for, phenanthrene, fluoranthene, 0.06 µg/L for pyrene and 0.1 µg/L for naphthalene. The LOQ for total hydrocarbons was 50 µg/L, while the LOQs in for non-ionic, cationic and anionic detergents were 0.30, 0.20 and 0.20 µg/L, respectively.

2.3.3. Multisonde

Temperature, pH, conductivity, dissolved oxygen and redox potential were automatically measured every 10 min in the tank receiving tunnel wash water by a multi water quality sensor (HORIBA W21SDI) connected to an external data logger.

2.4. Fish samples

During each sampling 6 fish from the control group and 6 from the exposure group were sacrificed with a single blow to the head and immediately subjected to sample and treatment. A total of 6 rounds of sampling were conducted, one prior (−3 h), two during (1 and 3 h) and three after the tunnel wash (14, 38 and 86 h).

2.4.1. Fish sampling protocol

The fish sampling was carried out following the EMERGE protocol (Rosseland et al., 2001). Fish length and weight were recorded and later used in the calculations of the condition factor (*K*-factor = fish weight (g) × 100 / fish length $(cm)^3$). This was immediately followed by the collection of a small blood sample for in situ analysis of glucose, Cl, K, Na, pCO_2 and hematocrit. The blood sampling was conducted by puncturing the caudal vein using a small heparinised syringe. The blood was carefully transferred to an EC8+ cassette and analysed on a portable I-Stat blood analyser (Abbott, USA). A few measurements of blood Cl and K were above the instrument range. We decided to use the maximum recordable value (140 mmol/L) which we considered being a valid approach.

The second gill arch on the right hand side was dissected and stored at −20 °C until analysis of trace metal accumulation. Finally,

the abdomen was opened and the liver was carefully withdrawn. A small piece from the distal part of the liver was snap frozen in liquid nitrogen for later analysis of the mRNA transcription of selected biomarkers.

2.4.2. Determination of trace metals in gills

The accumulation of the trace metals Al, As, Ba, Cd, Co, Cr, Cu, Fe, Ni, Pb, Sb, V and Zn in gill tissue were determined utilising an ICP-MS instrument (Perkin Elmer ELAN 6000). Prior the quantification the gills were freeze-dried and weighed, and finally digested in Teflon beakers containing suprapure concentrated HNO_3 and internal standard (20 µg/L of In). The digestion procedure was fulfilled by utilising a MLS-Milestone UltraClave (MLS GmbH). In addition, reference material (DOLT-3 (dogfish liver) and DORM-2 (dogfish muscle), National Research Council Canada) and blank samples were included as quality assurance. The detection limits (LOD) for trace metals in digested samples expressed as µg/L were 3.5 (Al), 0.1 (As), 0.6 (Ba), 0.003 (Cd), 0.01 (Co), 0.3 (Cr), 0.3 (Cu), 3.0 (Fe), 0.1 (Ni), 0.1 (Pb), 0.005 (Sb), 0.6 (V) and 3.3 (Zn). The measurements of the trace metals in DOLT-3 and DORM-2 were all within 10% of the certified values, beside Ni (16%) and Zn (13%) in DOLT-3 and As (13%), Ni (12%) and Pb (11%) in DORM-2, respectively. The results are given in µg/g dry weight (dw).

2.4.3. Gene expression

Cell lysates from liver tissue were obtained by homogenising approximately 20 mg tissue samples in 500 µL of RLT lysis buffer, using a Precellys orbital shaker bead mill (Bertin, Montigny-le-Bretonneux, France). Samples were homogenised for 3×10 s at 6500 rpm with (Precellys CK14 beads), and cell debris was removed by centrifugation at 8000 *g* for 1 min. DNA-free total RNA was then isolated from 350 µL of the supernatant using the RNeasy mini kit and RNase free DNase kit, according to the producers instructions (Qiagen, Hilden, Germany).The RNA was quality controlled by photometric analyses of 260/230 and 260/280 nm ratio, and RNA used for qPCR had 260/230>1.8 and 260/280>2 (data not shown). The samples were also inspected by electrophoresis using RNA 6000 nano chips in a Bioanalyzer instrument (Agilent technologies, Santa Clara, California, USA). RNA was then reverse transcribed using high capacity cDNA archive kit according to the producer's instructions (Applied Biosystems, Foster City, California, USA), before being assayed for gene expression in an absolute quantification protocol by real-time PCR (ABI 7500, Applied Biosystems, Foster City, California, USA). CYP1A primers sequences were from work of (Chung-Davidson et al., 2004), VTG primer sequences were obtained from Korner et al. (2008), primer sequences for MT-A were obtained from Hansen et al. (2007), whereas GCS sequences were from Finne et al. (2007). TRX primers were designed with PrimerExpress software (Applied Biosystems), using Genbank EST sequence CA054594 as template. Overview of primer sequences are listed in Table 2. Twenty five microliters Supermix with ROX (Quanta Biosciences, Gaithersburg, Maryland, USA) PCR reactions were run in triplicate and each reaction contained cDNA from 50 ng of RNA and 300 pmol forward/reverse primer. Standard curves were run on 0.8, 4, 20 and 500 ng cDNA, and relative expression compared to the control was then determined from the threshold cycle (Ct) value and the slope of the standard curves, after normalisation to the amount of RNA in each sample. No template controls (NTC) were included in the real-time PCR analyses and no signal was detected.

2.5. Statistics

The multivariate statistics (principal response curves) on the biological data were performed by utilising CANOCO 4.55 whereas the graphics were performed with CanoDraw 4.14, both being part of the CANOCO software package. All descriptive statistics and Pearson product moment correlations were performed with Minitab 15 software.

2.5.1. Principal response curves

In the present study we utilised principal response curves (PRC), a multivariate statistical method based on partial redundancy analysis (pRDA), to better display the time dependent multivariate data (Borcard et al., 1992; Van den Brink and Ter Braak, 1999). The sampling time was used as categorical covariables and the interaction between sampling time and treatment was used as explanatory variables. Hence, the focus was put on the deviation of the selected endpoints (*y*-axis), i.e. metals in gill tissue, blood physiology and gene expression along a time gradient (*x*-axis) in the exposed group from that in the control group. In addition, the final diagram is accompanied with the endpoint weights (i.e. species weights in CANOCO terminology) which are interpreted as follows: endpoints with high positive weight are likely to follow the pattern in the PRC, while high negative weights follow the opposite pattern. As a rule of thumb, endpoints with weight between -0.5 and 0.5 indicate no response or a response unrelated to the pattern in the displayed PRC. As the PRC is based on a pRDA the explainable variance in % can be divided in a time effect $((1-\text{sum all eigenvalues})\times100)$, a chemical exposure effect (sum all canonical eigenvalues $\times100$) and how much of the latter exposure effect is captured by the first canonical axis of the PRC $((\text{eigenvalue first canonical axis}/\text{sum all canonical eigenvalues})\times100)$.

The endpoints were $\text{Log}(x+1)$ transformed, and in addition centred and standardised prior the analysis. The significance of the PRC analyses was tested by running Monte Carlo permutation tests. The permutation tests were designed as a split-plot consisting of 12 whole plots, each consisting of fish sampled through time in its respective exposure tank. The whole plots were randomly permuted (499 permutations under reduced model), whereas the individual fish forming the whole plots were not. In order to run the permutation tests of the split-plot design properly the data matrix need to be balanced. Hence, samples with missing values were removed and the final data matrix contained four fish from each exposure tank at each time point.

The three different groups of endpoints; accumulation of trace metals in gills, blood physiology and gene expression of selected biomarkers were analysed separately. Hence, three PRC diagrams were made for interpretation.

3. Results

3.1. Water quality

The water quality data obtained by the multi water probe are presented in Fig. 1. The incoming tunnel wash water caused a rapid

Table 2
Corresponding primer sequences for MT-A, CYP1A, VTG, GCS and TRX used for real time PCR.

Gene	Genbank (acc no)	Forward primer	Reverse primer
Metallothionein-A (MT-A)	X97274	CCTTGTGAATGCTCCAAAACTG	CAGTCGCAGCAACTTGCTTTC
Cytochrome *P450 1A* (CYP1A)	AF539415	CCAAACTTACCTCTGCTGGAAGC	GGTGAACGGCAGGAAGGA
Vitellogenin (Vtg)	AF454750	AACGGTGCTGAATGTCCATAG	ATTGAGATCCTTGCTCTTGGTC
γ-glutamyl-cystein synthetase (GCS)	CA050524	TGATGGACAACACATTCATTAATTGA	GCGATGCCCGGAACTTATT
Thioredoxin (TRX)	CA054594	ACCGTGCAGCCTAGAATGCT	GTGATGTCTCTCTTTGCAGTTCCTT

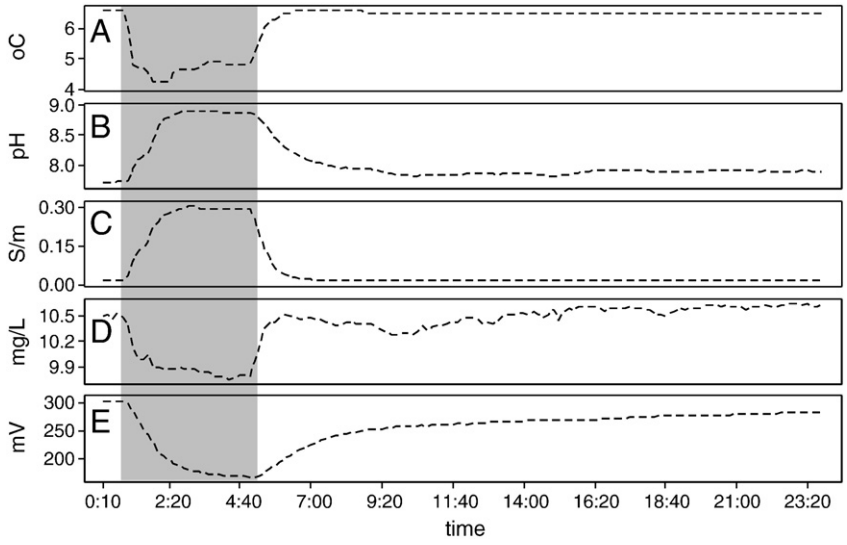

Fig. 1. Variation of temperature (A), pH (B), conductivity (C), dissolved oxygen (D) and redox potential (E) based on the automatic water logging system. Measurements were recorded every 10 min during the experiment. The tunnel wash is indicated by a grey vertical bar.

change in the general water quality. All the measured parameters either increased (pH and conductivity) or decreased (temperature, dissolved oxygen and redox potential) sharply within 30–60 min and stabilised until the washing was stopped and clean tap water was reintroduced in the exposure tanks. The pH increased with over a unit during the washing, from 7.7 to 8.8. The concentration of dissolved oxygen decreased from 10.5 to 9.8 mg/L during the washing. This decrease was followed by a slow drop in the redox potential. Both variables were back to normal levels within the next 24 h. The ionic strength, measured as conductivity, showed a substantial increase and was 25 times higher in the tunnel wash water compared to the tap water. This increase was a result of the road salt associated components Cl and Na, which appeared in high concentrations during the wash (Table 3). Important variables regarding protective mechanisms against metal toxicity such as TOC/DOC and Ca were roughly 5 and 3 times higher in the end of the tunnel wash period compared to the tap water, respectively (Table 3).

Not surprisingly the tunnel wash water contained high concentrations of trace metals compared to tap water (Fig. 2). The highest concentrations were measured in the last sampling round in the end of the tunnel wash period but we do not know if this was the peak of the concentrations or not. This pattern was evident for all the measured trace metals. In addition, the metals were highly associated with particles (>0.45 µm). Arsenic (As) was the only trace element that was more or less equally distributed between the particle fraction and the dissolved fraction. As was mainly present as anionic or neutral species in the dissolved fraction.

PAH and hydrocarbons were below the detection limit in the tap water but showed elevated concentrations during the tunnel wash (Table 4). The concentrations of the various PAH components are displayed in Fig. 3. Pyrene, chrysene and fluoranthene were the single PAH components that appeared with the highest concentrations (52% of the total 16PAH concentration) and all are classified as being high molecular mass PAHs (HMM>4 rings). In addition, chrysene is classified as carcinogenic. Contrary to PAH and hydrocarbons, only one of the water samples contained tensides above quantification level (i.e. non-ionic tenside). However, it should be stressed that the limit of quantification for tensides was relatively high 0.2 and 0.3 mg/L.

Table 3
Concentrations of TOC, DOC, hardness and major ions in tunnel wash water and in tap water during the in situ experiment.

Variable	Unit	Tap water ($n=4$)	Tunnel wash water		
		Mean ± S.E.M	01:30	02:30	04:00
TOC	mg/L	2.9 ± 0.02	5.6	6.2	14.7
DOC	mg/L	2.9 ± 0.06	4.0	5.0	14.2
Ca	mg/L	19.7 ± 0.03	31.9	34.0	57.0
K	mg/L	2.5 ± 0.01	4.7	6.5	17.5
Mg	mg/L	2.8 ± 0.01	6.0	8.4	13.1
Na	mg/L	22.3 ± 0.25	227	320	926
Mn	mg/L	0.006 ± 0.0001	0.185	0.302	0.551
Cl	mg/L	23.0 ± 0.04	514	617	1280
Sulphate	mg/L	36.9 ± 0.18	37.5	29.0	34.8
Nitrate	mg/L	1.4 ± 0.001	1.36	1.03	1.34
Total Hardness	mg/L	60.7	104.3	119.5	196.3

3.2. Accumulation of trace metals and physiological responses

No mortality occurred during the experiment, and there were no differences in the condition factor (K) between the two groups neither in the start nor in the end of the experiment ($K=1.1$ in both groups). However, the fish exposed to tunnel wash water clearly changed their behaviour compared to the control fish, i.e. the swimming performance was slow and they seemed to prefer staying close to the water surface and they were more easily caught during netting.

The accumulation of trace metals in gill tissue is presented in Table 5, together with the response in blood variables and the gene expression of selected biomarkers.

The PRC analysis of trace metal accumulation in gills revealed that 30% of the total variance could be attributed to time and 20% to the exposure of tunnel wash water (Fig. 4A and Table 6). The first PRC axis described 56% of the variance explained by exposure, and it was significant ($p=008$). The acute exposure of tunnel wash water caused an immediate accumulation of trace metals on the gills, as displayed in Fig. 4A, especially Sb, Al, Fe, Co, Cu and Pb. The highest accumulation was observed during the initial phase of the tunnel wash (1 h). After 3 h the trace metal concentrations in the gills were lower than the concentrations measured after 1 h exposure, despite the fact that the highest pollutant loadings in the water were measured in the end of the washing. The trace metal concentrations in the gills were back to control levels within 38 h.

The results from the second PRC analysis on the blood physiology data showed that time and exposure explained 15% and 20% of the total variance, respectively (Fig. 4B and Table 6). Fifty one percent of the variance explained by the exposure regime was captured by the

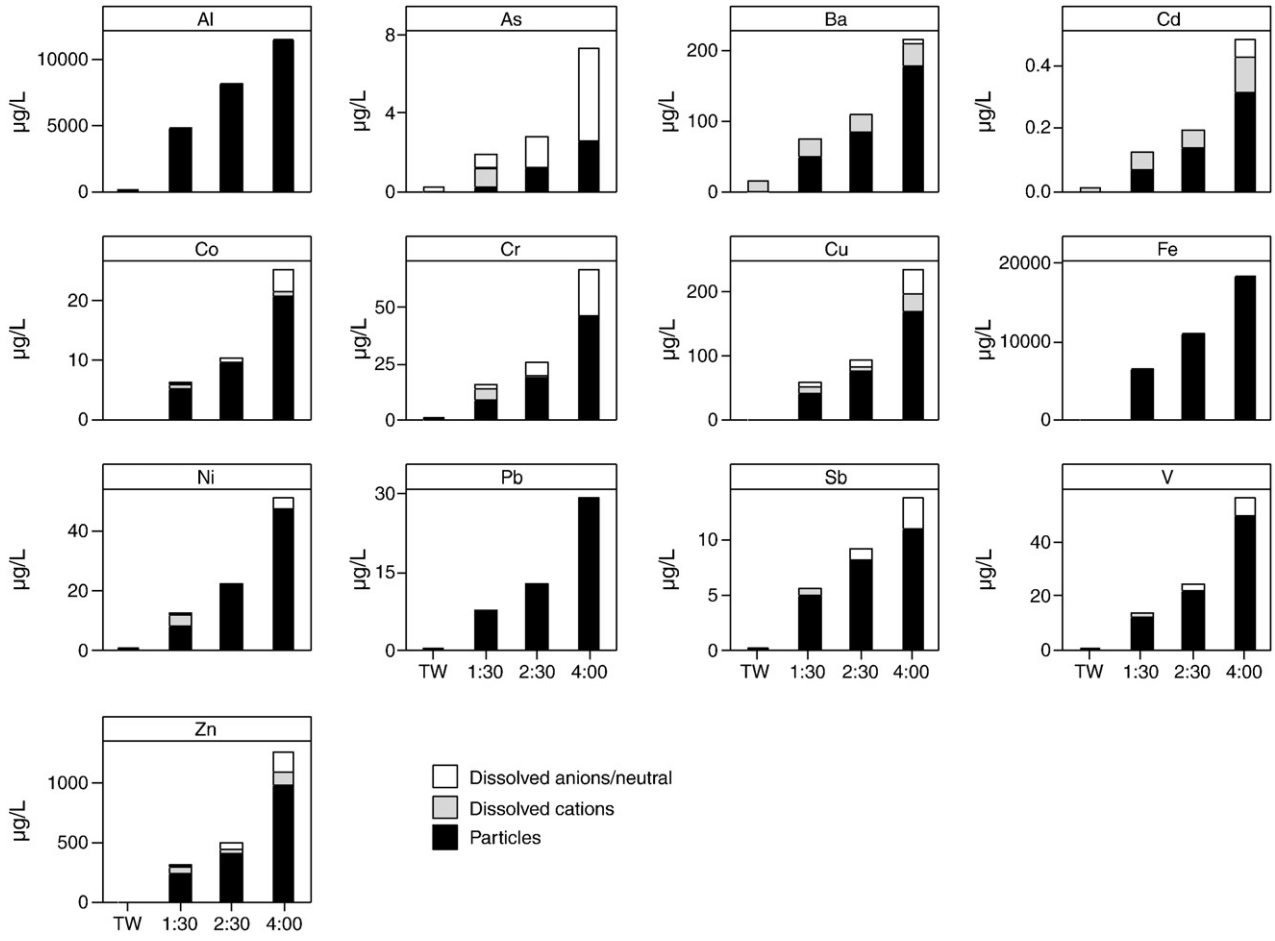

Fig. 2. Concentrations of trace metals in exposure water during the in situ experiment (TW = tap water).

first PRC ($p = 0.022$). The rapid accumulation of trace metals was followed by a rise in the glucose concentrations and a minor loss of the plasma ions Na and Cl (Fig. 4B). This pattern reached its maximum at 14 h after the tunnel wash start. These blood variables recovered to normal levels again within 38–86 h post wash.

The third PRC, analysing the gene expression data revealed that the time factor explained 18% of the total variance (Fig. 4C and Table 6). Twenty three percent of the total variance was attributed to the exposure regime and of that 72% was significantly captured by the first PRC ($p = 0.006$). Contrary to the rather quick responses seen in the accumulation of trace metals in gills and in blood parameters, the response pattern observed in the gene expression of biomarkers was delayed and the increase was not observed before 14 h post wash. The increase could, however, have occurred anywhere between the wash period and 14 h later (Fig. 4C). The expression rate continued to increase and did not reach maximum level until 86 h post wash. Hence, we cannot say whether the gene expression would still increase, stabilise or decrease beyond this sampling point. The observed pattern in PRC diagram was mainly caused by the expression of the biomarker for planar hydrocarbons (CYP1A) and oxidative stress (TRX) and to a minor extent GCS, being another biomarker for oxidative stress.

Table 4
Concentrations of PAH, hydrocarbons and tensides in tunnel wash water and in tap water during the in situ experiment.

Variable	Unit	Tap water ($n = 4$)	Tunnel wash water		
		Mean ± S.E.M	01:30	02:30	04:00
16PAH	µg/L	Not detected	1.26	2.76	2.42
Carcinogenic PAH[a]	µg/L	Not detected	0.63	1.14	1.03
Total hydrocarbons	µg/L	Not detected	3500	3870	6180
Non-ionic tensides	mg/L	<0.30	<0.30	<0.30	1.1
Cationic tensides	mg/L	<0.20	<0.20	<0.20	<0.20
Anionic tensides	mg/L	<0.20	<0.20	<0.20	<0.20

[a] USEPA (2009a).

4. Discussion

4.1. Water quality assessment

The pollution level in the wash water runoff was quite high, both for inorganic and organic contaminants and was comparable with other Norwegian tunnel studies (Paruch and Roseth, 2008a,b). In addition, several metals and PAHs exceeded existing water quality criteria for chronic exposure (e.g. CCME, 2007; EC, 2006; USEPA, 2009b). According to USEPA's and EC criteria for brief exposures, which is an estimate of the highest concentration that should not give any unacceptable effects to an aquatic community, the wash water could still be characterised as polluted due to the high concentrations of Cu, Zn and some PAH compounds (e.g. benzo(a)pyrene). The exceeding of the PAH benchmarks is of special concern, as PAH as a group is classified to be among the eleven "priority hazardous substances" in the EWFD's list of priority substances, meaning that emissions and the use of this group of compounds is to be stopped within 2020. This also supports the concern promoted by Napier et al. (2008) in their review regarding the increased automobile emissions of PAH to the environment.

The speciation of metals and metalloids depends on water quality variables such as pH, ionic strength, redox potential, temperature, suspended solids and the presence of inorganic and organic ligands

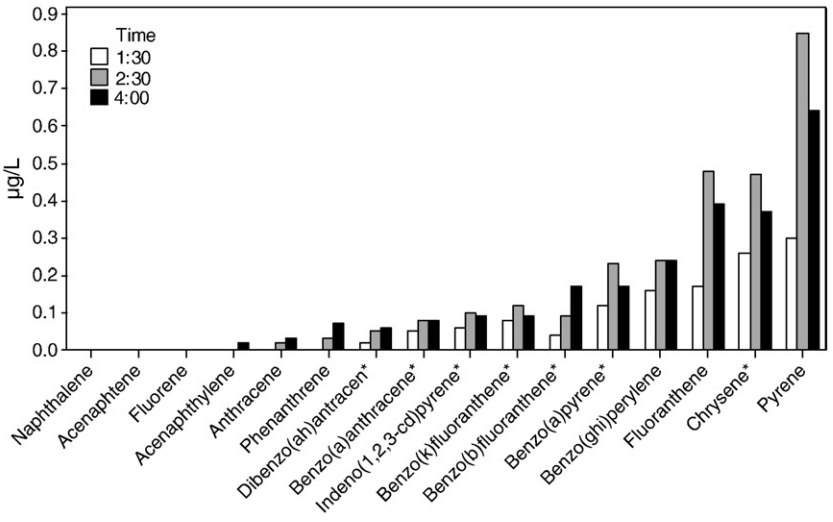

Fig. 3. The concentrations of various PAH compounds in exposure water during the in situ experiment. Carcinogenic PAHs are marked with *.

(Salbu and Oughton, 1995). In this context, pH and conductivity were the variables that showed the highest fluctuation in the present exposure study. Although they rapidly changed in the exposure tank receiving wash water, this did not seem to alter the partitioning and speciation of the metals which were strongly associated to particles (>0.45 µm) throughout all three sampling rounds. The high association between metals and particles may reflect the major contribution from wear of tires, vehicle body, asphalt and soot particles. In contrast, As was mainly found as negatively/neutral charged species in the dissolved fraction reflecting its solubility under oxidizing conditions (Weiner, 2008). Sb has many of the same properties as As, i.e. being a metalloid and anionic under normal oxic conditions. The concentrations of Sb in our study were at least 10 times higher than what could be expected in unpolluted natural waters (Filella et al., 2002). However, in the present study, Sb was found to be associated to particles rather than being present as dissolved species. The major source of Sb is wearing of brake linings (Thorpe and Harrison, 2008). Beside Sb, brake linings are also a significant source for Cu and Ba (Sternbeck et al., 2002), and both appeared in the tunnel wash water at high concentrations.

The tunnel wash water contained several different PAH compounds and the majority are classified as carcinogenic and mutagenic (e.g. benzo(a)pyrene and chrysene). Boxall and Maltby (1997) pointed out that PAH is one of the most, if not the most, important group of highway contaminants contributing to aquatic toxicity. In the present study, a clear dominance of HMM PAHs was observed, indicating a pyrogenic origin which includes incomplete combustion of fossil fuels (Brown and Peake, 2006; Karlsson and Viklander, 2008). However, the high content of pyrene might as well indicate contribution from tire wear (Karlsson and Viklander, 2008; Lindgren, 1998). For example Kose et al. (2008) concluded that tire wear was the most important source of PAH in road dust followed by road pavement asphalt as the second largest contributor.

According to the contractor the tunnels in this area are washed with a detergent, named REMI KRAFT 703 which contains both non-ionic and cationic tensides. Only non-ionic tensides were detected and quantified in the present study. Based on information listed in the material safety data sheet (Remitek, 2008) provided with REMI KRAFT 703 this non-ionic tenside is most likely a fatty-alcohol ethoxylates (FAE). According Corneliussen (2007), the REMI KRAFT 703 detergent is toxic to bacteria (*Pseudomonas* sp.) when mixed with water in the range of 0.5–1 %, which is approximate the concentration used during washing, while Jurado et al. (2009) showed that FAEs were toxic in the range 0.47–12.27 mg/L (EC_{20} (30 min)). However, the toxicity normally decreases within few days due to biodegradation.

4.2. Biological effects

Our results clearly showed that there was a rapid precipitation of Sb on the gills and the highest mean concentration was measured within one hour of the tunnel wash (mean 0.05 µg/g, (max. value 0.11 µg/g)). These concentrations are comparable to what Heier et al. (2009) found in their study of brown trout exposed to runoff from a military shooting range. Despite that Sb is considered toxic and has no known biological function (Filella et al., 2002), little data exists on the bioavailability and toxicity of Sb in the environment. However, a toxicity study exposing larvae of Japanese medaka (*Oryzias latipes* L.) to Sb for 24, 48, 72 and 96 h resulted in a NOEC of 120 mg/L and LC_{50} ranging from 261 to 173 mg/L (Nam et al., 2009). These reported concentrations are far above concentrations found in most natural waters (<1.0 µg/L, (Filella et al., 2002)) as well as in this study (5.8–13.9 µg/L). Other studies focusing on various macroinvertebrate taxa indicate that Sb has the potential of being readily taken up by amphipods (e.g. *Gammarus pulex* L.), isopods (*Asselus aquaticus* L.) and caddis larvae (*Hydropsyche pellucidula* L.) living in anthropogenic affected environment (Duran et al., 2007; Haus et al., 2007). There also exists some evidence that Sb at sub-lethal concentrations (<8.0 mg/L) can cause both respiratory problems and disturbance of the ion homeostasis in fish (Chen and Yang, 2007; Lin and Hwang, 1998). The present study showed that the accumulation of Sb in fish gills was associated with a modest increase in glucose and a small drop of the Na and Cl levels in the blood plasma, but no signs of respiratory problems (like coughing and hyperventilation) were observed.

The accumulation of Sb on gills of exposed trout was similar to the accumulation of Al and to some extent Fe, Co, Cu and Pb. As an example, Lauren and McDonald (1985) found that Cu concentrations as low as 12.5 µg/L, which is about 1/3 of the average dissolved concentration in the present study, lead to substantial loss of plasma ions and increased glucose concentrations in rainbow trout (*Oncorhynchus mykiss* L.), even in water at neutral pH and high Ca concentrations (approximately 40 mg/L). Other trace metals such as Al, Fe, and Pb have proven to cause both ion regulatory dysfunctions (e.g. decreased plasma Na and Cl concentrations) and to rapidly set off the glucose levels in the blood plasma of salmonids (e.g. Heier et al., 2009; Lauren and McDonald, 1985; Peuranen et al., 1994; Rogers et al., 2005; Rosseland et al., 1992). Hence, the observed ion loss and increased glucose concentrations in fish exposed to tunnel wash water were most likely caused by multiple stressors and not by a single factor. Based on the current experimental design it is impossible to reveal whether the altered blood physiology was set off by metals (or other

Table 5
Trace metal accumulation in gills together with physiological data (mean ± S.E.M. (S.E.M. given below the mean value), $n = 4–6$). The data is expressed with the following units; trace metal accumulation in gills (μg/g); blood plasma ions (mmol/L); blood hematocrit (Hct%); pCO_2 in blood (kPa), gene expression data are expressed in fold change relative to the 8 fish sampled at −3 h.

Var/Group	Control						Exposed					
Time (h)	−3	1	3	14	38	86	−3	1	3	14	38	86
Al-gill	16.3	21.9	33.1	22.0	26.4	35.5	27.8	101.1	67.0	40.8	47.2	48.2
	1.2	1.9	4.6	3.3	3.7	2.0	4.7	22.5	8.6	13.2	8.0	8.7
As-gill	1.53	1.61	1.67	1.67	1.69	1.85	1.65	1.58	1.59	1.54	1.77	1.67
	0.05	0.05	0.06	0.04	0.11	0.09	0.05	0.04	0.07	0.07	0.05	0.07
Ba-gill	1.68	1.77	1.52	1.32	1.03	1.36	1.86	1.97	2.25	1.28	1.28	1.61
	0.16	0.15	0.11	0.08	0.07	0.15	0.18	0.12	0.15	0.09	0.17	0.08
Cd-gill	0.45	0.58	0.63	0.57	0.60	0.57	0.58	0.65	0.68	0.62	0.58	0.68
	0.04	0.06	0.03	0.04	0.03	0.04	0.03	0.07	0.08	0.03	0.05	0.07
Co-gill	0.22	0.20	0.23	0.25	0.26	0.24	0.20	0.26	0.24	0.26	0.23	0.23
	0.02	0.01	0.02	0.02	0.02	0.03	0.01	0.02	0.01	0.02	0.01	0.01
Cr-gill	0.79	1.22	0.86	0.29	0.35	0.24	1.31	0.83	0.99	0.36	0.49	0.40
	0.08	0.35	0.06	0.05	0.05	0.05	0.22	0.10	0.06	0.12	0.08	0.21
Cu-gill	1.66	1.58	1.65	1.70	1.57	1.56	1.56	1.89	2.24	1.70	1.84	1.60
	0.04	0.04	0.04	0.12	0.02	0.02	0.04	0.07	0.32	0.10	0.26	0.06
Fe-gill	417	364	365	292	313	382	354	439	466	416	270	331
	37	12	21	27	17	35	13	53	24	25	15	31
Ni-gill	0.57	0.66	0.60	1.05	1.11	0.84	0.62	0.60	0.83	0.82	1.19	0.95
	0.03	0.08	0.03	0.06	0.08	0.04	0.03	0.03	0.18	0.02	0.04	0.04
Pb-gill	0.03	0.03	0.04	0.02	0.01	0.03	0.03	0.06	0.10	0.04	0.02	0.02
	0.01	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.05	0.01	0.00	0.00
Sb-gill	0.003	0.005	0.007	0.003	0.002	0.004	0.003	0.051	0.032	0.017	0.006	0.004
	0.001	0.001	0.002	0.001	0.000	0.001	0.000	0.014	0.004	0.005	0.002	0.001
V-gill	0.77	0.86	0.87	0.44	0.53	0.35	1.10	1.00	1.04	0.60	0.48	0.52
	0.10	0.05	0.05	0.06	0.08	0.04	0.12	0.08	0.04	0.06	0.03	0.07
Zn-gill	568	507	584	616	451	499	588	448	465	520	583	598
	52	41	72	51	14	35	72	78	54	76	48	79
Glu-blood	4.2	3.9	3.9	5.1	3.4	3.7	3.8	4.9	5.1	6.7	5.4	3.8
	0.5	0.1	0.2	0.7	0.2	0.3	0.2	0.3	0.2	1.1	0.9	0.3
Na-blood	143	144	147	143	144	144	142	143	142	138	145	146
	2.2	1.3	1.0	1.2	0.7	1.4	0.9	1.4	1.4	1.9	0.9	2.4
K-blood	5.7	5.4	4.4	5.3	5.7	4.9	6.6	6.1	6.2	5.5	5.5	5.1
	0.9	0.5	0.4	0.4	0.2	0.3	0.3	0.6	0.4	1.0	0.3	0.3
Cl-blood	135	137	136	136	133	136	138	137	135	133	135	139
	1.3	1.6	0.8	1.4	0.8	1.1	0.7	0.7	1.0	1.2	0.6	0.6
Hct-blood	30	31	27	31	32	31	29	30	34	31	29	27
	1.3	2.1	1.4	1.5	1.8	1.9	1.9	1.8	1.0	2.5	0.7	2.6
pCO_2-blood	0.61	0.59	0.58	0.63	0.58	0.64	0.66	0.56	0.51	0.64	0.51	0.55
	0.05	0.03	0.04	0.05	0.03	0.03	0.04	0.03	0.02	0.02	0.03	0.03
MT-A-liver	1.00	0.5	0.9	0.6	0.6	0.8		0.8	1.1	0.9	0.7	1.3
	0.15	0.1	0.1	0.2	0.2	0.1		0.3	0.3	0.1	0.2	0.6
GCS-liver	1.00	0.8	0.9	1.1	1.0	1.0		0.7	0.9	1.0	1.3	2.2
	0.07	0.1	0.1	0.2	0.2	0.2		0.1	0.1	0.1	0.2	0.8
TRX-liver	1.00	0.6	1.0	1.1	0.7	0.9		0.6	1.1	1.0	1.1	1.7
	0.16	0.1	0.2	0.4	0.1	0.1		0.1	0.2	0.1	0.1	0.5
CYP1A-liver	1.00	0.9	1.0	0.9	0.6	1.1		0.7	0.9	2.6	4.2	6.9
	0.21	0.2	0.1	0.1	0.1	0.3		0.1	0.1	0.3	0.4	1.3
VTG-liver	1.00	1.0	1.3	1.6	1.2	0.6		1.0	1.5	1.1	0.3	0.8
	0.27	0.4	0.7	0.5	0.5	0.4		0.5	0.6	0.2	0.1	0.5

stressors) acting in an antagonistic, additive or synergistic mode of action. However, according to a review by Norwood et al. (2003) evaluating 191 published tests on the toxicological effects of metal mixtures, there was a tendency toward metals acting in an antagonistic way (less than additive, 43%). On the other hand synergistic effects, which often attract most political attention, are rarely found and seem to play only a minor role in an environmental context (Syberg et al., 2009).

The trace metals were more or less associated with particles, which are considered rather inert with respect to active biological uptake. In addition, the wash water contained relatively high concentrations of DOC (the last water sample contained 14 mg/L) and Ca (32–57 mg/L) in terms of being protective against metal toxicity through providing alternative binding sites for the trace metals, and according to the Biotic Ligand Model (BLM) being a strong competitor for available binding sites on the gill surface, respectively (Fairbrother et al., 2007; Hollis et al., 1997; Niyogi and Wood, 2004). Hence, the high concentrations of DOC and Ca in the end of the tunnel wash might explain why the accumulation of trace metals on the gills was lower in the end (3 h) compared to the beginning of the washing (1 h), although the highest total metal concentrations in the wash water were measured in the end. It should be stressed, however, that the presence of detergents can be a source of high DOC concentrations in wash water (Roseth and Søvik, 2005). Hence, the high DOC content in the wash water could be attributed to the application of various soap components, which instead of binding dissolved metals could increase the bioavailability of particle bound contaminants through remobilization. However, a series of experiments conducted by Tao et al. (1999a,b, 2000) has indicated that particle bound Cd, Pb and Cu can be available for uptake through the gills. Their theory is that particle bound metals adhere to the negatively charged mucus layer covering the gill surface followed by desorption processes where the trace metals ions are removed from the particle and into the mucus due to the lower pH in the gill microenvironment compared to the surrounding water body. Findings in our study indicate that Al and Pb were rapidly accumulated in the gills of the runoff exposed fish,

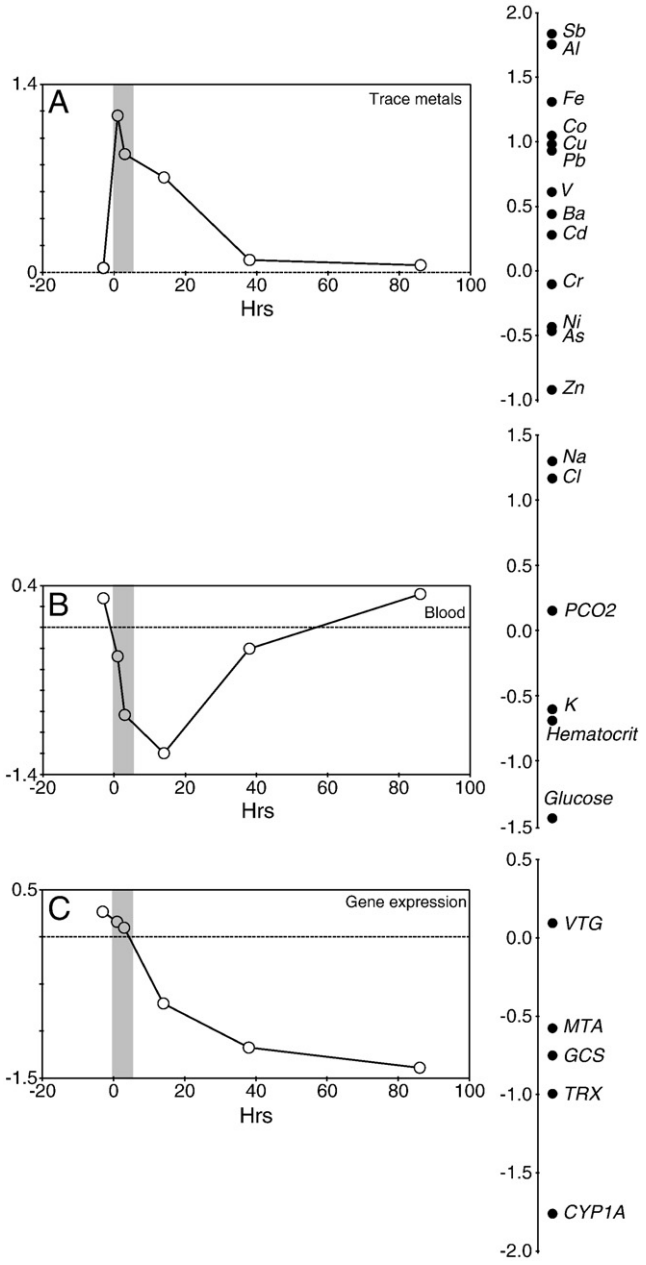

Fig. 4. Diagrams showing the first component of the principal response curves (PRC) of trace metals in gills (A), blood physiology (B) and gene expression over selected biomarkers (C). Each diagram has its corresponding set of endpoint weights displayed on the right hand side. The control group in each diagram is displayed with a dotted line through zero. The tunnel wash is indicated by a grey vertical line. Interpretation of the diagrams is described in the method section.

although the dissolved concentration of these two elements both in the control water and the tunnel wash water were almost identical throughout the experiment. Thus, the gill accumulation of at least Al and Pb must have been attributed to the retention of particles containing these metals in gill mucus. The subsequent release could be attributed to sloughing of mucus filled with retained particles.

Table 6
Statistical summary of the Monte Carlo permutation tests obtained from the PRC analyses. In addition, the percentage of the total variance which can be attributed to time and exposure are displayed together with the fraction of the exposure variance captured by the first PRC axis.

Monte Carlo test statistics				% explained variance		
PRC analysis	Eigenvalue	*F*-ratio	*p*-value	Time	Exposure	1st PRC axis
Gills	0.110	6.734	0.008	30	20	56
Blood	0.101	4.889	0.022	15	20	51
Gene expression	0.163	8.913	0.006	18	23	72

The rapid accumulation of trace metals caused a small, but rapid effect in the blood while the gene expression of selected genes in the liver showed a delayed response in time as would be expected, being dependent upon a "physiological disorder signal" to be activated. MT which is frequently utilised as a biomarker for trace metals, and which additionally is considered as an important protector against oxidative stress (Halliwell and Gutteridge, 2007; Sato and Bremner, 1993; van der Oost et al., 2003), was not induced in the liver of the exposed trout. This lack of mRNA MT-A transcription, despite increased gill-metal concentrations of MT binding metals (e.g. Cu), could indicate that the exposure was too low in terms of both duration, concentrations or both for contaminants to be taken up into the blood and to trigger the transcription. Alternatively, that the particle associated trace metals were mainly retained in mucus and subsequently released from the mucus, as indicated by the rapid decrease in gill-metal concentration after the washing event. Or that the primary defence at the gills was sufficiently protective so that the need for increased MT in the liver was absent.

Opposite to MT-A, the transcription of CYP1A increased steadily after 14 h (10 h after exposure stop) and this increase was still apparent at the end of the study (86 h). The expression of CYP1A is not a toxic effect per se, but is considered a biomarker for the activation of the aryl hydrocarbon receptor (AhR) after exposure to planar aromatic hydrocarbons like PAH, which in fish has been correlated to various toxic effects including narcosis, mortality, decrease in growth, reduced condition factor, edema, cardiac dysfunction, deformities, lesions and tumors, cataracts, immune system dysfunctions and estrogenic effects (Logan, 2007). In addition, the expression of CYP1A is proved to be robust in a mixture exposure situation (Finne et al., 2007). The increased CYP1A expression indicated that the waterborne PAHs and hydrocarbons in the tunnel wash water were readily bioavailable, despite that PAH/hydrocarbons normally are strongly attached to particles and/or organic matter (e.g. Beasley and Kneale, 2002; Krein and Schorer, 2000). The detergent used during washing may enhance this bioavailability as suggested by Ramachandran et al. (2006) and Schein et al. (2009) who studied the effect of dispersion of crude oil and diesel. Hence, our results clearly indicated that PAH/hydrocarbons were rapidly absorbed by fish, although a time lag between exposure and expression of CYP1A in liver was evident. Such time lag between exposure and effect has been documented by others, e.g. that biotransformation of benzo(a)pyrene in gills occurred at an earlier phase compared to the liver (e.g. Jonsson et al., 2006; Levine and Oris, 1999).

In our study, the gene expression profile of TRX and GCS in the exposed fish followed, although notably less up-regulated, the pattern of CYP1A. The correlation coefficients between the mRNA expression of CYP1A and GCS and TRX in the group of exposed fish were 0.82 ($p = 0.000$) and 0.77 ($p = 0.000$), respectively (not shown). A correlation between CYP1A and TRX has previously been documented in rainbow trout exposed to diesel (Mos et al., 2008). Additionally, an exposure study with car tire leachates conducted by Stephensen et al. (2003) revealed an activation of the CYP1A system along with increased oxidative stress, possibly due to leakage of PAH and aromatic nitrogen compounds from the tires. Hence, the correlation observed in our study indicates that ROS was produced, and the concentrations of Zn and pyrene in the tunnel wash water runoff indicated a substantial contribution from tire wear. Therefore, we cannot exclude that various tire additives such as aromatic nitrogen compounds contributed to the increased antioxidant responses in the exposed fish. Nor can we exclude the contribution from trace metals like Cu and Fe which are, at elevated concentrations, known to cause ROS through Haber Weiss and Fenton reactions (Halliwell and Gutteridge, 2007; Martinez-Alvarez et al., 2005; Mason and Jenkins,

1995).This and similar studies focusing on multiple stressors and multiple effects face the challenge of interpreting huge and complex data sets, which certainly call for some simplification. Multivariate statistics is in this context a promising tool, and can reveal patterns of responses and inter-individual relationships between endpoints that otherwise might be undetected by univariate statistics (Galloway et al., 2006). Multivariate statistics also overcome the problem with multiple statistical testing, i.e. increasing the probability of committing "type I error (false positive)" and the risk of misinterpreting random noise. Hence, the use of PRC appeared to be an appropriate way of summarising the overall picture regarding biological effects of tunnel wash water runoff in the present study.

In summary, the present study shows that tunnel wash water runoff can be toxic to fish. However the severity will be largely dependent on the pollutant levels, duration of the exposure and the range of dilution in the receiving recipient. Our findings showed that most of the contaminants were associated with particles which are normally considered as biologically inert. In addition, the wash water had high concentrations of Ca and DOC which most likely minimized the toxicity. We observed accumulation of trace metals in gills immediately after the wash water entered the exposure tank. This was followed by a modest, but rapid, change in blood plasma ions and glucose. Both the accumulation of trace metals and the blood parameters recovered to control levels within 38–86 h. In contrast, the mRNA transcription of the CYP1A and the oxidative stress related biomarkers TRX and GCS did not increase until 14 h after the exposure start and did not return to background levels when the experiment was terminated 86 h after exposure start. As this is one of the few scientific papers based on in situ experiments showing that discharges of polluted tunnel wash water could be a potential threat to organisms living in receiving water bodies, further in situ research should be performed under various hydrological regimes such as low flow vs. high flow (i.e. dilution series) and with other organisms as well (e.g. Atlantic salmon). Additionally, long term effects of polluted tunnel wash water from tunnels carrying a lot of traffic should be addressed.

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Ecotoxicological impact of highway runoff using brown trout (*Salmo trutta* L.) as an indicator model

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The ecotoxicological impact of highway runoff on brown trout (*Salmo trutta* L.) was studied in an *in situ* experiment consisting of four 24 h simulated runoff episodes. Fish were maintained in 5 tanks and exposed to highway runoff from a sedimentation pond close to E6 outside the city of Oslo, Norway. The tanks had the following contaminant loadings during the episodes: stream water (control), pond inlet, pond outlet, pond inlet + stream water and pond outlet + stream water. Opposite to road salt and compared to earlier findings, the first two episodes had rather low concentrations of trace metals, hydrocarbons and polycyclic aromatic hydrocarbons. A heavy rainfall before episode 3 increased the concentrations of all the contaminants except road salt which was diluted. In addition, lowered oxygen levels led to hypoxic conditions. Overall the fish exposed to highway runoff had, compared to the control fish, higher concentrations of trace metals in gills and liver, increased activity of the antioxidant defense system represented by superoxide dismutase, catalase and metallothionein, problems with the regulation of plasma Cl and Na, as well as increased levels of blood glucose and pCO_2. Finally, this seemed to affect the metabolism of the fish through reduced condition factor. The observed effects were likely caused by multiple stressors and not by a single contaminant. The sedimentation pond clearly reduced the toxicity of the highway runoff. But even in the least polluted exposure tank (pond outlet + stream water) signs of physiological disturbances were evident.

Introduction

Urbanization and an ever increasing traffic density during the last decades represent a potential threat to both public and environment health. Traditionally air and noise pollution has been of key interests, however, highway runoff as a potential hazard to receiving water bodies has gained more attention. As pointed out by Glenn *et al.*[1] highway runoff is a major challenge because of its variable and stochastic nature of processes such as traffic density, rainfall runoff, *etc.* Typically, the chemical composition of highway runoff can be very different between as well as within episodes.

Highway runoff consists of a mixture of several contaminants, such as inorganic metal species, organic micropollutants and road salt. The most important vehicle contributions of metals[2,3] are wearing from tires (*e.g.* Fe, Ni, Cu, Zn and Cd), brake linings (*e.g.* Cu and Zn), engines (*e.g.* Al, Cu and Ni) and the vehicle body (*e.g.* Fe, Al and Zn). Tire wear, petroleum spillage and leakage together with incomplete combustion are sources for organic micropollutants like polycyclic aromatic hydrocarbons (PAHs).[2,4,5] Wearing of asphalt includes, besides mineral particles, a certain amount of bitumen being a source of PAH.[5,6]

As a result, various treatment systems have been implemented along the roads to protect the receiving aquatic ecosystems from highway runoff. A major fraction of the contaminants is associated with particles and most of the treatment systems are therefore based on removing the contaminants by sedimentation. However, the toxicity of contaminants, at least for metals, is mostly related to the dissolved fraction which includes colloids as well as the low molecular mass positively charged metal species.[7,8] In a survey conducted by Sansalone and Glenn[9] it was shown that the dissolved fraction of several metals, measured as heavy metal transfer (partitioning analysis), was dominating the

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Environmental impact

Highway runoff contains an orchestra of pollutants which can cause multiple stressor reactions to aquatic biota. By using an omnipresent fish species like brown trout (*Salmo trutta* L.), we have been able, through a broad physiological response characterization and *in situ* chemical fractionation techniques, to quantify traffic related pollutants and to study the effectiveness of mitigation through sedimentation ponds. Hence, our results contribute to increased knowledge about environmental effects regarding highway runoff and it will gain knowledge to the scientific community as well as to the public authorities (*e.g.* road authorities).

runoff water at the edge of the road. Hence, treatment systems solely based on sedimentation might be inappropriate to protect the aquatic biota. This is in accordance with results found by Marsalek *et al.*[10] that documented only minor reduction in toxicity in the effluent of two storm water treatment ponds.

In northern countries, like Norway, road salt is frequently used during winter maintenance to achieve good friction on the road. As an example, approximately 137 000 tons of salt (mainly sodium chloride (NaCl)) were applied on Norwegian roads during the winter season 2006–2007.[11] Thus, the sodium and chloride concentrations can be rather high during runoff episodes. It has previously been documented that road salt at concentrations as low as 180 mg L^{-1} may cause alteration in the blood physiology of rainbow trout (*Oncorhynchus mykiss* L.).[12] In addition road salt can lead to increased mobility, remobilization and finally increased bioavailability of the metals.[13–17] Such processes have earlier been observed during sea salt episodes on the west coast of Norway.[18,19]

Already in 1984 a Norwegian study examined the toxicity of highway runoff by using aquatic organisms such as bacteria, fungi, algae and fish.[20] They concluded that the acute toxicity was moderate. However, it should be stressed that the traffic density in 1980s was rather low (annual average daily traffic (AADT) < 10 000 vehicles). In the recent years the number of papers investigating the toxicity of highway runoff has increased. Small-scale toxicity tests utilizing organisms such as bacteria, algae, rotifers and daphnids are probably the most frequently used approach in studying highway toxicity.[21,22] Macro-invertebrates are also frequently used as pollution indicators in recipients receiving highway runoff.[23,24–26] These two approaches have both advantages and disadvantages. For example, small-scale toxicity tests have their strength in being simple, cost effective and reproducible through standardized results,[27] while the major advantage of conducting macro-invertebrate surveys is their ecological relevance. However, detecting effects on a population level are challenging as the effects tend to appear only after long time of exposure.[28] Typically drawbacks regarding small-scale toxicity tests are that they are conducted in the laboratory with short time span (*e.g.* 24, 48 or 96 h) and with end points such as effect concentration (EC_{50}, often linked to growth) and/or lethal concentration (LC_{50}).

Hardly any former studies have utilized a holistic approach, including series of biomarker responses from organisms likely to be found in most ecosystems affected. Highway runoff represents multiple stress exposure and will most likely cause multiple effects. Hence, the present study utilizes an alternative approach for studying the toxicity of highway runoff and includes an *in situ* experiment to account for episodic events and also includes a presumed sensitive and omnipresent species such as brown trout (*Salmo trutta* L.). The study covers aspects ranging from water quality assessment to accumulation and uptake of pollutants *via* the gills, which could harm and disturb important physiological processes at a cellular level (*e.g.* protection against reactive oxygen species (ROS), ion homeostasis, and gas exchange) and finally could induce physiological effects at an individual level.

The objectives of this study were to: (1) quantify chemical concentrations of contaminants in the four simulated consecutive highway runoff episodes; (2) characterize the associated physiological responses in exposed brown trout; (3) assess whether treatment ponds based on sedimentation are able to reduce the toxicity of highway runoff.

The end points included metal accumulation in gills and liver tissue (Al, Cd, Cu, Fe, Ni, Pb, Zn), blood physiology and biomolecules that are known to be linked to the free radical defense system:[29,30] superoxide dismutase (SOD), catalase (CAT) and metallothionein (MT). MT is also essential in the processes of detoxifying metal ions.[31] In addition the liver somatic index (LSI) and the condition factor (*K*-factor) were included, being good indicators of assessing overall pollution effects at the individual level.[32,33]

These kinds of studies dealing with multiple stressors and multiple end points have high complexity, and synergetic or antagonistic effects can occur.[34] Hence, multivariate statistics being successfully applied within the field of ecotoxicology[35,36–38] have been utilized for data interpretation purposes.

Material and methods

Study site

The experiment was conducted in spring 2007 by using a sedimentation pond receiving runoff from a four-lane motorway (E6) at Skullerud just outside the city of Oslo, Norway. The motorway has an annual average daily traffic (AADT) around 45 000 vehicles. In brief, the pond has a closed pre-sedimentation basin in front of the main pond (Fig. 1). The main pond has a wet volume of approximately 800 m^3 and is heavily vegetated from the midpoint and towards the outlet. The pond receives runoff from an area of 3.4 ha, where 2.2 ha are paved, and the retention time is dimensioned to be minimum 72 h.

Discharge from the pond enters the River Ljanselva approximately 4.8 km upstream the Oslo fjord. The river has several fish species such as brown trout and Atlantic salmon (*Salmo salar* L.) and is thus an important locality for fish recruitment to the Oslo fjord.

Fig. 1 Overview of the Skullerud sedimentation pond including parts of the catchment area and the recipient River Ljanselva: A = closed pre-sedimentation basin, B = pond inlet (PI), C = pond outlet (PO) and D = stream water (SW) upstream the pond outlet.

Experimental setup

The field experiment was planned according to animal welfare considerations (3Rs: replace, reduce and refine), and was approved by the National Animal Research Authority. Veterinary approved one year old brown trout from a nearby hatchery (OFA), weighing 71.0 ± 20.4 g, were transported for 1 h in aerated tanks and randomly placed in 5 experimental circular PVC tanks (90 L) housed in a field laboratory at site. Hence, the biomass was 24.4 ± 0.5 g L^{-1} in the beginning of the experiment. The fish were maintained in continuously flowing stream water pumped from upstream the pond outlet (5–6 L min^{-1}) with temperature 9.7 ± 0.1 °C, pH 7.5 ± 0.1 and dissolved oxygen 10.5 ± 0.2 mg L^{-1}, corresponding to approximately 95% saturation (measured in the SW head tank, $N = 18$). The fish were acclimated for 5 days prior to the runoff. Three fish died during the acclimation period.

To simulate realistic runoff episodes the fish were exposed to four consecutive episodes during a period of 12 days, each of 24 h duration (*i.e.* 30 fish experienced one episode, 30 fish experienced two episodes and so on, Fig. 2). The runoff water was pumped from the inlet (after the pre-sedimentation basin) and the outlet of the pond, respectively, into two separate head tanks by using drainage pumps and additional hoses. From the head tanks the runoff water was further drained to the fish tanks at controlled flow rate (Fig. 2). Thus, the five tanks had the following pollutant loadings: SW (stream water control), PI (pond inlet), PO (pond outlet), PISW (pond inlet + stream water (mix ratio 50 : 50)) and POSW (pond outlet + stream water (mix ratio 50 : 50)).

Water quality

Water from each experimental tank was sampled into 2 acid prewashed plastic bottles (60 mL): one for the analysis of total metal concentrations and one for the dissolved metal concentrations (0.45 μm, Millipore). The latter was obtained by filtering the water *in situ* using a 0.45 μm Millipore filter, and will include hydrolysis products, complexes and polymers and colloids, as well as low molecular mass species.[8] The samples were immediately acidified with 1% ultrapure HNO_3. In addition, two samples (100 mL) for the analysis of total organic carbon (TOC), dissolved organic carbon (DOC, measured as TOC in a 0.45 μm filtered water sample) and anions, and two samples (1000 mL) for the analysis of hydrocarbons and 16 PAH were collected, respectively. All samples were stored cold (4 °C) and dark until analysis.

The concentration of Al, Cd, Cu, Fe, Ni, Pb and Zn was determined in the total and filtered samples, while calcium (Ca), magnesium (Mg), sodium (Na) and manganese (Mn) concentrations were only determined as total concentration. Both ICP-MS (Perkin Elmer ELAN 6000) and ICP-OES (Perkin Elmer Optima 5300DV) were used for the analysis. Internal standards (indium and yttrium), blanks and reference material (SRM 1640 and 1643e, NIST) were used as quality control. Due to relatively high salt concentrations, the samples were diluted with Barnstead water to reduce interferences in the measurements. The detection limit (LOD) for the trace metals was 3.0, 0.002, 0.14, 5.0, 0.02, 0.015 and 0.12 μg L^{-1} for Al, Cd, Cu, Fe, Ni, Pb and Zn, respectively. The measurements of the trace metals in the reference material were all within 10% of the certified values.

Chloride (Cl^- (ISO 10304-1 and 2)), nitrate (NO_3^- (ISO 10304-1 and 2)), sulfate (SO_4^{2-} (CZ_SOP_D06-02-068-01)), TOC/DOC (EN 1484), hydrocarbons (EN ISO 9377-2) and 16 PAH (EPA-8270-C) were analyzed by the commercial laboratory ALS Scandinavia. Conductivity, pH, temperature and oxygen were measured at least once a day by using a multi-water quality sensor (HORIBA W21SDI).

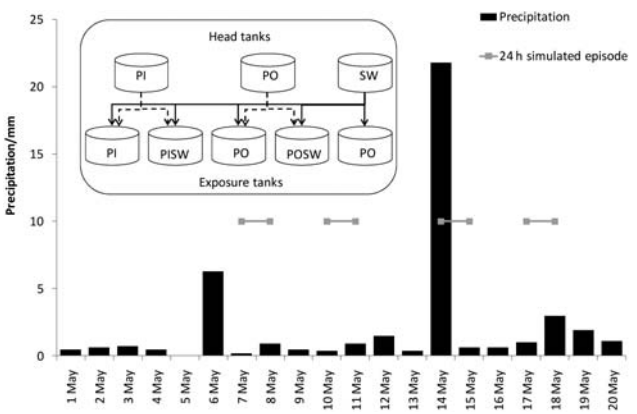

Fig. 2 Daily precipitation (bars) measured at a nearby meteorological station (Valle Hovin, Oslo municipality). The 24 h consecutive simulated runoff episodes are indicated by grey lines. An overview over the experimental setup including head tanks and exposure tanks is given in the inserted box in the upper left corner of the diagram: PI = pond inlet, PO = pond outlet, SW = stream water, PISW = pond inlet + stream water (mix ratio 50 : 50), POSW = pond outlet + stream water (mix ratio 50 : 50). Water was sampled from the different exposure tanks and filtrated *in situ* within 3–4 h after the pumps were started, while the fish were randomly sampled immediately after the pumps were turned off. The fish sampling ($N = 30$) was completed within 5 to 6 h.

Fish sampling

Six fish from each of the five exposure tanks were randomly sampled after every 24 h simulated episode. The fish were killed by a single blow to the head, and sampled according to the EMERGE protocol.[39] The total weight and length were measured at site, while the liver weight was measured at the laboratory.

Blood was sampled from the caudal vein by using a 1.0 mL syringe. A small volume of the blood was transferred to an I-Stat cassette (EC8+) and analyzed by a portable I-Stat analyzer (Abbot). A total of eight different haematological parameters were obtained: plasma ions (Na, K and Cl), pH, glucose, haematocrit, partial pressure CO_2 (pCO_2) and bicarbonate (HCO_3^-).

The second right gill arch was dissected and stored at −20 °C prior to analysis of accumulated trace metals. Then, the liver was carefully dissected and divided into two equally sized parts. The anterior part was stored at −20 °C prior to analysis of accumulated trace metals, while the posterior part was snap frozen in liquid nitrogen and stored at −80 °C. The latter part was analyzed for Cd/Zn–metallothionein (MT), superoxide dismutase (SOD), catalase (CAT) and total protein content.

Analysis of fish samples

The liver somatic index (LSI) and the condition factor (*K*-factor) were calculated from the obtained measurements of total fish and liver weight together with total fish length. LSI in % was obtained by the following formula: LSI (%) = liver weight × 100/fish weight. The *K*-factor was obtained by the following formula: $K =$ fish weight × 100/fish length.[3]

Accumulation of trace metals in gill and liver tissue. Gill and liver samples were freeze dried and weighed before digestion in Teflon beakers by adding 3 mL ultrapure concentrated HNO_3, 2 mL double distilled water (Barnstead) and 0.25 mL indium (In) as internal standard (final concentration 20 μg L^{-1}). The final digestion procedure was done by an MLS-Milestone UltraClave (MLS GmbH). The digested samples were diluted with Barnstead water to a final sample volume of 50 mL (Falcon). Finally, the concentrations of Al, Cd, Cu, Fe, Ni, Pb and Zn were determined by using ICP-MS (Perkin Elmer ELAN 6000). Several blanks and reference material (DOLT-3 and DORM-2, National Research Council Canada) were included as quality assurance. The detection limit (LOD) for the trace metals measured in digested gill and liver samples was 6.5, 0.01, 0.27, 6.4, 0.01, 0.05 and 0.61 μg L^{-1} for Al, Cd, Cu, Fe, Ni, Pb and Zn, respectively. The measurements of the trace metals in DOLT-3 and DORM-2 were all within 10% of the certified values, besides Cd in DOLT-3 (11%), Cu in DORM-2 (11%) and Zn that was 20 and 25% lower than the certified values, respectively. The results are given in μg per g dry weight (dw).

Superoxide dismutase (SOD). Liver samples were homogenized in 5 mM Tris–HCl buffer (pH 7.4) (1 : 5 w/v) by using a Potter-Elvehjem homogenizer. The homogenate was then centrifuged at 10 000 g for 12 min (4 °C), and the obtained supernatant was divided into three aliquots and stored at −80 °C until analysis of MT, SOD and CAT.

The superoxide dismutase (SOD) inhibition activity was determined by using an assay provided by Kamiya Biomedical Company (Cat. No. KT-019). This assay is an indirect method using the highly water soluble tetrazolium salt (WST-1) which produces a water soluble formazan dye upon reduction with the superoxide anion. The xanthine oxidase (XO) activity, which is inhibited by SOD, is linearly related to the reduction rate of the superoxide anion.

20 μL of sample/standards and 200 μL of working solution (WST) were added in triplicates in a 96 well microplate. The reaction was set off by adding 20 μL of enzyme working solution (EWS) to the samples and standards. In addition, two blanks were made by using 20 μL of Milli-Q water, 200 μL WST and 20 μL EWS. In blank no. 2 the EWS was replaced with dilution buffer. The plate was incubated at 37 °C for 20 min before reading the absorbance at 450 nm on a Multiskan Ascent 96 well plate reader (Thermo Labsystems).

Eleven SOD standards made from bovine liver (Sigma Aldrich) were included in the assay with concentrations ranging from 0.001–200 U mL^{-1}. One unit (U) of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide anion.[40] The standard curves obtained are normally not linear, thus a linearization was necessary. Based on the linearized standard curve the concentrations (U mL^{-1}) of the samples were calculated and multiplied with the dilution factor used in the assay.

Catalase (CAT). The assay, including formaldehyde standards, positive catalase control and samples, was performed on a 96 well microplate with a final volume of 240 μL in each well (CAT Cayman kit). In brief, 100 μL phosphate buffer (100 mM, pH 7.0), 30 μL methanol and 20 μL sample/control/standards diluted in phosphate buffer (25 mM, pH 7.5) containing 1 mM EDTA and 0.1% BSA (bovine serum albumin) were added in triplicates. The reaction was initiated by adding 20 μL of hydrogen peroxide (35 mM) to the wells followed by incubating the plate on a shaker for 20 min in room temperature. The reaction was stopped by adding 30 μL of potassium hydroxide (10 M) followed up by adding 30 μL of purpald (34.2 mM in 0.5 M HCl) to every well, followed by incubating the plate on a shaker for 10 min in room temperature. Finally, 10 μL of potassium periodate (0.5 M) were added to the wells and the plate was incubated for 5 min on a shaker at room temperature. The absorbance was read at 540 nm using a Multiskan Ascent photometric plate reader (Thermo Labsystems). The CAT activity (consumed H_2O_2) in μmol min^{-1} $liver^{-1}$ fresh weight was calculated based on the obtained formaldehyde standard curve.

Cd/Zn–metallothionein (MT). The MT concentrations in liver tissue were assayed by using a method described by Olsvik *et al.*[41] The method is a slightly modified version of the original method described by Bartsch *et al.*[42] In brief, 100 μL of the supernatant were incubated with 100 μL acetonitrile and vortexed before 1 mL of buffer (10 mM Tris–HCl, 1 M NaCl, pH 7.4) was added. The sample was then incubated for 5 min with 40 μL cadmium solution consisting of both radioactive (^{109}Cd) and stable Cd isotopes. After the incubation, 100 μL of chelex-100 (Bio-Rad, Hercules, CA, USA) were added to the sample and rotated slowly for 15 min. This was followed by a centrifugation step (10 000 g, 4 °C for 5 min). Finally 0.9 mL of the supernatant was pipetted into a 20 mL scintillation vial and the ^{109}Cd activity was measured on a NaI detector (Wallac, Perkin Elmer, Wizard 3, 1480 Automatic gamma counter). Three parallels of total activity (buffer instead of sample) and background activity (buffer instead of sample and chelex-100) were included in the assay. The activity of the ^{109}Cd tracer represents the amount of Cd bound MT. It is assumed that the MT molecular mass is 7000 Da with a molar ratio of 7 g atoms of Cd per mole protein.

Protein content. The amount of MT and the enzymatic activity of SOD and CAT were normalized by the protein concentration in the liver homogenate. The protein concentration was measured by using the Quick Start Bradford protein assay purchased from Bio-Rad Laboratories. This assay is based on the method of Bradford.[43]

Data analysis

Univariate statistics. The statistics and graphs were conducted by using MINITAB 15. The data are presented as mean ± SEM. To test any significant differences between the concentrations of dissolved metals in the different exposure tanks during the

simulated episodes, one-way ANOVA followed by Tukey's method were applied. Data were log transformed and tested for both normality and homogeneity of variance, $p < 0.05$ was considered as significant. Correlation data given in brackets in the text refer to Pearson product moment correlations.

Multivariate statistics. The multivariate analyses were conducted by using the software CANOCO 4.5, and the ordination plots were created by the software CanoDraw 4.14 supplied with the CANOCO package.

The data matrix contained measurements from a total of 120 fish and 23 different physiological variables giving the possibility for a total of 2760 unique measurements (Pb and Al in gill tissue together with Pb and Ni in liver tissue were excluded in the final data analysis due to the high number of samples below limit of detection (LOD)). Due to reasons such as lack of tissue samples, 30 single measurements were missing. Hence, it was decided to insert dummy values for the missing measurements in order to run the permutation tests of the split-plot design properly (*i.e.* the number of samples in the split-plot design needs to be balanced). The selected dummy values were obtained by taking the average value of that specific variable in the entire dataset. The results obtained from this analysis were then compared with the results from the analysis where the selected dummies were a factor of 10 lower and analysis where the samples with missing values were omitted and ran without any permutation restrictions. The overall results from these analyses were quite similar both with respect to the % explained variation and in the graphical output. Hence, we considered substituting missing values with dummy values based on the average value of that specific variable in the entire dataset to be a conservative approach. In addition, the data were log transformed (log $(x + 1)$) to reduce the effects of extreme values, and then centred and standardized (*i.e.* bringing their means to zero and their variances to one).

To evaluate the maximum variation in the dataset principal component analysis (PCA) was applied. PCA is an unconstrained linear method (indirect gradient analysis) which seeks to describe the explanatory variable by ordination axes that best explain the measured variability. The axes can thus be interpreted as the best obtained theoretical explanatory variables.

A redundancy analysis (RDA) which seeks to describe the variability with a set of explanatory variables was used as a second run. RDA is a constrained linear method (direct gradient analysis) and the counterpart of the unconstrained linear method (PCA). The ordination axes are thus weighted sums of explanatory variables, *i.e.* the constrained ordination axes correspond to the directions of the greatest dataset variability that can be explained by the explanatory variables.

By using RDA combined with Monte Carlo permutation tests we were able to test the following hypothesis: "there is no effect of highway runoff on the brown trout". In our model the physiological variables were used as response variables, while the exposure tanks during different episodes were used as categorical explanatory variables (*i.e.* dummy variables coded by 0 and 1). The four consecutive episodes were given dummy values from 1 to 4 and used as co-variables when testing the effect of exposure (*i.e.* removing the effect of the time). The permutation scheme was set as follows: 20 whole plots, each plot consisting of 6 sampled fish from a specific exposure tank and episode number (*i.e.* whole plot 1 consists of 6 fish sampled from the SW tank in episode 1). The whole plots were randomly permuted whereas the individual fish samples from each whole plot were not permutated. We used 499 permutations under reduced model, which better maintains the type I error in small datasets.[44] $p < 0.05$ was considered as significant.

The output of the RDA is displayed in ordination diagrams (bi-plot) showing the physiological variables as arrows and the categorical explanatory variables (episodes) as centroids. The diagram is focused on inter sample distance to better display the differences between the various exposure groups. The arrows point in the direction of steepest increase of values for the corresponding physiological variable and the angle between them indicates the sign of the correlation. Arrows in same direction are positively correlated and arrows pointing in opposite directions are negative correlated. Arrows having an angel close to 90° have zero correlation. The distance between the different centroids approximates the average dissimilarity (Euclidean distance) of the measured physiological variables between the various exposures and episodes. By projecting the centroids onto the arrow (90°) of physiological variables, it is possible to determine the internal ranking among the centroids on a specific physiological variable. Centroids being close to the origin are predicted to have the average value of that physiological variable. A more comprehensive explanation of PCA and RDA and the interpretation of ordination diagrams can be found in books written by Lepš and Šmilauer[45] and ter Braak and Šmilauer.[44]

Results and discussion

Water quality

The contaminant loadings in highway runoff are determined by numerous factors including traffic density, age of the road construction, precipitation frequency and intensity and runoff area. This experiment was conducted shortly after the winter season. Hence, the use of studded tires (*ca.* 20%) and road salt during the winter would also be important factors most likely. In addition, the traffic density is quit high with AADT being approximately 45 000 vehicles. The concentrations of the main contaminants believed to be associated with traffic and highway pollution are presented in Fig. 3, while general water quality parameters are presented in Table 1.

The pH in all the exposure tanks was circumneutral, which is in accordance with previous measurements.[46] The stream water contained high concentrations of the main cations such as Ca, K, Mg and Na, as well as TOC/DOC (Table 1). As high concentrations of Ca is protective against metal toxicity due to Ca being a powerful competitor with high binding strength to biological membranes, and TOC/DOC can complex dissolved metal ions,[47,48] the recipient was considered to be rather robust in terms of preventing negative effects in aquatic organisms. The concentrations of the main cations were even higher in the pond water compared to the stream water. The Ca and Mg concentrations in the PI tank were in average 2 and 8 times higher compared to the SW tank, respectively, probably reflecting the geochemical composition of the asphalt. Na and conductivity are good fingerprints for road salt and they showed in average 49 and 17 times higher values in the PI tank compared to the SW, respectively.

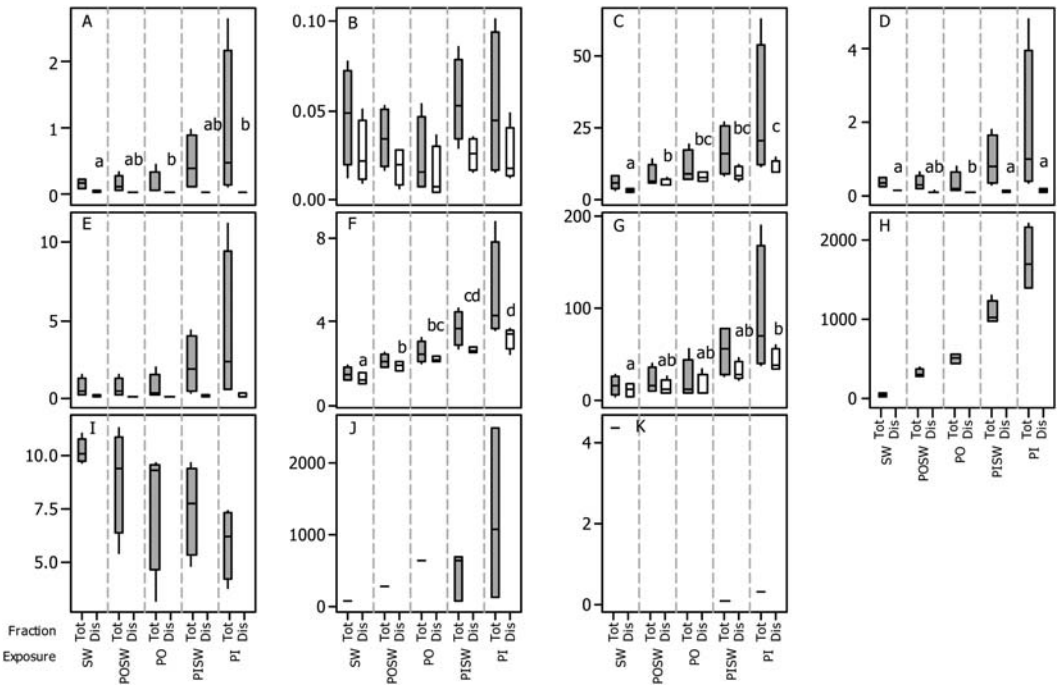

Fig. 3 Box plots showing the total and dissolved concentrations of trace metals (A = Al, B = Cd, C = Cu, D = Fe, E = Pb, F = Ni, G = Zn), road salt measured as Cl (H), dissolved oxygen (I), hydrocarbons (J), and 16 PAH (K) in the various exposure tanks during the 4 simulated consecutive runoff episodes. Al, Fe, Cl and dissolved oxygen are expressed in mg L^{-1}, and the others are expressed in μg L^{-1}. The rectangular box for each group represents the interquartile range of the data including the median value displayed as a horizontal line, while the whiskers extending from the boxes represent the upper and lower 25% of the distribution. The significant differences ($p < 0.05$) between the concentrations of dissolved metals in the various exposure tanks during the episodes are indicated by different small letters.

Measurements done in 2003/2004 by Åstebøl[46] showed that the annual average concentrations measured as event mean concentration (*i.e.* a flow average concentration for the event) in the pre-sedimentation basin were found to be 0.21, 86, 17.1 and 273 μg L^{-1} for the trace metals Cd, Cu, Pb and Zn, respectively. In addition, the annual average concentrations of PAH, hydrocarbons and Cl were 1.8 μg L^{-1}, 5000 μg L^{-1} and 720 mg L^{-1}, respectively. Some precipitation was recorded prior to the experiment (Fig. 2) and based on the measurements given above, we expected that the pollutant loading, at least from the inlet, should be relatively high. However, the pollutant concentrations, except for road salt, were surprisingly low during the first two simulated episodes.

This is exemplified by the total concentrations of Cu and Cl (Fig. 4). The disagreement between our measurements and that done in 2003/2004 clearly demonstrates the contaminant variability between runoff events.

Between the second and the third simulated episodes there was a heavy rainfall leading to a substantial increase in the contaminant loadings in episodes 3 and 4, while the road salt variables were more diluted (Fig. 2 and 4). Due to the residence time of contaminants in the pond, the highest concentrations in the pond outlet (PO) were observed in the fourth and last episode.

A large part of the Cd, Cu, Ni and Zn concentrations could be considered as dissolved, while Al, Fe and Pb were highly associated with particles (Fig. 3). This is in accordance with a French

Table 1 General water quality variables presented as mean values (min and max) sampled during 4 simulated runoff episodes. SW = stream water, POSW = pond outlet + stream water (mix ratio 50 : 50), PO = pond outlet, PISW = pond inlet + stream water (mix ratio 50 : 50), and PI = pond inlet

Variable	SW	POSW	PO	PISW	PI
Ca/mg L^{-1}	23.0 (12.5–32.9)	27.3 (21.3–33.5)	30.0 (24.8–31.9)	38.1 (31.1–47.0)	46.0 (37.4–55.6)
K/mg L^{-1}	1.9 (1.0–2.8)	3.0 (2.4–3.4)	3.8 (3.4–4.0)	4.7 (4.1–5.5)	6.4 (5.4–7.2)
Mg/mg L^{-1}	3.9 (2.1–5.5)	13.6 (11.4–16.1)	20.2 (15.8–23.1)	21.1 (19.4–23.3)	32.2 (25.7–37.6)
Mn/mg L^{-1}	0.1 (0.0–0.1)	0.1 (0.0–0.1)	0.1 (0.0–0.2)	0.4 (0.4–0.6)	0.7 (0.5–0.9)
Na/mg L^{-1}	19.4 (5.2–35.0)	169.9 (144.1–213.1)	264.0 (230.4–302.4)	597.2 (530.6–721.1)	943.6 (758.3–1178.0)
pH	7.3 (7.0–7.5)	7.2 (7.0–7.5)	7.2 (7.0–7.5)	7.1 (7.0–7.2)	7.1 (7.0–7.2)
Conductivity/mS m^{-1}	27.1 (6.7–52.7)	80.4 (52.3–116.0)	119.3 (86.0–152.0)	268.3 (153.0–390.0)	446.8 (252.0–652.0)
Temperature/°C	10.0 (9.9–10.1)	10.6 (10.4–10.7)	10.9 (10.6–11.0)	9.9 (9.7–10.0)	9.8 (9.7–9.9)
Sulfate/mg L^{-1}	15.7 (8.9–23.5)	18.2 (13.3–24.0)	18.5 (14.6–20.6)	20.6 (13.0–28.2)	22.9 (16.7–27.7)
Nitrate/mg L^{-1}	6.1 (<2 to 6.5)	4.6 (<2 to 6.2)	<2	3.5 (<2 to 3.6)	<2
TOC/mg L^{-1}	6.1 (4.1–7.8)	6.9 (5.5–8.0)	8.5 (7.0–10.4)	7.8 (6.7–8.8)	8.4 (8.1–9.0)
DOC/mg L^{-1}	5.4 (3.8–6.3)	6.9 (3.4–11.0)	7.5 (6.2–8.5)	7.8 (6.6–9.7)	8.5 (7.8–9.2)

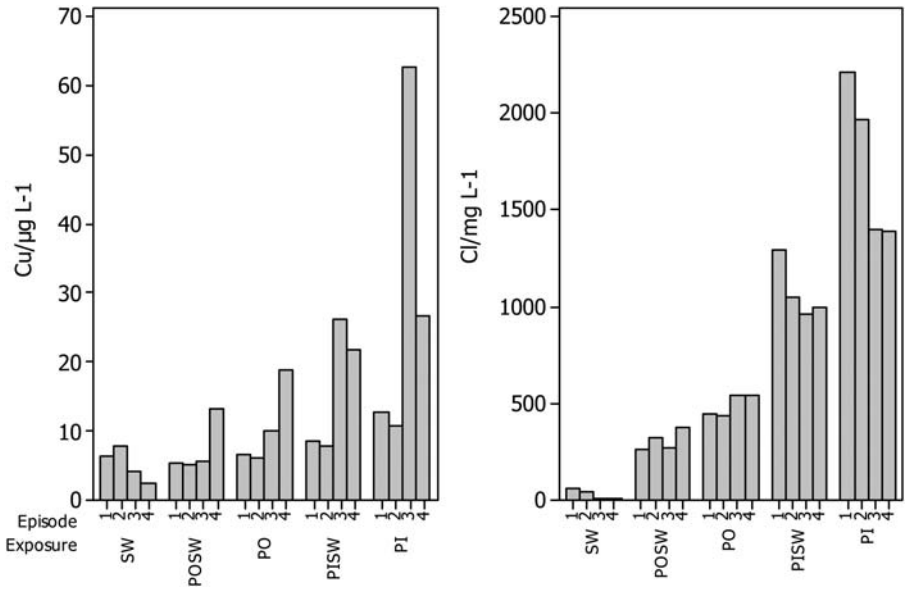

Fig. 4 Total concentrations of Cu and Cl in the various exposure tanks during runoff episodes 1 to 4.

study of a retention/infiltration pond receiving runoff from a multilane bridge with AADT of 90 000 vehicles.[49]

When the concentrations of the various dissolved trace metals in the different exposure tanks are compared (Fig. 3), the stream water (SW) had on average significantly lower dissolved concentrations of Cu and Ni compared to all the other exposure tanks. Dissolved Zn was significantly higher in the PI exposure tank compared to SW, while dissolved Al was significantly higher in the SW tank compared to the PI and PO exposure tanks. No significant differences between the tanks could be seen from the dissolved Cd and Pb concentrations. Novotny *et al.*[17] argued that the excessive use of road salt may increase the mobilization of trace metals. In our material we revealed highly significant positive correlations between road salt, measured as Cl, and the dissolved concentrations of Ni ($r = 0.96$, $p = 0.000$), Cu ($r = 0.88$, $p = 0.000$) and Zn ($r = 0.63$, $p = 0.003$). In contrast, the correlation between road salt and dissolved Al was a highly significant negative correlation ($r = -0.72$, $p = 0.001$).

The hydrocarbon concentrations were low and under the detection limit in several of the exposure tanks (Fig. 3). However, as for the metals the concentrations increased after the heavy rainfall between episodes 2 and 3. This was also the case for the 16 PAHs (Fig. 3). This probably reflects the nature of the PAH being hydrophobic and strongly associated with particles,[50] and hence easily removed from the water phase by sedimentation. Previous measurements have revealed high concentrations of PAH in the sediment.[51] To our surprise the highest concentration of 16 PAH was measured in the control tank containing only stream water. As the concentration in the mixing tanks PISW and POSW was low (mix ratio 50 : 50) contamination seems to have occurred.

The oxygen concentrations in the pond water were lower than in the control water, and the greatest difference was observed between the SW and PI tanks where the mean values were 10.3 ± 0.3 (*ca.* 90% saturation) and 6.0 ± 0.8 mg L^{-1} (*ca.* 50% saturation), respectively (Fig. 3). The lowest concentrations were measured during the third and fourth episodes after the heavy rainfall. Reduced oxygen concentrations and anoxic conditions in sedimentation ponds have also been observed elsewhere.[52,53] This phenomenon is often linked to stratification of water during wintertime due to road salting and ice cover. Hence, the low oxygen measurements could be due to disturbance of anoxic or hypoxic bottom water during heavy rainfall between episodes 2 and 3.

Accumulation of trace metals and physiological responses

Principal component analysis (PCA) and redundancy analysis (RDA). No mortality was observed during the exposure study. The results from the accumulation of trace metals in gills and liver, as well as the physiological measurements used in the multivariate statistics are listed in Table 2 showing the overall statistics including mean ± SEM and range (min and max values). Fig. 5 displays ordination diagrams presenting the outcome of the RDA. The interpretation of the diagrams is described in the "Material and methods" section.

The first four unconstrained axes in the PCA explained 42% of the total variation (not shown). The RDA revealed that the exposure regime explained 17.5% ($p = 0.002$), being almost 42% of the explanation provided in the PCA. As could be expected a great part of the variation on the first RDA axis was seen between the control groups (SW1–SW4) and the fish exposed to pond inlet water (PI1–PI4).

Decreasing values of the *K*-factor and decreasing values of Na and Cl in plasma imply negative effects. The decrease in plasma ions is typically linked to osmoregulation failure, while the *K*-factor is a more general health parameter. The majority of the other variables showed an increase towards fish sampled from the various exposure tanks receiving polluted runoff water.

Accumulation of trace metals in gills. Presently there is a broad acceptance that the primary site of action regarding acute toxicity of trace metals takes place in the gills.[54–56] Hence, the

Table 2 Summary statistics (mean ± SEM and min–max values) of trace metal accumulation in gills and liver together with physiological response variables in exposed brown trout obtained from the various exposure tanks during 4 consecutive simulated runoff episodes

Variable	Unit	SW ± SEM min–max	POSW ± SEM min–max	PO ± SEM min–max	PISW ± SEM min–max	PI ± SEM min–max
Cd gill	μg g^{-1} dw	0.59 ± 0.03 0.31–0.80	0.59 ± 0.04 0.25–1.15	0.54 ± 0.03 0.24–0.85	0.64 ± 0.03 0.42–1.00	0.56 ± 0.03 0.33–0.79
Cu gill	μg g^{-1} dw	1.47 ± 0.05 1.08–1.88	1.59 ± 0.10 0.87–3.36	1.71 ± 0.08 1.13–2.86	1.57 ± 0.06 1.04–2.41	1.79 ± 0.08 1.25–2.81
Fe gill	μg g^{-1} dw	405 ± 15 272–577	416 ± 32 175–908	415 ± 17 206–548	420 ± 18 302–662	442 ± 14 315–577
Ni gill	μg g^{-1} dw	0.58 ± 0.02 0.45–0.84	0.58 ± 0.03 0.29–1.11	0.55 ± 0.02 0.39–0.78	0.62 ± 0.03 0.47–1.07	0.58 ± 0.02 0.46–0.82
Zn gill	μg g^{-1} dw	379 ± 14 276–513	360 ± 24 181–724	357 ± 16 211–525	345 ± 15 240–553	357 ± 16 241–575
Al liver	μg g^{-1} dw	2.68 ± 0.38 1.29–11.08	2.52 ± 0.15 1.37–3.95	2.46 ± 0.13 1.45–3.49	2.63 ± 0.15 1.06–4.65	2.67 ± 0.12 1.89–4.42
Cd liver	μg g^{-1} dw	0.21 ± 0.01 0.09–0.34	0.20 ± 0.01 0.09–0.31	0.21 ± 0.01 0.13–0.32	0.20 ± 0.01 0.12–0.34	0.22 ± 0.01 0.13–0.38
Cu liver	μg g^{-1} dw	324 ± 31 105–676	325 ± 24 180–691	326 ± 24 191–644	310 ± 25 118–666	335 ± 24 133–573
Fe liver	μg g^{-1} dw	197 ± 12 100–312	162 ± 9 102–258	150 ± 8 92–250	166 ± 8 92–228	128 ± 7 84–210
Zn liver	μg g^{-1} dw	83 ± 3 44–103	83 ± 2 71–129	84 ± 2 69–103	83 ± 1 66–93	88 ± 2 66–109
Na blood	mM	143 ± 0.8 134–149	142 ± 1.3 116–147	141 ± 1.0 128–153	142 ± 0.8 131–148	141 ± 0.9 131–150
K blood	mM	4.8 ± 0.2 3.0–6.7	5.1 ± 0.2 2.4–7.5	4.9 ± 0.2 3.4–6.9	5.2 ± 0.2 3.5–8.2	5.3 ± 0.2 3.6–7.3
Cl blood	mM	134 ± 0.5 130–138	131 ± 1.7 99–140	133 ± 0.7 127–139	132 ± 0.6 123–136	131 ± 0.8 121–137
Glucose	mM	3.7 ± 0.2 2.3–5.4	4.9 ± 1.0 2.9–27.1	4.4 ± 0.2 2.5–6.5	3.9 ± 0.3 2.6–10.5	5.4 ± 0.5 3.0–12.5
Hct	%	29 ± 1.3 19–38	33 ± 1.1 23–46	31 ± 1.2 15–42	28 ± 1.0 16–36	32 ± 1.1 20–42
pH	—	7.75 ± 0.05 7.33–8.14	7.67 ± 0.04 7.30–8.08	7.73 ± 0.04 7.37–8.30	7.75 ± 0.05 7.42–8.40	7.76 ± 0.05 7.39–8.25
pCO_2	mmHg	6.8 ± 0.3 5.4–11.0	8.0 ± 0.3 5.6–11.8	7.3 ± 0.2 5.5–10.0	7.4 ± 0.2 5.2–9.5	8.2 ± 0.3 5.4–12.7
HCO_3	mM	10.3 ± 1.0 4.1–19.4	10.0 ± 1.0 5.3–25.2	10.5 ± 1.0 5.6–26.9	11.5 ± 1.4 4.9–36.1	12.7 ± 1.3 5.8–26.9
CAT	μM min^{-1} mg $protein^{-1}$	0.43 ± 0.02 0.27–0.64	0.46 ± 0.03 0.18–0.73	0.48 ± 0.02 0.37–0.65	0.57 ± 0.08 0.23–1.88	0.52 ± 0.04 0.25–1.22
SOD	U mg $protein^{-1}$	121.2 ± 7.4 86.7–196.4	133.4 ± 5.9 73.1–226.1	142.6 ± 8.6 64.0–232.7	141.9 ± 8.5 86.4–260.3	132.5 ± 5.6 81.0–197.3
MT	μg mg $protein^{-1}$	0.29 ± 0.02 0.13–0.43	0.25 ± 0.01 0.12–0.39	0.27 ± 0.01 0.16–0.38	0.31 ± 0.04 0.16–1.04	0.33 ± 0.03 0.21–0.98
LSI	%	1.0 ± 0.02 0.7–1.2	1.1 ± 0.04 0.7–1.7	1.0 ± 0.04 0.7–1.6	1.1 ± 0.04 0.8–1.8	1.1 ± 0.03 0.9–1.5
K-Factor	—	1.1 ± 0.02 0.8–1.2	1.1 ± 0.01 0.9–1.2	1.0 ± 0.01 0.9–1.1	1.0 ± 0.01 0.9–1.1	1.0 ± 0.02 0.9–1.2

accumulated concentrations of various metals in gill tissue reflect the presence of gill reactive metal species in the exposure. The metal concentrations in gill tissue found in our study (Table 2) are in accordance with other comparable exposure studies utilizing brown trout.[57,58]

From the RDA plot it can be seen that accumulation of Cu and Fe in gills is positively correlated with the first axis, and hence contributing to the separation of the exposure tanks along this axis (Fig 5), while Ni and Zn were more associated with the second axis. This implies that deposition of these trace metals occurred at a higher level in fish exposed to polluted runoff water compared to the control fish (SW). Overall, the sum of the weighted mean concentrations of Cu, Fe, Zn and Ni in gills increased in the following manner SW < POSW < PO < PISW < PI. Cd appeared to be negatively correlated with Ni and Zn. However, the short arrow of Cd indicates that this variable is less important in this model, which is in consistence with the low concentrations and variability in the water sampled from the various exposure tanks.

Blood physiology. Al, Cu, Pb and Zn are able to disturb the ion balance of fish by interfering with the gill surface resulting in reduced plasma concentrations of Cl and Na ions.[59–62] There are mainly two causes leading to loss of plasma ions: (1) increased passive efflux of ions across the gill due to increased membrane permeability or disruption of the membrane and (2) inhibition of active ion uptake by specialized chloride cells, *i.e.* reduced Na/K-ATPase.[55,63]

Such ion losses were also observed in this study. Besides K, both Na and Cl ions were negatively correlated with the accumulation of Zn, Ni and Cu in gills, *i.e.* increased accumulation of these trace metals corresponded with a loss of Na and Cl ions

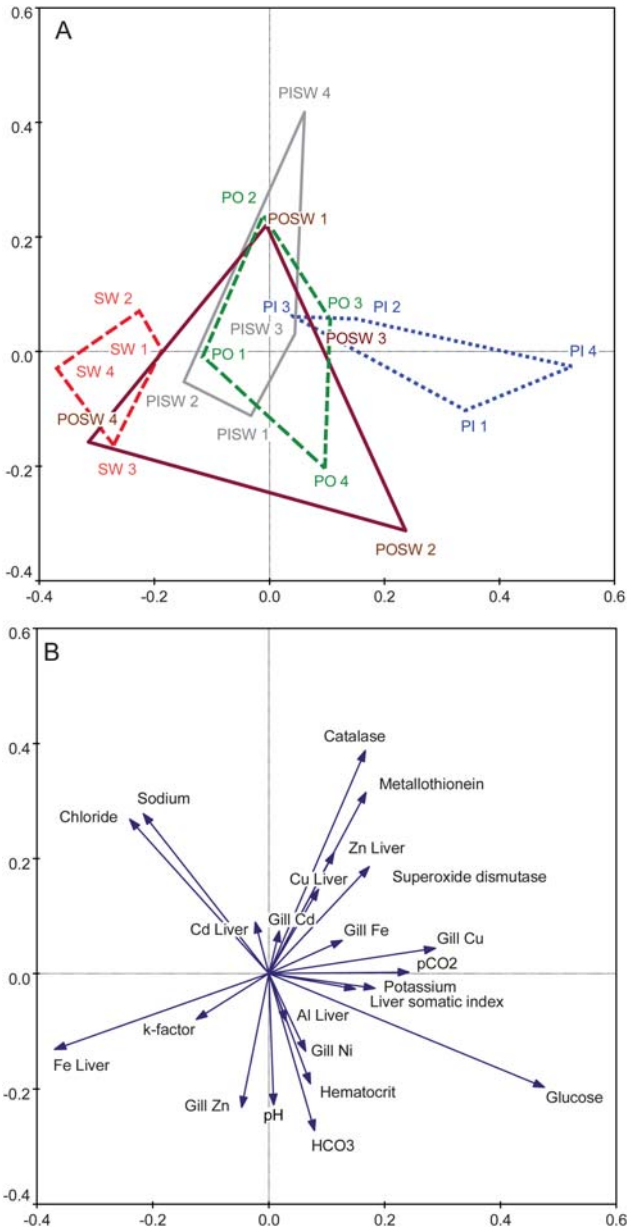

Fig. 5 The results from the RDA displayed in ordination diagrams showing the first two RDA axes. The exposure regime significantly explained 17.5% of the total variability ($F = 1.429$, $p = 0.002$). For clarity the samples (episodes 1–4) and the fish variables are displayed in separate diagrams. (A) Ordination of the various exposure tanks and episodes (*i.e.* PO 2 = centroid of samples from pond outlet in episode 2). Samples from same exposure tanks are internally connected with envelopes. (B) The accumulation of trace metals in gills and liver together with the physiological variables is visualized as arrows (summary statistics are listed in Table 2). The interpretation of the diagrams is described in "Material and methods" section.

(Fig. 5B). These trace metals appeared also at the highest dissolved concentrations and they were highly correlated with the amount of road salt (measured as Cl). Thus, mobilization of trace metals due to road salt may have occurred. Based on present knowledge, only Cu and Zn are able to cause osmoregulatory problems on trout, while Ni is linked to respiratory dysfunctions.[64] According to a classification system in Norwegian freshwaters[7] primarily related to salmonids, the measured concentrations of Ni in this experiment were rather low (<10 μg L^{-1}). The observed decrease in plasma ions in our study is thus more linked to Cu and Zn rather than Ni.

The ion losses were observed both early (episode 1) and at the end (episode 4) of the exposure study in fish exposed to the untreated water (PI). The high salt concentrations in the pond led to a substantial and immediate increase in the ionic strength in the exposure tanks, creating unstable mixing zones due to ion exchange processes.[18,62,65] Hence, gill reactive metal species will accumulate on gill surface causing a loss of plasma ions. The plasma concentrations of Cl and Na recovered to pre-exposure levels before episodes 3 and 4 where highly polluted water caused a second plasma ion loss in fish contained in the PO and PI exposure tanks.

Along with problems in maintaining a normal level of plasma ions, the fish also had increased levels of blood glucose (Fig. 5B). Elevated glucose levels are relatively rapid responses (minutes to hours) and are considered as an important indicator of general stress.[66] The glucose levels were well correlated with the loss of plasma ions, suggesting that the underlying processes were the same. Increased glucose levels together with substantial loss in plasma ions have been seen in salmonids exposed to Al, Cu and Pb.[58,62,67–69]

The increased accumulation of trace metals in gill tissue together with loss of plasma ions and elevated glucose concentrations strongly indicates multiple stress responses. This is interesting, as the high concentrations of TOC/DOC and Ca measured in all the exposure tanks (Table 1) should be sufficient to protect the trout against metal toxicity due to complexion between dissolved metal ions and TOC/DOC, and due to Ca being a strong competitor with high binding strength to biological membranes.[47,48] Tao *et al.*[70] argued that particle bound Cu likely would be a source of Cu toxicity due to the lowered pH in the gill mucus compared to surrounding water, and thus favoring a transfer of Cu ions from particles to the mucus matrix and finally into the gill tissue by diffusion. Similar processes have been found for Cd and Pb,[71,72] and we cannot exclude such processes in our study as a lot of metals were associated with particles (>0.45 μm filtrate).

The fish being exposed to highway runoff also experienced severe fluctuations in oxygen levels ranging from normoxic to hypoxic conditions (Fig. 3). Blood acidosis is often observed during severe hypoxic conditions, with high pCO_2 (hypercapnia) followed by decrease in blood pH and increase in HCO_3.[73] However, there was hardly any correlation between pCO_2 and the latter two variables pH and HCO_3 in our experiment (Fig. 5B). This may indicate that although the O_2 levels in the water were low, it might not have been sufficiently critical for hyperoxia to occur in the fish. This is because many aquatic organisms will have a partial pressure of oxygen (pO_2) in their arterial blood of 20–30% saturation compared to atmospheric condition to avoid oxidative stress.[74] Some authors like Pilgaard *et al.*[59] have also argued that many metals have a higher threshold for causing respiratory effects than for osmoregulatory effects.

Biomolecules. To assess the effects of induced ROS in the fish the antioxidant enzymes CAT and SOD together with MT were measured. SOD enzymes are highly efficient in catalytic removal of the superoxide by dismutation of the superoxide anion into

hydrogen peroxide and oxygen. The created hydrogen peroxide can be further removed by CAT which catalyzes hydrogen peroxide into water and oxygen.[29]

The enzymatic activity of CAT and SOD was positively correlated with the amounts of MT (Fig. 5B). All three biomolecules were associated with higher enzymatic activity and concentrations in fish exposed to PI and PISW sampled in episode 4 (Fig. 5A). All the fish from SW had a lower activity than the average of the whole dataset. By projecting the exposure centroids in the ordination diagram onto the MT, CAT and SOD arrows it can be seen that there is a tendency towards less effect of ROS during the first two episodes compared to the last two.

Fe and Cu are known to be inducers of ROS[29,75,76] through Fenton and Haber–Weiss reactions. Hence, the observed correlation between the biomolecules and the concentrations of Cu and Zn in the liver and Cu and Fe in gills may indicate the presence of reactive oxygen species (ROS) in fish exposed to PI, PISW and PO water (Fig. 5B). Furthermore, the same fish experienced additional stress due to repeated fluctuations in oxygen concentrations ranging from normoxic to severe hypoxic conditions. It has been documented that fish activates their antioxidant defense system during anoxic/hypoxic conditions to prepare themselves against oxidative stress during reoxygenation,[75,77] and that might also have contributed to the observed increase of the biomolecules linked to the antioxidant defense system.

Accumulation of trace metals in liver. The liver is considered as the main organ for detoxifying organic xenobiotoics and it also plays a crucial role in the excretion of toxic metals. The concentrations of Zn and Cu in liver appeared to be negatively correlated with the concentration of Fe indicating accumulation of the former two trace metals in fish exposed to road runoff, while the latter indicated higher concentrations in the control fish (SW). Cu and Zn were also well correlated with the MT levels and to some extent the enzymatic activity of SOD and CAT indicating that the antioxidant defense system was triggered in fish exposed to runoff water.

Liver somatic index (LSI) and condition factor (*K*-factor). Increased values of LSI, which have been linked to pollution of organic contaminants such as PAH,[66] were observed in the fish exposed to the untreated PI water (Fig. 5). However, studies have concluded that increased LSI might also be related to metal pollution.[78] The concentrations of organic contaminants such as PAH and hydrocarbons were rather low during this study. Hence, the observed increase in LSI probably reflects the effects of metal pollution rather than from organic pollutants.

We experienced that the fish exposed to the PI and PISW water had reduced *K*-factors at the end of the study (Fig. 5). There were no feeding of the fish through the experiment, and very little food was expected to follow the inflowing waters. A reduced *K*-factor would then most probably reflect an increased metabolism in fish exposed to PI and PISW water. Rosseland[79] found a clear increase in ventilation rate and metabolism in brown trout exposed to low pH and aluminium. As reviewed by Perry[80] there are evidence that acute hypoxia leads to increased hyperventilation which will increase the metabolic costs associated with breathing. There is also evidenced that fish exposed to at least some metals will be stimulated to produce more chloride cells in gill epithelium as a response to osmoregulatory problems (*e.g.* to compensate the loss of plasma ions) or to replace necrotic and apoptotic chloride cells.[63] An increased number of chloride cells (hyperplasia) will increase the blood to water diffusion barrier and fortify the problems in maintaining the internal body oxygen and CO_2 levels.[80] This together with increased metabolic costs associated with detoxification processes to handle ROS through antioxidant defense system would likely affect the overall energy budget. Olsvik *et al.*[81] found a negative correlation between Cd/Zn–MT and *K*-factor in different brown trout populations living in Cu and Cd/Zn contaminated streams in Norway. The reduced *K*-factor found in the end of our study can most probably be connected to the aspects described above.

Conclusions

Fish exposed to highway runoff had higher concentrations of trace metals in gill and liver tissue, increased activity of antioxidant defense system represented by SOD, CAT and MT, problems with the regulation of plasma ions as well as increased levels of glucose and pCO_2. The exposure with runoff water seemed to affect the metabolism of the fish manifested by reduced *K*-factor.

The observed effects are likely caused by several pollutants (multiple stressors) and not by a single factor. The high concentrations of dissolved species of Cu, Ni and Zn found in the water were in accordance with the accumulation of these metals in gill and liver tissue. The accumulation was probably enhanced by the high salt concentrations, increasing the ionic strength and creating unstable mixing zones. In addition the fluctuations in oxygen saturation probably contributed to the observed effects.

The sedimentation pond clearly reduced the toxicity of the highway runoff. But even in the least polluted exposure tank (POSW) where pond outlet water was mixed with stream water, signs of physiological disturbances were evident.

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Hepatic gene expression profile in brown trout (Salmo trutta) exposed to traffic related contaminants

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ABSTRACT

In the last decades there have been a growing concern regarding highway runoff as a potential threat and a significant source of diffuse pollution to the aquatic environment. However, identifying ecotoxicological effects might be challenging, especially at sites where the traffic density are modest to low. Hence, the need for alternatives to for instance small-scale toxicity tests using conventionally endpoints such as mortality and growth are warranted. The present paper presents result from a DNA microarray screening performed on liver from brown trout (Salmo trutta) acutely exposed (4 h) to traffic related contaminants during washing of a highway tunnel outside the City of Oslo, Norway. The results clearly demonstrated that traffic related contaminants may cause a plethora of molecular changes extending several hours after the exposure. At time 38 h (i.e. 34 h recovery), several gene ontology (GO) terms were significantly suppressed in exposed fish including GOs within the immune system. In addition, several enzymes involved in the biosynthesis of cholesterol were apparently strongly inhibited. Readily bioavailable polycyclic aromatic hydrocarbons (PAHs) or other traffic related organic pollutants may have caused these alterations in exposed fish. The initial responses were subsequently followed by up-regulation of xenobiotic biotransformation and antioxidant defense system at time 86 h (i.e. 82 h recovery), including classical biomarkers such as cytochrome P450 (CYP1A1 and CYP1B1), cytosolic sulfotransferase 3 (SULT) and glutathione peroxidase 1 (GPX). Of special interest was the apparently up-regulation of the paraoxonase (PON) enzyme indicating

the presence of organophosphorus compounds (OPs) in the runoff water. In addition, the apparently up-regulation of the arsenite methyltransferase (AMT) may indicate that metalloids such as arsenic (As) and antimony (Sb) were readily bioavailable despite the fact that no liver accumulation of these metalloids was observed.

KEY WORDS: Microarray, brown trout, highway runoff, immunotoxicity, cholesterol, paraoxonase, CYP1A, oxidative stress

1 Introduction

A modern and well functioning transportation network is fundamental for a modern living, e.g. maintaining vital settlements in rural areas, ensuring proper security for the road users as well as ensuring a safe and secure flow of goods and services. In Norway, the road network increased from approximately 45 000 km in 1948 to 93 000 km today, while the transportation load increased from 2.5 to 60.6 million passenger km in the same period (OVF, 2008). This increased traffic load has undoubtedly a major negative effect on the environment, and contributes to emissions of greenhouse gasses, noise, local air-, soil- and water pollution.

From the 1970s there has been a growing concern regarding highway runoff as a potential threat and a significant source of diffuse pollution to the aquatic environment (Hedley and Lockley, 1975). Since then, several papers have been published regarding chemical characterization and quantification of traffic related pollutants (e.g. Sansalone and Buchberger, 1997; Glenn et al., 2001; Desta et al., 2007), however, relatively few have investigated the bioavailability and toxicity of highway runoff. Most of these reported toxicity studies have been conducted by utilizing small-scale laboratory toxicity tests with conventional endpoints such as mortality, growth and reproduction (e.g. Gjessing et al., 1984; Kayhanian et al., 2008; Waara and Farm, 2008), or by conducting in situ surveys of biological communities, e.g. periphyton and macroinvertebrates (e.g. Maltby et al., 1995; Boisson and Perrodin, 2006; Woodcock and Huryn, 2008).

Identifying ecotoxicological effects of highway runoff might be challenging, especially at sites where the traffic density are modest to low. For example, in a Swedish study no toxicity was observed when using a battery of small-scale toxicity tests in 65 untreated highway runoff samples from a road having an annual average daily traffic (AADT) around 20 000 vehicles (Waara and Farm, 2008). Hence, alternatives to the more conventionally endpoints mentioned above is warranted for at least two reasons, firstly; the lack of sensitivity as effects at higher hierarchical levels are always preceded by earlier changes in biological processes (Bayne et al., 1985), and secondly; they do not provide any mechanistic understanding of the toxicants mode of action (MoA) (Snape et al., 2004). In this context, toxicogenomics utilizing high content DNA

69 microarray technology is promising to perform genome-wide screening approaches to elucidate
70 the MoA of multiple contaminants.

71 DNA microarray technology allows rapid measurements of transcriptional changes of thousands
72 of genes simultaneously. Hence, complex pathways and strategies that an exposed organism
73 applies in response to environmental stressors may be revealed (Steinberg et al., 2008). This
74 technology has provided knowledge on toxicant exposure, disease state and cellular metabolism
75 (Lettieri, 2006; Prunet et al., 2008). For example, Walker et al. (2008) clearly demonstrated that
76 microarray technology was able to disclose several novel genes in gill epithelial cells from rainbow
77 trout (Oncorhynchus mykiss) responding to metal toxicity, while Finne et al. (2007)
78 demonstrated that cultured hepatocytes from rainbow trout exposed to a chemical mixture gave a
79 significant alteration of the transcriptomic signature compared to hepatocytes exposed to the
80 single chemicals. Hence, the use of a holistic genome-wide screening approach such as that
81 provided by high-content ecotoxicogenomics should therefore be an important tool in assessing
82 multiple effects induced in organisms by multiple stressors. The U.S. Environmental Protection
83 Agency (US EPA) currently accepts toxicogenomics data as part of a weight-of-evidence
84 approach for establishing mechanisms of toxicity for regulated substances (Van Aggelen et al.,
85 2010).

86 Recent exposure studies on highway and tunnel wash water runoff, using brown trout (Salmo
87 trutta) as model organism, have documented sublethal effects following short term exposures (4
88 – 24 h) (Meland et al., 2010a; Meland et al., 2010b; Meland et al., 2010c). This included alterations
89 in blood physiology and activating of the antioxidant defense system. The latter was manifested
90 by increased levels of the metal sequestering protein metallothionein and the antioxidant enzymes
91 superoxide dismutase and catalase. In addition, an increased hepatic mRNA expression of the
92 mixed function oxidase biotransformation enzyme cytochrome P450 1A (CYP1A) was evident.

93 The present paper presents results from a DNA microarray screening performed on liver samples
94 obtained from brown trout acutely exposed to traffic related contaminants during washing of a
95 highway tunnel. The aim was to increase the mechanistic understanding of the effects of traffic
96 related contaminants, common in highway- as well as in tunnel wash water runoff, on brown
97 trout, a salmonid species being both geographically abundant and pollutant sensitive. In addition,
98 the focus was put on biological and molecular pathways by the means of gene ontology (GO)
99 assessment rather than studying single gene expression.

2 Materials and methods

The current research is part of a larger experiment and a full description of the study site and experimental setup are published elsewhere (Meland et al., 2010a). In brief, brown trout were exposed in situ to tunnel wash water runoff during washing of the 3.8 km long Nordby tunnel (located at highway E6 about 30 km southeast of the City of Oslo, Norway) which has a traffic load of approximately 25 000 vehicles per day. A control tank (70 L) and an exposure tank (70 L) supplied with municipal tap water in a flow through system (ca. 5 L/min) contained approximately 40 fish each. The fish had an average weight of 47 ± 3 g and 49 ± 3 g, and an average length of 16 ± 0.4 cm and 16 ± 0.3 cm in the control and the exposure tank, respectively. The condition factors were identical in the two groups (K = 1.1 ± 0.01). Immediately after the tunnel wash had started, the water supply to the exposure tank was switched from tap water to the polluted wash water runoff at the same flow rate (ca. 5 L/min). The exposure lasted for 4 h, where after the water supply to the wash water exposed tank was switched back to high-quality tap water. Fish from the exposure tank and the control tank were sampled before (-3 h), during (1 and 3 h) and after (14, 38 and 86 h) the tunnel wash episode, respectively. The fish was killed by a single blow to the head before the dissection, following the EMERGE protocol (Rosseland et al., 2001). The liver was carefully withdrawn from the abdominal cavity and divided in two pieces. One piece of tissue from the distal part of the liver was snap frozen in liquid nitrogen and stored at -80°C for later DNA microarray analysis, while the second piece were stored at -20°C until analysis of trace metal accumulation. DNA microarray analyses were performed on a subset of these samples, based on knowledge gained from the qrtPCR assessment of stress-specific genes which clearly indicated physiological responses in the exposure groups 38 and 86 h after the beginning of the exposure, i.e. 34 and 82 h recovery (henceforth just termed 38 and 86 h) (Meland et al., 2010a). Hence, DNA microarray analyses were performed on 4 random samples from exposed fish and control fish at time 38 and 86 h (total of 16 samples).

A detailed characterisation and quantification of the water quality during the tunnel wash is published elsewhere (Meland et al., 2010a), but a brief summary is presented in Table 1.

2.1 Determination of trace metals in liver

The accumulation of aluminium (Al), arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), cupper (Cu), iron (Fe), nickel (Ni), lead (Pb), antimony (Sb), vanadium (V) and zinc (Zn) in liver were determined utilising an ICP-MS instrument (Perkin Elmer ELAN 6000). Prior to the quantification, the liver samples were freeze-dried and weighed, and finally digested in Teflon beakers containing suprapure concentrated HNO_3 and internal standard (20 μg/L of

In). The digestion procedure was completed by utilising a MLS-Milestone UltraClave (MLS GmbH). Reference material (DOLT-3 (dogfish liver) and DORM-2 (dogfish muscle), National Research Council Canada) and blank samples were included as quality assurance. The detection limits (LOD) for trace metals in digested samples expressed as µg/L were 3.5 (Al), 0.1 (As), 0.6 (Ba), 0.003 (Cd), 0.01 (Co), 0.3 (Cr), 0.3 (Cu), 3.0 (Fe), 0.1 (Ni), 0.1 (Pb), 0.005 (Sb), 0.6 (V) and 3.3 (Zn). The measurements of the trace metals in DOLT-3 and DORM-2 were all within 10 % of the certified values, beside Ni (16 %) and Zn (13 %) in DOLT-3 and As (13 %), Ni (12 %) and Pb (11 %) in DORM-2, respectively. The results are given in µg/g dry weight (dw).

To test for any significant differences between the concentrations of trace metals in liver from the exposed fish and the control fish, one way ANOVA was applied (MINITAB 15). The data were tested for normality and homogeneity of variance prior to the analysis.

2.2 Microarray design

Microarray analysis was performed with an Atlantic salmon custom array from Agilent (Agilent technologies, Santa Clara, California, USA), after re-annotation by the cGRASP consortium (http://web.uvic.ca/grasp/) and selective in-house re-annotation by Blast2Go (http://www.blast2go.org/) using default parameters with minor modifications. In short, sequences were blasted against the NCBI non-redundant (nr) and the swissprot database using blastX (EV=1.0E-3, min. 20 Hits). Sequences with blast hits were mapped against the Blast2Go database and resulting mapped sequences annotated in a sequential manner according to decreasing cut-off values (1: EV=1.0E-6, cut-off: 55, HSP coverage cut-off: 75; 2: EV=1.0E-6, cut-off: 55, HSP coverage cut-off: 0 and 3: EV=1.0E-6, cut-off: 60, HSP coverage cut-off: 0, Evidence code weight: ISS=1.0, IEA=1.0). Gene ontology (GO) were enriched by merging Interpro annotations to existing GOs and the GOs augmented by ANNEX before 1. level annotations were removed.

2.3 RNA extraction and microarray hybridisation

Cell lysates from liver tissue was obtained by homogenising approximately 20 mg tissue samples in 500 µl of RLT lysis buffer, using a Precellys orbital shaker bead mill (Bertin, Montigny-le-Bretonneux, France). Samples was homogenised for 3 x 10 sec. at 6000 rpm with Precellys CK14 beads and cells debris were removed by centrifugation at 8000 g for 1 min. DNA-free total RNA was then isolated from 350 µl of the supernatant using the RNeasy mini kit and RNase free DNase kit, according to the producer's instructions (Qiagen, Hilden, Germany). The RNA was quality controlled by photometric analyses of 260/230 and 260/280 nm ratio, and RNA used for the microarray had 260/230 > 1.8 and 260/280 > 1.5 (data not shown). The samples were also inspected by electrophoresis using RNA 6000 nano chips in a Bioanalyzer instrument (Agilent

technologies, Santa Clara, California, USA), and RIN values ranged from 7.4 to 9.7. Microarray cDNA synthesis, linear amplification/cRNA synthesis/ Cy-3 labelling, hybridization, washing and scanning were performed as described by Agilent's protocol "One-color Microarray-Based Gene Expression Analysis (Quick Amp Labeling), Version 5.7 March 2008". All chemicals used in the process were bought from Agilent, as parts of the kits: Agilent One-color RNA Spike-In Kit, Agilent Low RNA Input Linear Amplification Kit PLUS, One-Color, Gene Expression Hybridization Kit, and Wash Buffer 1 and 2. Briefly, for each microarray slide, 200 ng of total RNA sample was mixed with a spike-in standard (Agilent One-color RNA Spike-In Kit) for later verification of the dynamic range and linearity of fluorescence signal. A T7 promotor primer was then added and annealed to the RNA template at 60ºC for 10 minutes before immediate cooling on ice. First strand cDNA was then synthesized by incubating the template with first strand buffer, 0.1 M DTT, 10 mM dNTP mix, MMLV-RT enzyme and RNase Out for 2 h at 40 ºC. The cDNA was then denatured for 10 minutes at 65 ºC, before being cooled to 4 ºC. The resulting cRNA was then synthesised from the cDNA template using NTPs as well as Cyanine 3-CTP together with a T7 RNA Polymerase for 2 h at 40 ºC. The samples was then kept at - 20 ºC over night before the labelled and amplified cRNA was purified by Qiagen's RNeasy mini spin columns according to suppliers protocol (Qiagen). After careful washing, the eluate was measured by nanodrop to ensure a cRNA yield of > 1.65 μg and specific activity of > 9.0 pmol Cy3/μg cRNA. Hybridisation was finally prepared by fragmentation of 1.65 μg of the labelled cRNA for exactly 30 minutes at 60ºC, a process which was stopped by addition of 2x Gene Expression Hybridization Buffer. The hybridisation mix was then carefully pipetted on to the gasket slides, before placing the arrays with the active side down on top of the gasket slides, and fixing the two together in the hybridisation chamber. After 17h hybridization at 65°C, the slides were washed with Agilent wash buffers, and scanned (5 μm resolution) immediately (5 μm resolution), using an Agilent microarray scanner. A total of 16 arrays were used, leaving one array per individual (n=4).

2.4 Microarray data analysis

Scanned images were analysed with Agilent fature extraction Ver 10.7 (Agilent Technologies). Resulting raw data were normalised (25 Quantile, median to baseline of all samples), outlier flagged and significantly regulated genes across treatments identified in a two way ANOVA ($p < 0.05$, Benjamini-Hochberg FDR correction) by GeneSpring GX 10.0.2 (Agilent Technologies). Data from individual treatments (Treatments versus control) were subjected to a two class statistical analysis using Bayes statistics in GEPAS 4.0 (http://gepas.bioinfo.cipf.es/) followed by

gene set (functional) enrichment by FatiGo and Fatiscan (http://babelomics3.bioinfo.cipf.es/). Resulting enriched GO terms were visualised by Blast2GO.

3 Results

3.1 Water quality and accumulation of trace metals in liver

The exposure water contained high concentrations of both inorganic and organic contaminants (Table 1). However, no fish died during the exposure. The concentration of trace metals in liver of exposed fish compared to the liver of control fish are presented in Table 2. There appeared to be no statistically difference (one way ANOVA, $p > 0.05$) in trace metal concentrations between the exposed fish and the control fish in neither of the sampling times, i.e. before, during and after the exposure.

3.2 Microarray analysis

Over 38700 of totally 43663 features were successfully identified as being of sufficient quality across individual arrays after filtering for expression (20-100%) and removal of feature outliers. A two-way ANOVA using time and exposure as co-factors revealed that 1697 features were significantly regulated (Fig. 1). Around 1660 features were associated with the parameter time, 41 features were differentially regulated in comparison to the control as a function of exposure and 9 features were significantly regulated as a consequence of both time and exposure (Fig. 1 and Supplemental Table S1). Of the 28 features being robustly regulated at both sampling times, 17 genes with known identity were identified. While several of the significantly expressed genes had no known functions, a few were well known (e.g. cytochrome P450 1A (CYP1A1), cytochrome P450 1B (CYP1B1), cytosolic sulfotransferase 3 (SULT)) and cytosolic Acetyl-CoA acetyltransferase). Despite that 41 differently expressed genes were associated with the exposure to traffic related contaminants at one or both of the sampling times (38 and 86 h), no significant GO terms could be identified being overrepresented in functional enrichment analysis by Fatiscan or FatiGo. Hence, it was decided to evaluate the microarray data by analyzing significantly overrepresentation of GO terms at each sampling time, i.e. comparing the enrichment of functional GO terms between the exposed fish and the control fish at 38 and 86 h separately.

Functional enrichment analysis of all 38762 features by Fatiscan identified 327 and 48 differentially regulated GO terms between the exposed and the control fish at 38 h and 86 h, respectively. A clearly higher number of GO terms were found to be differentially regulated short

time after the exposures, whereas the transcriptional response after 86 h was considerably lower. The results clearly demonstrated transcriptional changes in hepatic tissue of juvenile brown trout exposed for 4 h to traffic related contaminants in tunnel wash water runoff.

Functional differently expressed GO terms at 38 h and 86 h related to biological processes and molecular functions are depicted in Fig. 2 and Fig. 3, while the corresponding figures for cellular compartments are depicted in Supplemental Fig. S1. For clarity, only GO terms at level 4 (biological process) and 3 (molecular function) were displayed. However, a selection of GO terms and genes of special interest was identified (Tables 3 and 4) to aid the interpretation of the data. A complete presentation of all significantly expresses GO terms are provided elsewhere (Supplemental tables S2 – S7).

After 38 h there was an overrepresentation of down-regulated genes linked to various metabolic processes (Fig. 2 and Table 3). More detailed, this included several processes related to the immune system such as down-regulation of immune system process and adaptive immune response. Looking at the gene transcription level, these GO terms involved an apparent down-regulation of genes coding for the signaling protein Sam and sh3 domain containing 3 and the lectin fucolectin-4 precursor. Similar to several of the immunological associated functions, GO functions related to the biosynthesis of lipids and steroids were significantly down-regulated in exposed fish at time 38 h (Fig. 2 and Table 3). As an example, the major metabolic intermediates and the enzymes involved in the cholesterol biosynthesis pathway were affected (Fig. 4). Several enzymes involved in this pathway were apparently largely inhibited, e.g. mevalonate decarboxylase and squalene epoxidase.

The exposure of brown trout to traffic related contaminants caused an evident overrepresentation of GO terms involving chemotaxis and cytokine activity (e.g. locomotory behavior and protein binding) (Fig. 2 and Table 3). The by far most apparently up-regulated gene, in this respect, was that coding for the cytokine (signaling molecule) interleukin-8 (IL-8). In addition, cell communication and transmembrane- and substrate specific transporter activity involving terms such as symporter activity, notch binding and regulation of synaptic plasticity transporter activity were up-regulated in exposed fish. Examples of genes within these GO-terms were the solute carrier family 6 (neurotransmitter GABA (γ-aminobutyric acid)) members 13 and complexin 4 precursor.

Although 19 of the significantly expressed GO functions at 38 h also appeared significant at 86 h, it seemed to be a shift in processes during the recovery period from those linked to sensing and

immunity towards those involved in xenobiotic biotransformation and redox conditions (Fig. 3 and Table 4). GO terms of special interest after 86 h were the overrepresentation of processes associated with xenobiotic stimulus, glutathione metabolic process (e.g. sulfur metabolic process), oxidoreductase activity and cell redox homeostasis (e.g. homeostatic process). Several classical biomarker genes were found to be apparently up-regulated in these GO terms, although not necessary being identified as differentially expressed by the two-way ANOVA, including cytosolic sulfotransferase 3 (SULT), microsomal glutathione S-transferase 3 (GST), CYP1A1 and thioredoxin domain-containing protein 14 precursor.

4 Discussion

In Meland et al. (2010a) it was documented that the tunnel wash water contained high concentrations of trace metals, PAHs and road salt (measured as Cl), and that this cocktail of contaminants affected the exposed fish several hours after the acute exposure (4 h). This were manifested by a rapid alteration in blood physiology (e.g. plasma ions and glucose) and a subsequent up-regulation of the planar aromatic hydrocarbon biomarker CYP1A and the oxidative stress related biomarkers thioredoxin (TRX) and γ-glutamylcysteine synthetase (GCS) in liver of exposed fish. The blood variables recovered back to control levels within 38 h, while the hepatic biomarkers did not reach their maximum level until 86 h.

The key objective of the present paper was to potentially disclose effects on biological functions and pathways which were undetected by the means of blood physiology measurements and hepatic mRNA transcription of a selection of biomarkers previously conducted by qPCR (Meland et al., 2010a). To extend the mechanistic understanding of sublethal effects in brown trout exposed to traffic related contaminants in tunnel wash water runoff a high-content transcriptomic screening approach using a high density oligoarray was introduced. The oligoarray that was used for the analysis is a custom, off the shelf product, where annotations were enriched by a combination of the effort by the cGRASP consortium and additional annotation enrichment by a local installation of Blast2GO. Another important aim in the present paper, was to potentially disclose effects on biological functions and pathways which were undetected by the means of blood physiology measurements and hepatic mRNA transcription of a selection of biomarkers previously conducted by qPCR. Hence, the focus was more on functional pathways rather than on single gene expression. In fact, several studies have advocated the use of functional pathways instead of focusing on individual genes as they do not operate alone in the cell, but in a sophisticated network of interactions (e.g. Al-Shahrour et al., 2006; Luebke et al.,

294 2006). This is mainly due to two reasons; firstly, the expression of individual genes may vary from
295 case to case, however, alterations in the pathways in which these genes are involved are usually
296 consistent, and secondly; when the transcriptional changes are minimal or moderate (Olsvik et al.,
297 2008), e.g. pollution episodes of modest character, single gene expression may be confounded by
298 temporal variation in responses.

299 The outcome of the microarray study revealed that the traffic related contaminants caused a
300 plethora of molecular changes in liver of acutely exposed fish several hours after the exposure.
301 Perhaps the most pronounced effect observed at time 38 h, was the inhibition of GO functional
302 terms related to lipid metabolic processes including biosynthesis of cholesterol steroids. Toxic
303 effects on the cholesterol biosynthesis in mammals have been linked to exposure of organic
304 toxins such as pyrethroids, organophosphates (OPs) and dioxins (Fletcher et al., 2005; Sato et al.,
305 2008; Elhalwagy and Zaki, 2009), and to Cu (Huster et al., 2007).

306 In the present study, several genes encoding for enzymes involved in the cholesterol biosynthesis
307 pathway were apparently strongly inhibited, e.g. isopentenyl-diphosphate delta isomerase,
308 mevalonate decarboxylase and 7-dehydrocholesterol reductase. In addition, squalene epoxidase
309 which is the main precursor of the steroid biosynthesis were strongly inhibited (Fig. 4). A similar
310 inhibition of genes involved in the biosynthesis of cholesterol, measured in liver of Cu exposed
311 stickleback (Gasterosteus aculeatus), has recently been published by Santos et al. (2010).
312 Cholesterol, which in vertebrates is produced mainly in the liver, is important for various cellular
313 processes including membrane stability, steroid hormone production and bile acid biosynthesis.
314 In fact, the inhibition of the cholesterol biosynthetic pathway in exposed fish seemed to
315 subsequently affect the bile acid biosynthesis, as an apparently strong inhibition of the CYP7A1
316 enzyme, which catalyzes the conversion of cholesterol into bile acid (Davis et al., 2002), was
317 observed after 86 h. In a toxicological context, the inhibition of CYP7A1 may indicate that the
318 exposed fish could also experience problem with detoxification, as bile acid synthesis and
319 secretion are vital for the elimination of endogenous metabolic byproducts and xenobiotics
320 (Hinton et al., 2008).

321 Cu is one of the major contaminants in highway runoff, and the total concentration in the
322 present study (130 μg/L, Table 1) was within the same range causing suppression of the
323 cholesterol pathway in stickleback (Santos et al., 2010). Cu together with other metals
324 accumulated rather rapidly onto the gills of exposed fish (Meland et al., 2010a). Although no
325 differences in the concentrations of Cu and other trace elements in liver of exposed and

unexposed fish were observed (Table 2), it cannot be totally excluded that Cu crossed the gill membrane and potentially interfered with the cholesterol and steroid pathways.

Similar to Cu, there is evidence that also organophosphorus compounds (OPs) may inhibit the biosynthesis of cholesterol (Elhalwagy and Zaki, 2009). OPs appear to be abundant in urban runoff water and snow banks along roads due to their presence in flame retardants, plasticizers and additives in lubricants and hydraulic fluids (Marklund et al., 2005; Regnery and Puttmann, 2010). Their presence, although not measured in the present study, may thus have caused the observed suppression of the cholesterol biosynthesis. This hypothesis is, in fact, supported by the apparent up-regulation of the serum paraoxonase/arylesterase 2 (PON) enzyme at time 86 h (expressed in the GO xenobiotic stimulus, Table 4). PON is essential in the detoxification of OPs (Costa et al., 2005), and is currently considered as the main protector against OP mediated neurotoxicity (Goswami et al., 2009). Hence, the inhibition of the cholesterol pathway together with an apparent up-regulation of serum PON in exposed fish in the present study may thus indicate the presence of OP compounds originating from oil spill/leakage from vehicles. In addition, the observed inhibition may be an early stage in toxicity related to both metal and organic pollutants present in highway and tunnel wash water runoffs, which subsequently could impair the overall health of aquatic organisms.

The immune system is normally divided into an innate- and an adaptive component, whereas the former include cell- and humoral mediated responses, the latter includes all the defense mechanisms that are present prior to the appearance of pathogens including initiating of inflammation (Zelikoff, 1998; Reynaud et al., 2008). The very notable, although modest, down-regulation of several immunological processes at time 38 h, included both genes associated to the innate immune system and the adaptive immune system. Suppression of the immune system caused by various organic toxins has previously been observed in fish (Dunier and Siwicki, 1993; Carlson et al., 2004a; Carlson et al., 2004b; Koskinen et al., 2004; Krasnov et al., 2005; Mos et al., 2008; Nakayama et al., 2008). For example, Carlson et al. (2004a) demonstrated that the PAH compound benzo(a)pyrene reduced the lymphocyte proliferation, phagocyte-mediated superoxide generation and antibody-forming cell (AFC) in Japanese medaka (Oryzias latipes). They concluded that the suppression was mediated by the catalytic production of reactive PAH metabolite by the CYP1A biotransformation enzyme, rather than the parent compound. A similar immunomodulatory mechanism would likely also be present in the current study, as the tunnel wash water contained high concentrations of several PAHs (e.g. pyrene, benzo(a)pyrene, fluoranthene) and exposed fish had induced levels of hepatic CYP1A mRNA transcription

(Meland et al., 2010a). In addition to the suppression of the humoral immune system, Krasnov et al. (2005) observed down regulation of several genes involved in the genetic apparatus of the cell in liver of juvenile rainbow trout (Oncorhynchus mykiss) exposed to sublethal doses (25 – 100 μg/L) of pyrene for 4 days. The pyrene concentrations in that study were quit high compared to the concentrations in the present study (max. 0.9 μg/L). However, the findings in the present study where gene expression related processes such as RNA processing and splicing and DNA dependent transcription was down-regulated in exposed fish (see Fig. 2A and Supplemental Table S2) were coherent with those presented by Krasnov et al. (2005).

Although several immunological processes were suppressed in the exposed fish after 38 h, genes associated to chemotaxis and cytokine activity were apparently up-regulated (e.g. interleukin-8 (IL-8) as well as tumor necrosis factor receptor superfamily member 11B precursor (TNF)) indicating that the immune system was only partly inhibited. Cytokines, which are produced and secreted from cells of the immune system as well as from non-immune cell types, are mediating chemotaxis in the innate immune system and is essential in inflammatory responses (Engelsma et al., 2002). For example, it is reported that IL-8 and TNF stimulate chemotaxis of neutrophiles which have phagocytotic function (Nakayama et al., 2008). In addition, they are involved in hypersensitivity, i.e. responding to irritants, allergens etc (Luebke et al., 2006). Similar to the present study, Nakayama et al. (2008) observed alterations in several immunological processes together with increased cytokine activity in Japanese flounder (Paralichthys olivaceus) exposed to heavy oil for 3 days and sampled 4 days post exposure. They argued that the heavy oil exposure suppressed pathogen resistance causing bacterial growth and infections in exposed fish, which the immune system subsequently reacted upon. This may be a plausible explanation also in the present study. However, taking into account the rather short time frame between exposure and sampling in the present study, a more likely explanation for the inflammation may be that gill epithelia cells were physically irritated due to the highly turbid tunnel wash water. Additionally, inflammation may have been triggered by gill reactive metals such as Al, Co, Cu, Fe, Pb and Sb, being able to cause structural damages (Evans, 1987; Rosseland et al., 1992).

Along with the up-regulation of chemotaxis and cytokines, the exposed fish also showed up-regulation of processes related to cell signaling and communication. These processes included e.g. notch binding and the activity of various symporter proteins localized in cell membranes (e.g. organic acid: sodium symporter and cation: amino acid symporter). The symporter activity involved genes coding for GABA (Table 3) which is an inhibitory neurotransmitter causing opening of ion channels. GABA is widely distributed in nonneural tissue, including liver

(Tillakaratne et al., 1995; Erlitzki et al., 2000), and is not restricted to synaptic transmission but may also be a tool of intercellular communication (Erdö and Wolff, 1990). Interestingly, this was also observed in the previous discussed study by Nakayama et al. (2008) exposing Japanese flounder to heavy oil. In addition, there was an overrepresentation of up-regulated genes associated to synaptic plasticity and transmission of nerve impulses in exposed fish. These findings may imply that the tunnel wash water had an unspecific effect on cell communication lasting several hours after the exposure. Such influence might oppose a threat to fish if these changes reduce their mobility and predator avoidance, thus playing an ecological significant role.

Nevertheless, the up- and down-regulation of various immunological processes together with increased expression of cell communication and signaling at 38 h may be the initial responses to alterations of the surrounding environment (e.g. pollution). They are without doubt essential in maintaining normal homeostasis, and alterations of such responses are normally observed prior to the more classical biomarkers for chemical stress and toxicity (Carlson and Zelikoff, 2008). This is in line with the results obtained from the present exposure study, as at time 86 h the initially observed molecular responses related to immunity and cell signaling were no longer of significance. Instead, processes related to biotransformation and cellular redox homeostasis were more evident in the exposed fish. In addition, there was a marked decrease in significantly expressed GO terms between the two time points. This may in fact suggest that early and initial responses in exposed fish (e.g. suppression of immunological processes and cholesterol biosynthesis) were followed by subsequent compensatory mechanisms mitigating the effects of traffic related contaminants.

One of the significantly up-regulated functional terms in the exposed fish at time 86 h was response to xenobiotic stimulus. This term included mRNA coding for cytosolic sulfotransferase 3 (SULT) which is a so-called phase II enzyme involved in conjugation of xenobiotics including carcinogenic PAH metabolites (Shimada, 2006), and glutathione peroxidase 1 (GPX). SULT was also one of the significantly expressed genes in the two-way ANOVA (Supplemental Table S1). Finally, this GO functional group contained apparently increased transcriptions of the genes serum PON and arsenite methyltransferase (AMT). The former takes, as previously discussed, part in the detoxification of OPs, while the latter being part of the biotransformation process of inorganic metalloid arsenic (As). Interestingly, most of the As in the exposure water was present as dissolved species but no accumulation were observed, neither on gills (Meland et al., 2010a) nor in liver (Table 2). As earlier discussed, the lack of accumulation of metals in the liver does not necessarily exclude the presence of bioreactive As. Alternatively, Sb which have many of the

425 similar chemical and toxicological properties as As accumulated on gills of exposed fish.
426 Although the methylation of Sb is low (Gebel, 1997), at least in mammals, an appealing thought
427 could be that the AMT also is involved in the detoxification of Sb. Hence, a combination of these
428 two metalloids, acting in a additive or synergistic fashion, could have triggered the AMT enzyme
429 in the present study.

430 Glutathione peroxidase (GPX) is an essential enzyme in the antioxidant defense system, and is
431 beside catalase, the main pathway for detoxifying H_2O_2 (hydrogen peroxide) caused in the redox
432 cycling of free radicals. In addition, GPX is crucial in terms of protecting membranes against
433 lipid peroxidation (van der Oost et al., 2003). Increased hepatic mRNA transcriptions of GPX
434 linked to oxidative stress has previously been documented in brown trout living in metal polluted
435 areas (Hansen et al., 2006). The apparent induction of GPX in exposed fish may thus indicate
436 increased reactive oxygen species (ROS) production in the liver of exposed fish. The presence of
437 ROS in exposed fish is further supported by the up-regulation of other glutathione associated
438 genes in the GO function glutathione metabolic processes. Genes associated with the glutathione
439 (GSH) rate limiting enzyme GCS (i.e. glutamate cysteine ligase regulatory- and catalytic subunits)
440 (the 7^{th} and 11^{th} most regulated genes in the GO glutathione metabolic processes (not shown))
441 and microsomal glutathione S-transferase 3 (GST) are examples in this respect (Table 4). GSH is
442 the most abundant cellular antioxidant preventing oxidation of protein thiol groups either directly
443 by reacting with ROS or indirectly through the GST pathway (Limon-Pacheco and Gonsebatt,
444 2009). This is also in line with the increased up-regulation of GCS in exposed fish measured by
445 qPCR (Meland et al., 2010a), and verifies the microarray analysis performed in the present study.

446 Genes having protective properties against oxidative stress were also present in the functional
447 term oxidoreductase activity and cell redox homeostasis, e.g. TRX, TRX-reductase and TRX
448 domain containing protein 14 precursor. However, only the latter was among the five most
449 regulated genes (Table 4). Similar to GCS, TRX was, according to the qPCR analysis, found to be
450 up-regulated in tunnel wash water exposed fish (Meland et al., 2010a). This is also in line with a
451 recent published study by Mos et al. (2008), who observed that TRX was up-regulated in rainbow
452 trout upon diesel exposure. TRX contributes to the cell antioxidant defense not only due to their
453 capability to repair the catalytic activity of peroxiredoxins and GPXs, decomposing
454 hydroperoxides and H_2O_2, but also by directly reducing both H_2O_2 and oxidized glutathione
455 (GSSG) (Kalinina et al., 2008). In addition, glutathione peroxidases, lipid hydroperoxides and
456 H_2O_2 can be directly reduced by TRX-reductase posing an alternative pathway of enzymatic
457 detoxification of lipid hydroperoxides (Kalinina et al., 2008). In addition to be essential in the

antioxidant defense system, TRX proteins are involved in several functions such as cell viability, cell cycle and antiapoptotic mechanisms.

Several different contaminants, commonly found in polluted highway runoff (e.g. trace metals and PAHs), are well known inducers of oxidative stress. For example, increased ROS generation may appear through Fenton chemistry involving transition metals such as Cu and Fe (Halliwell and Gutteridge, 2007). In addition, Fenton chemistry is believed to be involved in ROS generation during activation of the CYP1A metabolic pathway when PAH quinones are produced during redox cycling of the parent PAH (Flowers et al., 1997; Xue and Warshawsky, 2005). Finally, ROS may be produced by uncoupling of the electron transfer and O_2 reduction during the CYP1A biotransformation cycle (Di Giulio and Meyer, 2008). Hence, the coherent up-regulation of antioxidant defense mechanisms and the increased expression of CYP1A1 mediated by the aryl hydrocarbon receptor (AhR), pose strong evidence of uptake of PAHs or other organic contaminants in exposed fish. For example, Stephensen et al. (2003) revealed activation of the CYP1A system along with increased oxidative stress due to leakage of PAHs and aromatic nitrogen compounds from tires. Additionally, this suggests that ROS production was elevated during biotransformation and detoxification of PAHs or other organic contaminants, rather than caused by uptake of transition metals. A rather sharp decline in gill metal concentrations after the tunnel wash exposure and a unchanged hepatic mRNA transcription of the metal binding protein metallothionein (MT-A) (Meland et al., 2010a), may further strengthen this theory. In addition, the inflammatory response manifested by e.g. IL-8 and TNF induction in exposed fish at 38 h, may have indirectly contributed to oxidative stress as neutrophiles through the "respiratory burst" produces large quantities of superoxide (Fatima et al., 2000; Luster et al., 2001). For example, Fatima et al. (2000) showed that pollution induced over-activation of phagocytes caused peroxidative damage in fish tissue, a typical effect of ROS (van der Oost et al., 2003).

5 Conclusion

In summary, the short term sub-lethal exposure (4 h) of brown trout to traffic related contaminants caused a plethora of previously undetected molecular changes several hours after the exposure (i.e. during recovery). At time 38 h the most notably changes were the down regulation of several immunological processes, including genes related to the innate and the adaptive system. In addition, several enzymes in the cholesterol biosynthesis were apparently strongly inhibited. After 86 h these initial responses triggered were subsequently followed by an up-regulation of genes involved in xenobiotic biotransformation and redox conditions. Several classical genes such as CYP1A1, CYP1B1, SULT and GST were expressed indicating that

oxidative stress was induced in the exposed fish. The observed responses were most likely caused by PAH or other organic micro pollutants present in the exposure water. In that respect, the apparent up-regulation of the enzyme PON was interesting as it is essential in detoxifying OPs, and may therefore indicate the presence of OPs in the runoff water. In addition, the apparent up-regulation of the metalloid specific enzyme AMT may indicate that As and Sb were taken up and metabolized in liver of exposed fish, despite that no metal accumulation was observed in liver.

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7 Tables

Table 1. Water quality variables measured in tap water and tunnel wash water during the exposure experiment (mean ± SEM).

Variable	Unit	Tap water (n=3 – 4) mean ± SEM	Tunnel wash water (n=3) mean ± SEM
TOC	mg/L	2.9 ± 0.02	8.9 ± 2.9
DOC	mg/L	2.9 ± 0.06	7.7 ± 3.2
Ca	mg/L	19.7 ± 0.03	41.0 ± 8.0
K	mg/L	2.5 ± 0.01	9.6 ± 4.0
Mg	mg/L	2.8 ± 0.01	9.2 ± 2.1
Na	mg/L	22.3 ± 0.25	491 ± 219
Mn	mg/L	0.006 ± 0.0001	0.35 ± 0.1
Cl	mg/L	23.0 ± 0.04	804 ± 240
Sulphate	mg/L	36.9 ± 0.18	33.8 ± 2.5
Nitrate	mg/L	1.4 ± 0.00	1.2 ± 0.11
Hardness	mg/L	60.8 ± 0.1	140.0 ± 29
Al-Tot	µg/L	48.8 ± 3.2	8103 ± 1922
As-Tot	µg/L	0.3 ± 0.01	3.9 ±1.7
Ba-Tot	µg/L	15.8 ± 0.2	132.1 ± 42.6
Cd-Tot	µg/L	0.01 ± 0.00	0.26 ± 0.11
Co-Tot	µg/L	0.1 ± 0.00	14.1 ± 5.8
Cr-Tot	µg/L	0.6 ± 0.04	36.1 ± 15.5
Cu-Tot	µg/L	1.5 ± 0.4	129.8 ± 54.7
Fe-Tot	µg/L	82.6 ± 5.7	12063 ± 3449
Ni-Tot	µg/L	1.0 ± 0.01	28.1 ± 11.8
Pb-Tot	µg/L	0.02 ± 0.01	16.4 ± 6.6
Sb-Tot	µg/L	0.1 ± 0.00	9.6 ± 2.3
V-Tot	µg/L	0.3 ± 0.01	31.4 ± 12.7
Zn-Tot	µg/L	3.7 ± 1.4	701 ± 296
16PAH	µg/L	n.d.	2.2 ± 0.5
THC	mg/L	n.d.	4517 ± 838
Non-ionic tensides	mg/L	<0.30	1.1
Cationic tensides	mg/L	<0.20	<0.20
Anionic tensides	mg/L	<0.20	<0.20

692 Table 2. Trace metal concentrations in liver (µg/g dw) of brown trout (mean ± S.E.M. (S.E.M. given
693 below the mean value), n = 1 – 6). The microarray analyses were performed with liver samples
694 obtained at time 38 and 86 h.

Var/Group	Control						Exposed					
Time (h)	-3	1	3	14	38	86	-3	1	3	14	38	86
Al-liver	1.0	2.1	4.1	0.9	1.1	3.6	4.2	1.1	2.3	1.3	2.4	1.0
	0.1	0.5	1.8	0.1	0.2	1.7	2.8	0.2	0.8	0.1	-	0.1
As-liver	2.6	2.3	2.8	2.4	3.2	3.2	2.6	2.7	3.0	2.6	2.7	2.6
	0.1	0.1	0.2	0.1	0.3	0.2	0.1	0.1	0.2	0.3	0.1	0.1
Ba-liver	0.06	0.07	<LOD	0.06	<LOD	<LOD	0.09	0.05	0.10	0.08	<LOD	0.06
	0.02	0.01	-	0.03	-	-	0.03	0.01	0.04	0.02	-	0.02
Cd-liver	0.13	0.13	0.12	0.14	0.14	0.14	0.13	0.13	0.14	0.13	0.14	0.13
	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.01	0.01
Co-liver	0.14	0.12	0.14	0.14	0.14	0.14	0.13	0.14	0.14	0.14	0.13	0.14
	0.008	0.007	0.008	0.010	0.004	0.006	0.008	0.009	0.004	0.007	0.006	0.008
Cr-liver	0.18	0.19	0.35	0.25	0.19	0.19	0.26	0.32	0.12	0.20	0.48	0.18
	0.01	0.02	0.10	0.04	0.01	0.01	0.06	0.14	0.02	0.02	0.23	0.01
Cu-liver	269	289	196	309	341	276	296	181	301	326	347	269
	44	35	16	29	112	47	31	26	46	63	90	44
Fe-liver	141	142	136	177	162	136	157	132	158	131	160	141
	17	12	7	15	19	16	20	13	20	14	22	17
Ni-liver	0.03	0.03	0.12	0.06	0.08	0.07	0.04	0.03	0.03	0.02	0.49	0.03
	0.004	0.005	-	0.033	-	-	0.015	0.006	0.002	0.002	-	0.004
Pb-liver	0.002	0.004	<LOD	0.002	<LOD	<LOD	0.004	0.003	<LOD	0.003	<LOD	0.002
	0.000	0.001	-	0.000	-	-	0.001	0.001	-	0.001	-	0.000
Sb-liver	<LOD	<LOD	0.003	<LOD	0.002	0.003	0.003	<LOD	0.001	<LOD	<LOD	<LOD
	-	-	0.001	-	-	0.001	0.002	-	0.000	-	-	-
V –liver	0.30	0.31	0.38	0.33	0.41	0.41	0.37	0.34	0.42	0.41	0.40	0.30
	0.07	0.06	0.03	0.06	0.05	0.05	0.05	0.04	0.04	0.06	0.03	0.07
Zn-liver	103	95	110	102	105	106	102	96	106	102	114	103
	4	4	6	2	5	4	2	4	2	5	6	4

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Table 3. The 5 most regulated genes (fold change) in some important significantly expressed GO terms at time 38 h. The first column shows example of GO terms at level 3 and 4 and displayed in fig 2, while the second column gives examples of related GO terms at lower levels (complete GO lists are presented in Supplemental Table S2 – S3).

GO-term (level 3 - 4)	GO-term (level < 3 – 4)	Gene Id	Gene name	Fold change (38h)	Fold change (86h)
Cellular biosynthetic process	Steroid biosynthetic process	A_05_P424717	Squalene epoxidase	-7.3	-1.4
Lipid metabolic process	Lipid biosynthetic process	A_05_P460182	Inositol polyphosphate-5-phosphatase e	-4.3	1.4
Cellular alcohol metabolic process		A_05_P382912	Squalene synthetase	-4.1	1.1
		A_05_P365037	Acyl carrier protein, mitochondrial precursor	-3.6	1.4
		A_05_P490877	Mevalonate decarboxylase	-3.6	1.3
regulation of immune system process	Positive regulation of adaptive immune response	A_05_P391212	Sam and sh3 domain containing 3	-1.6	1.1
		A_05_P460497	Fucolectin-4 precursor	-1.6	-1.1
regulation of response to stimulus	Regulation of humoral immune response	A_05_P274954	Fucolectin-6 precursor	-1.5	1.5
Regulation of humoral immune response		A_05_P265764	Sam and sh3 domain containing 3	-1.4	1.2
		A_05_P331107	Complement factor D precursor	-1.3	1.0
Adaptive immune response					
Locomotory behavior	Chemotaxis	A_05_P286337	Interleukin-8	39.1	1.3
Protein binding	Cytokine activity	A_05_P358487	Chemokine (C-C motif) ligand 20	2.7	1.5
		A_05_P401667	Dedicator of cytokinesis protein 2	2.6	1.2
		A_05_P354192	Family with sequence similarity member c	2.4	1.3
		A_05_P372392	Tumor necrosis factor receptor superfamily member 11B precursor	2.0	2.1
Neurological system process	Organic acid: sodium symporter activity	A_05_P451902	Solute carrier family 6 (neurotransmitter gaba) member 13	3.4	1.3
Cell-cell signaling	Notch binding	A_05_P446267	P3 protein	3.2	3.5
Transmembrane transporter activity	Regulation of neurological process	A_05_P455192	Delta and Notch-like epidermal growth factor-related receptor precursor	3.0	1.2
Substrate-specific transporter activity	Regulation of synaptic plasticity				
		A_05_P466742	Complexin-4 precursor	2.6	1.9
		A_05_P304262	Calcium calmodulin-dependent protein kinase ii inhibitor 2	1.7	1.1

Table 4. The 5 most regulated genes (fold change) in some important significantly expressed GO terms at time 86 h. The first column shows example of GO terms at level 3 and 4 and displayed in fig 2, while the second column gives examples of related GO terms at lower levels (complete GO lists are presented in Supplemental Table S2 – S3).

GO-term (level 3 - 4)	GO-term (level < 3 – 4)	Gene Id	Gene name	Fold change (38h)	Fold change (86h)
Xenobiotic stimulus	Xenobiotic stimulus	A_05_P493012	Cytosolic sulfotransferase 3	8.4	2.9
		A_05_P387007	Serum paraoxonase/arylesterase 2	1.4	2.7
		A_05_P448722	Arsenite methyltransferase	1.1	2.3
		A_05_P367057	Glutaryl-CoA dehydrogenase, mitochondrial precursor	1.1	2.2
		A_05_P389542	glutathione peroxidase 1	1.0	2.0
Sulfur metabolic process	Glutathione metabolic process	A_05_P374972	Microsomal glutathione S-transferase 3	1.2	3.5
Cofactor metabolic process		A_05_P388822	Glutathione peroxidase 3 precursor	1.0	2.5
		A_05_P251384	Glutathione transferase omega-1	1.6	2.4
		A_05_P489127	Glutathione synthetase	1.4	2.0
		A_05_P364752	Glutathione s-transferase	1.2	1.9
Oxidoreductase activity	Oxidoreductase activity	A_05_P249154	Cytochrome P450 1A1	5.9	7.9
		A_05_P327962	Cytochrome P450 7A1	1.3	-7.3
		A_05_P333662	Short chain dehydrogenase reductase family member 5	1.5	5.0
		A_05_P265789	Oxidoreductase domain-containing protein	1.4	4.3
		A_05_P424002	Cytochrome P450 1B1	26.1	3.4
Homeostatic process	Cell redox homeostasis	A_05_P458342	DNA-(apurinic or apyrimidinic site) lyase	-1.8	3.0
Regulation of cellular process		A_05_P469457	thioredoxin domain-containing protein 14 precursor	2.8	2.9
		A_05_P345937	nebulette (actin-binding z-disk protein)	1.1	2.9
		A_05_P451837	chromosome 17 open reading frame 37	1.7	2.8
		A_05_P368740	sh3 domain binding glutamic acid-rich protein like 3	2.0	2.8

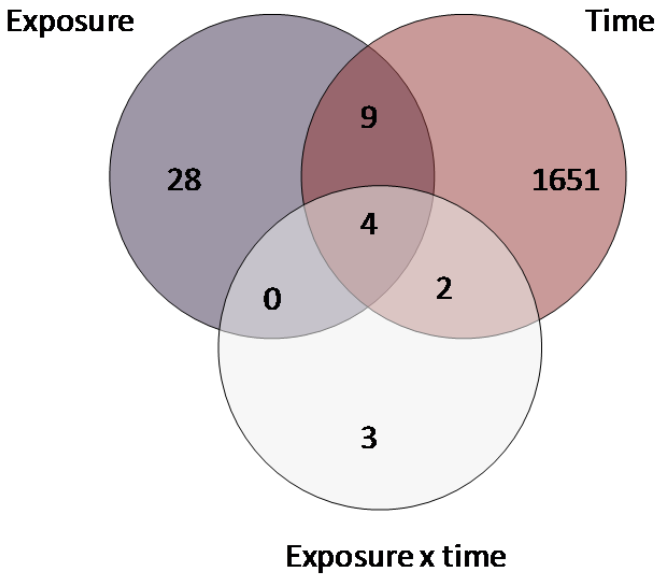
8 Figure caption

Figure 1. A Venn diagram showing the outcome of the two-way ANOVA on expressed genes using time and exposure as factors. Significantly different expressed genes between exposed and unexposed brown trout are listed in Supplemental Table 1.

Figure 2. Significantly expressed GO terms that were regulated after 38 h in brown trout exposed to 4 h of traffic related contaminants. A) biological process, and B) molecular process. Cellular components are displayed in Supplemental Fig S1. White and black bars represent down-regulated and up-regulated GO terms, respectively.

Figure 3. Significantly expressed GO terms that were regulated after 86 h in brown trout exposed to 4 h of traffic related contaminants. A) biological process, and B) molecular process. Cellular components are displayed in Supplemental Fig S1. White and black bars represent down-regulated and up-regulated GO terms, respectively.

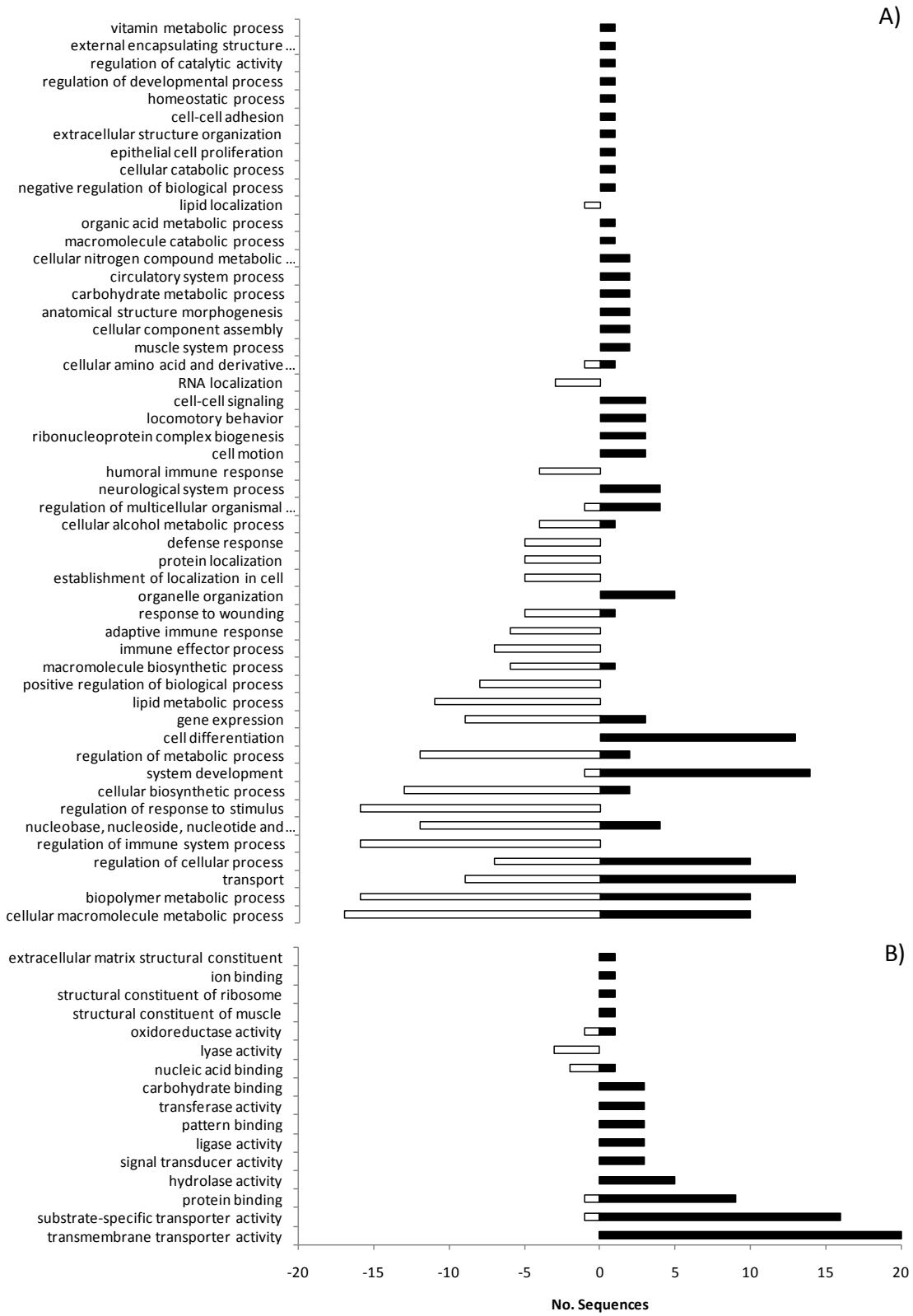
Figure 4. The fold change of down-regulated genes (→) involved in the cholesterol biosynthesis pathway after 38 h in brown trout exposed to 4 h of traffic related contaminants. These genes were expressed in significantly down-regulated GO terms such as cellular biosynthetic process, lipid metabolic process and cellular alcohol metabolic process (see also Fig. 2 and Supplemental Table S2). Acetyl-CoA acetyltransferase was in addition found to be significant in the two-way ANOVA.



723

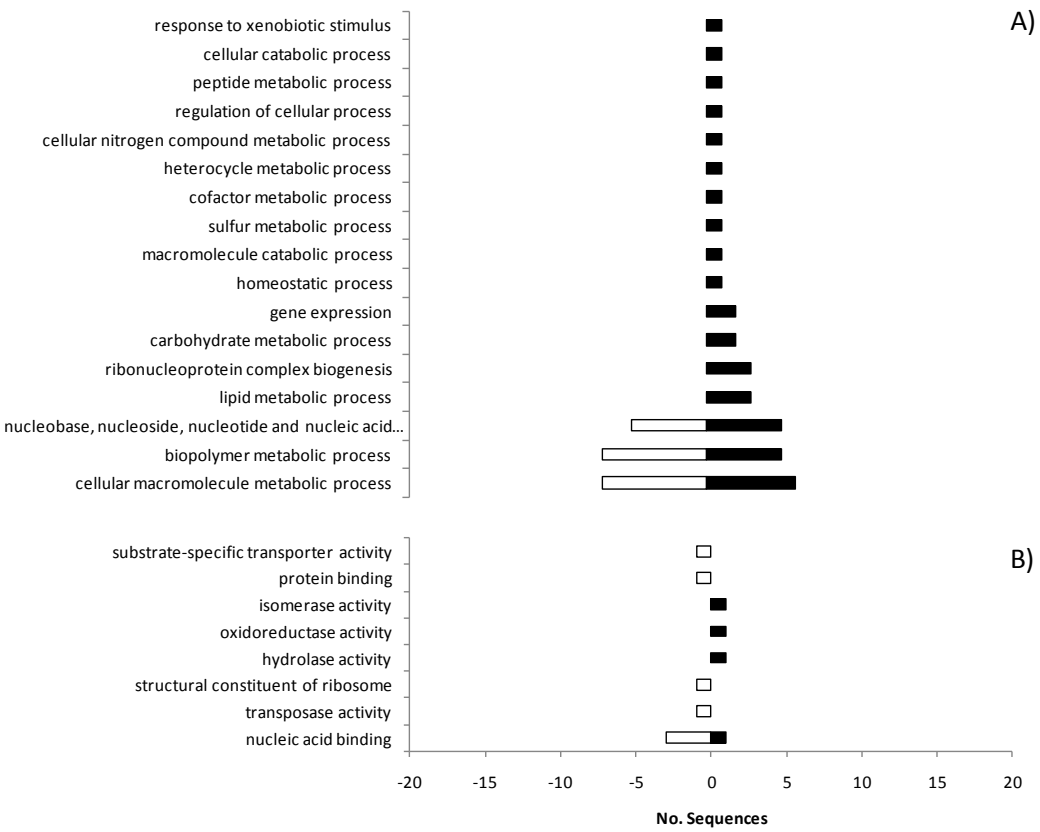
724 **Fig. 1** A Venn diagram showing the outcome of the two-way ANOVA on expressed genes using time
725 and exposure as factors. Significantly different expressed genes between exposed and unexposed
726 brown trout are listed in Supplemental Table 1.

727



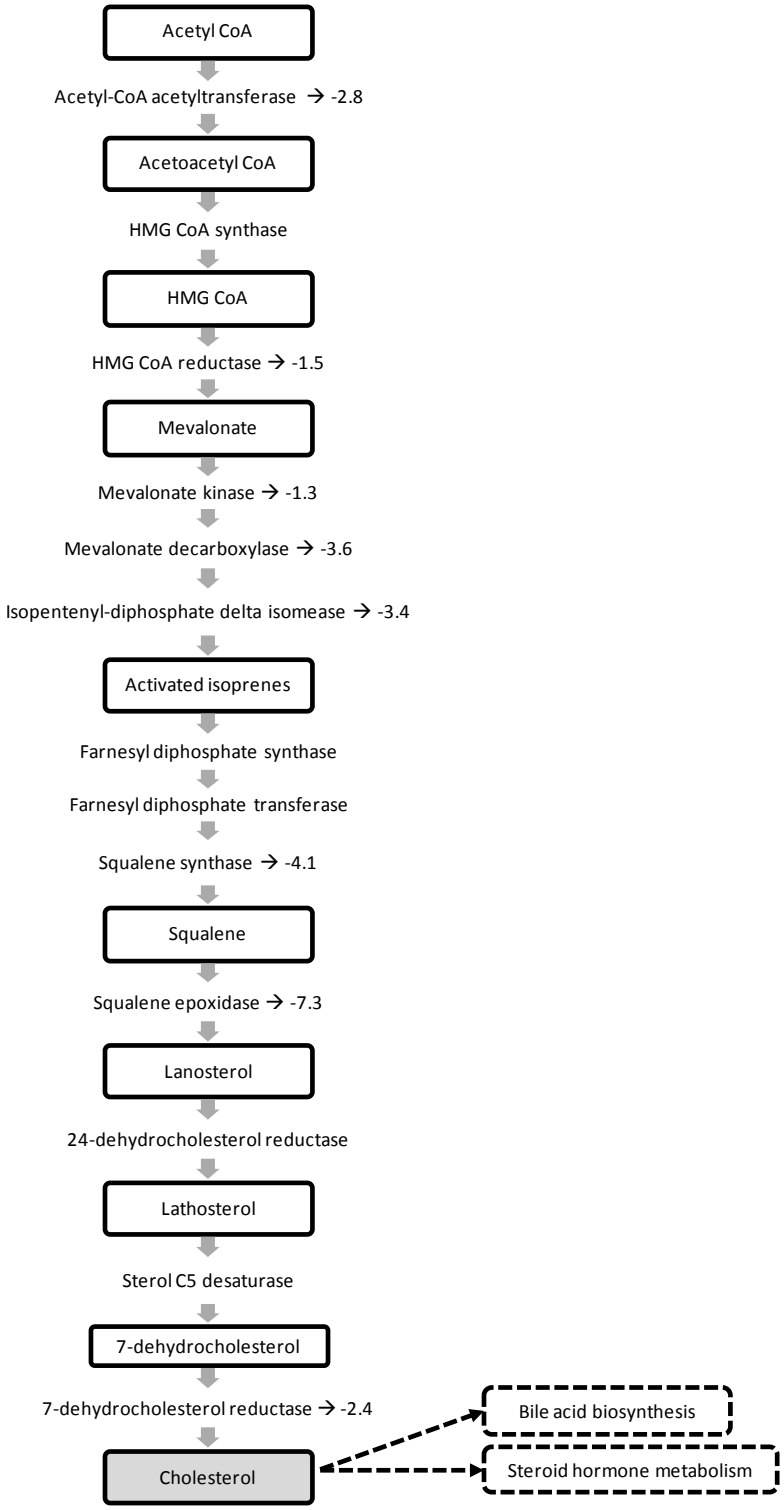
728

729 **Figure 2.** Significantly expressed GO terms that were regulated after 38 h in brown trout exposed to
730 4 h of traffic related contaminants. A) biological process, and B) molecular process. Cellular
731 components are displayed in Supplemental Fig S1. White and black bars represent down-regulated
732 and up-regulated GO terms, respectively.



733

734 **Figure 3.** Significantly expressed GO terms that were regulated after 86 h in brown trout exposed to
735 4 h of traffic related contaminants. A) biological process, and B) molecular process. Cellular
736 components are displayed in Supplemental Fig S1. White and black bars represent down-regulated
737 and up-regulated GO terms, respectively (Supplemental Table S1).



738

739 **Figure 4.** The fold change of down-regulated genes (→) involved in the cholesterol biosynthesis
740 pathway after 38 h in brown trout exposed to 4 h of traffic related contaminants. These genes were
741 expressed in significantly down-regulated GO terms such as cellular biosynthetic process, lipid
742 metabolic process and cellular alcohol metabolic process (see also Fig. 2 and Supplemental Table S2).
743 Acetyl-CoA acetyltransferase was in addition found to be significant in the two-way ANOVA.
744 (Supplemental Table S1).

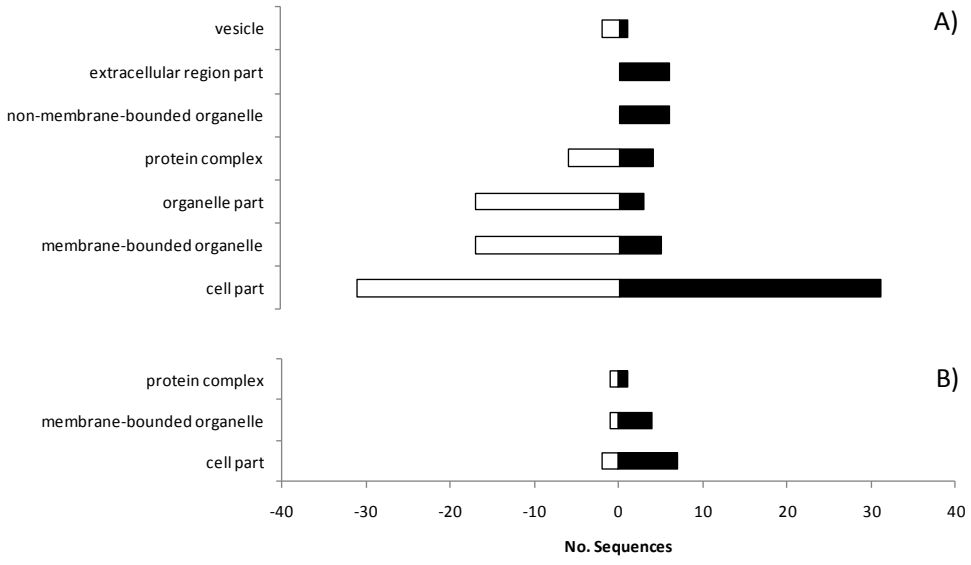
745

9 Supplementary material

Supplemental Figure S1. Significantly expressed GO terms related to cellular components that were regulated after 38 h (A) and 86 h (B) in brown trout exposed to 4 h of traffic related contaminants. White and black bars represent down-regulated and up-regulated GO terms, respectively.

Supplemental Table S1. The regulation of genes after 38 h and 86 h in liver of brown trout exposed to 4 h of traffic related contaminants. Only those genes significantly expressed as a function of exposure are displayed (two-way ANOVA, $p<0.05$).

Supplemental Table S2 – S7. The significantly expressed GO terms (biological process, molecular function and cellular component) in liver of brown trout exposed to 4 h of traffic related contaminants at time 38 h (biological process (S2), molecular function (S3) and cellular component (S3)) and 86 h (biological process (S4), molecular function (S5) and cellular component (S6)). The two first columns on the left hand side show the GO term id and GO-term level (3 and 4) used in Fig. 2 and Fig. 3 in the paper. The last two columns show the down- and up-regulation of various GO terms within the GO-terms at level 3 and 4.



Supplemental Figure S1. Significantly expressed GO terms related to cellular components that were regulated after 38 h (A) and 86 h (B) in brown trout exposed to 4 h of traffic related contaminants. White and black bars represent down-regulated and up-regulated GO terms, respectively.

Supplemental Table S1. The regulation of genes after 38 h and 86 h in liver of brown trout exposed to 4 h of traffic related contaminants. Only those genes significantly expressed as a function of exposure are displayed (two-way ANOVA, $p<0.05$).

Gene Id	Gene name	Fold change (38h)	Fold change (86h)
A_05_P418557	5-aminolevulinate synthase, nonspecific, mitochondrial precursor	1.6	1.6
A_05_P271584	Acetyl-CoA acetyltransferase, cytosolic	-2.8	-1.6
A_05_P417942	Acetyl-CoA acetyltransferase, cytosolic	-3.0	-1.9
A_05_P454147	AF058445 histone macroH2A1.1 {Gallus gallus}	-1.1	-1.8
A_05_P486052	Apolipoprotein B-100 precursor	-1.3	-2.0
A_05_P248994	Cytochrome P450 1A1	3.9	5.0
A_05_P249154	Cytochrome P450 1A1	5.9	7.9
A_05_P261934	Cytochrome P450 1A1	4.7	4.3
A_05_P431722	Cytochrome P450 1A1	4.1	5.1
A_05_P424002	Cytochrome P450 1B1	26.1	3.4
A_05_P493012	Cytosolic sulfotransferase 3	8.4	2.9
A_05_P298907	Dolichyl-P-Man:Man(7)GlcNAc(2)-PP-dolichyl-alpha-1,6-mannosyltransferase	-1.1	-1.6
A_05_P280912	Hypothetical protein Kpol_249p2 [Vanderwaltozyma polyspora DSM 70294]	-1.0	-3.9
A_05_P339072	Inositol polyphosphate-5-phosphatase e	-1.1	1.0
A_05_P254059	Loc446973 protein	3.6	5.8
A_05_P480297	Male-specific lethal 3-like 1	1.2	1.3
A_05_P443292	Mannosyl-oligosaccharide glucosidase	-1.4	-2.0
A_05_P302072	Novel protein bloodthirsty	-21.4	1.1
A_05_P294422	Organic solute transporter subunit alpha	1.5	1.8
A_05_P248884	PDZ and LIM domain protein 3	1.7	1.6
A_05_P409382	PDZ and LIM domain protein 3	2.0	1.9
A_05_P417077	Periodic tryptophan protein 2 homolog	-2.3	-2.0
A_05_P306317	Predicted protein [Nematostella vectensis]	10.3	-1.0
A_05_P423217	NADPH-dependent FMN reductase, partial	1.4	2.2
A_05_P419892	Chromosome undetermined SCAF9604, whole genome shotgun sequence	1.3	1.1
A_05_P427597	NADH dehydrogenase subunit 2, partial	-1.1	-1.9
A_05_P455452	SUGAR ABC TRANSPORTER PERMEASE PROTEIN, partial	1.5	2.0
A_05_P294152	Solute carrier family 24 (sodium potassium calcium exchanger) member 6	1.9	5.4
A_05_P427702	Solute carrier family 37 (glycerol-3-phosphate transporter) member 3	-1.0	-1.6
A_05_P452122	Ubiquitin specific protease 48	2.6	1.6
A_05_P298897	UNKNOWN	-1.4	-1.6
A_05_P302552	UNKNOWN	1.3	1.4
A_05_P322727	UNKNOWN	3.2	2.5
A_05_P336672	UNKNOWN	2.2	1.8
A_05_P347892	UNKNOWN	-1.1	-3.8
A_05_P354612	UNKNOWN	5.6	1.5
A_05_P358982	UNKNOWN	11.3	3.7
A_05_P359792	UNKNOWN	1.6	7.4
A_05_P419382	UNKNOWN	-1.9	-1.3
A_05_P420477	UNKNOWN	-1.6	-2.2
A_05_P393017	Zgc:153292 protein	1.3	1.3

Supplemental Table S2. The significantly expressed GO terms (biological process) in liver of brown trout exposed to 4 h of traffic related contaminants at time 38 h. The two first columns on the left hand side show the GO term id and GO-term level (3 and 4) used in Fig. 2 and Fig. 3 in the paper. The last two columns show the down- and up-regulation of various GO terms within the GO-term level 4, respectively.

GO-id	GO-term (L4) - Biological process	Down-regulated	Up-regulated
GO:0044237	cellular macromolecule metabolic process	activation of plasma proteins during acute inflammatory response	ncRNA metabolic process
		cellular macromolecule biosynthetic process	ncRNA processing
		cellular macromolecule metabolic process	negative regulation of protein modification
		cellular protein metabolic process	protein modification by small protein conjugation
		complement activation	protein ubiquitination
		mRNA metabolic process	regulation of cellular protein metabolic process
		mRNA processing	rRNA metabolic process
		protein maturation via proteolysis	rRNA processing
		regulation of complement activation	translation
		regulation of RNA metabolic process	ubiquitin-dependent protein catabolic process
		regulation of transcription	
		RNA biosynthetic process	
		RNA metabolic process	
		RNA processing	
		RNA splicing	
		transcription	
		transcription, DNA-dependent	
	biopolymer metabolic process	activation of plasma proteins during acute inflammatory response	ncRNA metabolic process
		cellular protein metabolic process	ncRNA processing
		complement activation	negative regulation of protein modification
		mRNA metabolic process	protein modification by small protein conjugation
		mRNA processing	protein ubiquitination
		protein maturation via proteolysis	regulation of cellular protein metabolic process
		protein metabolic process	rRNA metabolic process
		regulation of complement activation	rRNA processing
		regulation of RNA metabolic process	translation
		regulation of transcription	ubiquitin-dependent protein catabolic process
		RNA biosynthetic process	
		RNA metabolic process	
		RNA processing	
		RNA splicing	
		transcription	
		transcription, DNA-dependent	
	transport	intracellular protein transport	amine transport
		intracellular transport	aromatic amino acid transport
		lipid transport	carboxylic acid transport
		nuclear transport	cation transport
		nucleic acid transport	di-, tri-valent inorganic cation transport
		nucleocytoplasmic transport	gamma-aminobutyric acid transport
		oxygen transport	ion transport
		protein transport	L-amino acid transport
		RNA transport	organic acid transport
			polyamine transport
			sulfur amino acid transport
			tryptophan transport
	regulation of cellular process	epidermal growth factor receptor signaling pathway	cyclic-nucleotide-mediated signaling

		regulation of cellular biosynthetic process	G-protein coupled receptor protein signaling pathway
		regulation of cellular metabolic process	G-protein signaling, coupled to cyclic nucleotide second messenger
		regulation of complement activation	negative regulation of protein modification
		regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic pro	regulation of actin filament-based process
		regulation of RNA metabolic process	regulation of cellular component biogenesis
		regulation of transcription	regulation of cellular protein metabolic process
			regulation of synaptic plasticity
			regulation of synaptic transmission
			regulation of transmission of nerve impulse
	regulation of immune system process	activation of immune response	
		complement activation	
		positive regulation of adaptive immune response	
		positive regulation of adaptive immune response (sensu Gnathostomata)	
		positive regulation of immune response	
		positive regulation of immune system process	
		regulation of adaptive immune response	
		regulation of adaptive immune response (sensu Gnathostomata)	
		regulation of B cell mediated immunity	
		regulation of complement activation	
		regulation of humoral immune response	
		regulation of immune response	
		regulation of immune system process	
		regulation of immunoglobulin mediated immune response	
		regulation of leukocyte mediated immunity	
		regulation of lymphocyte mediated immunity	
	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	mRNA metabolic process	ncRNA metabolic process
		mRNA processing	ncRNA processing
		nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	rRNA metabolic process
		regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic pro	rRNA processing
		regulation of RNA metabolic process	
		regulation of transcription	
		RNA biosynthetic process	
		RNA metabolic process	
		RNA processing	
		RNA splicing	
		transcription	
		transcription, DNA-dependent	
	regulation of response to stimulus	activation of immune response	
		complement activation	
		positive regulation of adaptive immune response	
		positive regulation of adaptive immune response (sensu Gnathostomata)	
		positive regulation of immune response	
		positive regulation of response to stimulus	
		regulation of acute inflammatory response	
		regulation of adaptive immune response	
		regulation of adaptive immune response (sensu Gnathostomata)	
		regulation of B cell mediated immunity	

		regulation of complement activation	
		regulation of humoral immune response	
		regulation of immune response	
		regulation of immunoglobulin mediated immune response	
		regulation of leukocyte mediated immunity	
		regulation of lymphocyte mediated immunity	
	cellular biosynthetic process	cellular biosynthetic process	glutamine biosynthetic process
		cellular macromolecule biosynthetic process	translation
		cholesterol biosynthetic process	
		cholesterol metabolic process	
		regulation of cellular biosynthetic process	
		regulation of transcription	
		RNA biosynthetic process	
		steroid biosynthetic process	
		steroid metabolic process	
		sterol biosynthetic process	
		sterol metabolic process	
		transcription	
		transcription, DNA-dependent	
	system development	cellular biosynthetic process	adult heart development
			cardiac cell development
			cardiac cell differentiation
			cardiac muscle cell differentiation
			cardiac muscle development
			cardiac myofibril assembly
			erythrocyte development
			heart development
			muscle development
			myofibril assembly
			striated muscle cell development
			striated muscle cell differentiation
			striated muscle tissue development
			system development
	regulation of metabolic process	regulation of biosynthetic process	negative regulation of protein modification
		regulation of cellular biosynthetic process	regulation of cellular protein metabolic process
		regulation of cellular metabolic process	
		regulation of complement activation	
		regulation of gene expression	
		regulation of macromolecule biosynthetic process	
		regulation of macromolecule metabolic process	
		regulation of metabolic process	
		regulation of nitrogen metabolic process	
		regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic pro	
		regulation of RNA metabolic process	
		regulation of transcription	
	cell differentiation		cardiac cell development
			cardiac cell differentiation
			cardiac muscle cell development
			cardiac muscle cell differentiation
			cardiac myofibril assembly
			cell development
			cell differentiation
			erythrocyte development
			muscle cell development
			muscle cell differentiation
			myofibril assembly
			striated muscle cell development
			striated muscle cell differentiation
	gene expression	gene expression	ncRNA processing

		mRNA processing	rRNA processing
		protein maturation via proteolysis	translation
		regulation of gene expression	
		regulation of transcription	
		RNA processing	
		RNA splicing	
		transcription	
		transcription, DNA-dependent	
	lipid metabolic process	cellular lipid metabolic process	
		cholesterol biosynthetic process	
		cholesterol metabolic process	
		isoprenoid biosynthetic process	
		lipid biosynthetic process	
		lipid metabolic process	
		steroid biosynthetic process	
		steroid metabolic process	
		sterol biosynthetic process	
		sterol metabolic process	
		terpenoid biosynthetic process	
	positive regulation of biological process	activation of immune response	
		complement activation	
		positive regulation of adaptive immune response	
		positive regulation of adaptive immune response (sensu Gnathostomata)	
		positive regulation of immune response	
		positive regulation of immune system process	
		positive regulation of response to stimulus	
		regulation of complement activation	
	macromolecule biosynthetic process	cellular macromolecule biosynthetic process	translation
		macromolecule biosynthetic process	
		regulation of macromolecule biosynthetic process	
		regulation of transcription	
		transcription	
		transcription, DNA-dependent	
	immune effector process	complement activation	
		immune effector process	
		regulation of B cell mediated immunity	
		regulation of complement activation	
		regulation of immunoglobulin mediated immune response	
		regulation of leukocyte mediated immunity	
		regulation of lymphocyte mediated immunity	
	adaptive immune response	positive regulation of adaptive immune response	
		positive regulation of adaptive immune response (sensu Gnathostomata)	
		regulation of adaptive immune response	
		regulation of adaptive immune response (sensu Gnathostomata)	
		regulation of B cell mediated immunity	
		regulation of immunoglobulin mediated immune response	
	response to wounding	activation of plasma proteins during acute inflammatory response	wound healing
		acute inflammatory response	
		complement activation	
		regulation of acute inflammatory response	
		regulation of complement activation	
	organelle organization		actin cytoskeleton organization and

			biogenesis
			actomyosin structure organization and biogenesis
			cardiac myofibril assembly
			cytoskeleton organization and biogenesis
			myofibril assembly
	establishment of localization in cell	establishment of cellular localization	
		intracellular protein transport	
		intracellular transport	
		nuclear transport	
		nucleocytoplasmic transport	
	protein localization	cellular protein localization	
		establishment of protein localization	
		intracellular protein transport	
		protein localization	
		protein transport	
	defense response	activation of plasma proteins during acute inflammatory response	
		acute inflammatory response	
		complement activation	
		regulation of acute inflammatory response	
		regulation of complement activation	
	cellular alcohol metabolic process	cholesterol biosynthetic process	
		cholesterol metabolic process	
		sterol biosynthetic process	
		sterol metabolic process	
	regulation of multicellular organismal process	fucose metabolic process	cellular component biogenesis
			regulation of neurological process
			regulation of synaptic plasticity
			regulation of synaptic transmission
			regulation of transmission of nerve impulse
	neurological system process		regulation of neurological process
			regulation of synaptic plasticity
			regulation of synaptic transmission
			regulation of transmission of nerve impulse
	humoral immune response	complement activation	
		humoral immune response	
		regulation of complement activation	
		regulation of humoral immune response	
	cell motion		cell migration
			cell motility
			cell motility involved in cell locomotion
	ribonucleoprotein complex biogenesis		ribonucleoprotein complex biogenesis and assembly
			ribosome biogenesis and assembly
			rRNA processing
	locomotory behavior		chemotaxis
			locomotory behavior
			taxis
	cell-cell signaling		regulation of synaptic plasticity
			regulation of synaptic transmission
			regulation of transmission of nerve impulse
	RNA localization	establishment of RNA localization	
		RNA localization	
		RNA transport	
	cellular amino acid and derivative metabolic process	taurine metabolic process	glutamine biosynthetic process
	muscle system process		muscle contraction
			muscle system process
	cellular component assembly		cardiac myofibril assembly
			myofibril assembly

	anatomical structure morphogenesis		cardiac myofibril assembly
			myofibril assembly
	carbohydrate metabolic process		aminoglycan metabolic process
			fucose metabolic process
	circulatory system process		circulation
			circulatory system process
	cellular nitrogen compound metabolic process		aminoglycan metabolic process
			glutamine biosynthetic process
	macromolecule catabolic process		ubiquitin-dependent protein catabolic process
	organic acid metabolic process		glutamine biosynthetic process
	lipid localization	lipid localization	
	negative regulation of biological process		negative regulation of protein modification
	cellular catabolic process		ubiquitin-dependent protein catabolic process
	epithelial cell proliferation		epithelial cell proliferation
	extracellular structure organization		extracellular structure organization and biogenesis
	cell-cell adhesion		calcium-independent cell-cell adhesion
	homeostatic process		erythrocyte development
	regulation of developmental process		regulation of developmental pigmentation
	regulation of catalytic activity		activation of protein kinase activity
	external encapsulating structure organization		regulation of system process
	vitamin metabolic process		vitamin metabolic process

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Supplemental Table S3. The significantly expressed GO terms (molecular function) in liver of brown trout exposed to 4 h of traffic related contaminants at time 38 h. The two first columns on the left hand side show the GO term id and GO-term level 3 used in Fig. 2 and Fig. 3 in the paper. The last two columns show the down- and up-regulation of various GO terms within the GO-term level 3, respectively.

GO-id	GO-term (L3) - Molecular function	Down-regulated	Up-regulated
GO:0022857	transmembrane transporter activity		active transmembrane transporter activity
			amine transporter activity
			amino acid transporter activity
			carboxylic acid transporter activity
			cation transporter activity
			cation:amino acid symporter activity
			gamma-aminobutyric acid:sodium symporter activity
			ion transporter activity
			L-amino acid transporter activity
			L-gamma-aminobutyric acid transporter activity
			organic acid transporter activity
			organic acid:sodium symporter activity
			porter activity
			sodium:amino acid symporter activity
			solute:cation symporter activity
			solute:sodium symporter activity
			substrate-specific transmembrane transporter activity
			sulfur amino acid transporter activity
			symporter activity
			transmembrane transporter activity
GO:0022892	substrate-specific transporter activity	oxygen transporter activity	amine transporter activity
			amino acid transporter activity
			carboxylic acid transporter activity
			cation transporter activity
			cation:amino acid symporter activity
			gamma-aminobutyric acid:sodium symporter activity
			ion transporter activity
			L-amino acid transporter activity
			L-gamma-aminobutyric acid transporter activity
			organic acid transporter activity
			organic acid:sodium symporter activity
			sodium:amino acid symporter activity
			solute:cation symporter activity
			solute:sodium symporter activity
			substrate-specific transmembrane transporter activity
			sulfur amino acid transporter activity
GO:0005515	protein binding	protein binding	actin binding
			chemokine activity
			chemokine receptor binding
			cytokine activity
			cytokine receptor binding
			G-protein-coupled receptor binding
			insulin-like growth factor binding
			Notch binding
			receptor binding
GO:0016787	hydrolase activity		deoxyribonuclease activity
			DNA N-glycosylase activity
			helicase activity
			hydrolase activity, acting on glycosyl bonds (GO:0016798)
			hydrolyzing O-glycosyl compounds
GO:0004871	signal transducer activity		G-protein coupled receptor activity
			transmembrane receptor activity
			receptor activity
GO:0016874	ligase activity		small conjugating protein ligase activity

			ubiquitin-protein ligase activity
			glutamate-ammonia ligase activity
GO:0001871	pattern binding		polysaccharide binding
			glycosaminoglycan binding
			pattern binding
GO:0016740	transferase activity		phosphofructokinase activity
			6-phosphofructo-2-kinase activity
			phosphotransferase activity, phosphate group as acceptor
GO:0030246	carbohydrate binding		polysaccharide binding
			carbohydrate binding
			glycosaminoglycan binding
GO:0003676	nucleic acid binding	nucleic acid binding	translation factor activity, nucleic acid binding
		RNA binding	
GO:0016829	lyase activity	sulfinoalanine decarboxylase activity	
		carboxy-lyase activity	
		carbon-carbon lyase activity	
GO:0016491	oxidoreductase activity	7-dehydrocholesterol reductase activity	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, reduced flavin or flavoprotein as one donor, and incorporation of one atom of oxygen
GO:0008307	structural constituent of muscle		structural constituent of muscle
GO:0003735	structural constituent of ribosome		structural constituent of ribosome
GO:0043167	ion binding		calcium ion binding
GO:0005201	extracellular matrix structural constituent		extracellular matrix structural constituent

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Supplemental Table S4. The significantly expressed GO terms (cellular component) in liver of brown trout exposed to 4 h of traffic related contaminants at time 38 h. The two first columns on the left hand side show the GO term id and GO-term level 3 used in Fig. 2 and Fig. 3 in the paper. The last two columns show the down- and up-regulation of various GO terms within the GO-term level 3, respectively.

GO-id	GO-term (L3) - Cellular component	Down-regulated	Up-regulated
GO:0044464	cell part	coated membrane	actin cytoskeleton
		coated vesicle membrane	cell junction
		cytoplasm	cell part
		cytoplasmic part	cell surface
		cytosol	connexon complex
		endomembrane system	contractile fiber
		hemoglobin complex	contractile fiber part
		heterogeneous nuclear ribonucleoprotein complex	flagellum
		integral to plasma membrane	gap junction
		intracellular	I band
		intracellular membrane-bound organelle	intercellular junction
		intracellular organelle	intracellular non-membrane-bound organelle
		intracellular organelle lumen	intrinsic to membrane
		intracellular organelle part	lysosome
		intracellular part	lytic vacuole
		intrinsic to plasma membrane	membrane
		membrane coat	myofibril
		mitochondrial membrane part	myosin complex
		mitochondrion	nucleolus
		nuclear envelope	occluding junction
		nuclear lumen	peptidoglycan-based cell wall
		nuclear part	plasma membrane
		nuclear pore	plasma membrane part
		nucleoplasm	proteasome complex
		nucleoplasm part	proteasome core complex (sensu Eukaryota)
		nucleus	ribonucleoprotein complex
		organelle envelope	ribosome
		organelle membrane	sarcomere
		pore complex	secretory granule
		transcription factor complex	spliceosome
		vesicle coat	tight junction
GO:0043227	membrane-bounded organelle	coated vesicle membrane	lysosome
		heterogeneous nuclear ribonucleoprotein complex	lytic vacuole
		intracellular membrane-bound organelle	nucleolus
		membrane-bound organelle	secretory granule
		mitochondrial membrane part	spliceosome
		mitochondrion	
		nuclear envelope	
		nuclear lumen	
		nuclear part	
		nuclear pore	
		nucleoplasm	
		nucleoplasm part	
		nucleus	
		organelle envelope	
		organelle membrane	
		transcription factor complex	
		vesicle coat	
GO:0044422	organelle part	coated vesicle membrane	myosin complex
		heterogeneous nuclear ribonucleoprotein complex	nucleolus
		intracellular organelle lumen	spliceosome

		intracellular organelle part	
		mitochondrial membrane part	
		nuclear envelope	
		nuclear lumen	
		nuclear part	
		nuclear pore	
		nucleoplasm	
		nucleoplasm part	
		organelle envelope	
		organelle lumen	
		organelle membrane	
		organelle part	
		transcription factor complex	
		vesicle coat	
GO:0043234	protein complex	hemoglobin complex	connexon complex
		membrane coat	myosin complex
		nuclear pore	proteasome complex
		protein complex	proteasome core complex (sensu Eukaryota)
		transcription factor complex	
		vesicle coat	
GO:0043228	non-membrane-bounded organelle		actin cytoskeleton
			intracellular non-membrane-bound organelle
			non-membrane-bound organelle
			ribosome
			myosin complex
			nucleolus
GO:0044421	extracellular region part		extracellular space
			proteinaceous extracellular matrix
			extracellular region part
			extracellular matrix part
			basement membrane
			extracellular matrix
GO:0031982	vesicle	coated vesicle membrane	secretory granule
		vesicle coat	

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Supplemental Table S5. The significantly expressed GO terms (biological process) in liver of brown trout exposed to 4 h of traffic related contaminants at time 86 h. The two first columns on the left hand side show the GO term id and GO-term level 4 used in Fig. 2 and Fig. 3 in the paper. The last two columns show the down- and up-regulation of various GO terms within the GO-term level 4, respectively.

GO-id	GO-term (L4) - Biological process	Down-regulated	Up-regulated
GO:0044260	cellular macromolecule metabolic process	cellular macromolecule metabolic process	aminoglycan catabolic process
		DNA integration	ncRNA metabolic process
		DNA metabolic process	ncRNA processing
		DNA recombination	pseudouridine synthesis
		post-translational protein modification	rRNA metabolic process
		protein modification	rRNA processing
		transposition, DNA-mediated	
GO:0043283	biopolymer metabolic process	biopolymer modification	ncRNA metabolic process
		DNA integration	ncRNA processing
		DNA metabolic process	pseudouridine synthesis
		DNA recombination	rRNA metabolic process
		post-translational protein modification	rRNA processing
		protein modification	
		transposition, DNA-mediated	
GO:0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	DNA integration	ncRNA metabolic process
		DNA metabolic process	ncRNA processing
		DNA recombination	pseudouridine synthesis
		nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	rRNA metabolic process
		transposition, DNA-mediated	rRNA processing
GO:0006629	lipid metabolic process		cellular lipid metabolic process
			lipid metabolic process
			glycerolipid metabolic process
GO:0022613	ribonucleoprotein complex biogenesis		ribosome biogenesis and assembly
			rRNA processing
			ribonucleoprotein complex biogenesis and assembly
GO:0005975	carbohydrate metabolic process		carbohydrate metabolic process
			aminoglycan catabolic process
GO:0010467	gene expression		ncRNA processing
			rRNA processing
GO:0042592	homeostatic process		cell redox homeostasis
GO:0009057	macromolecule catabolic process		aminoglycan catabolic process
GO:0006790	sulfur metabolic process		glutathione metabolic process
GO:0051186	cofactor metabolic process		glutathione metabolic process
GO:0046483	heterocycle metabolic process		heterocycle metabolic process
GO:0034641	cellular nitrogen compound metabolic process		aminoglycan catabolic process
GO:0050794	regulation of cellular process		cell redox homeostasis
GO:0006518	peptide metabolic process		peptide metabolic process
GO:0044248	cellular catabolic process		aminoglycan catabolic process
GO:0009410	response to xenobiotic stimulus		response to xenobiotic stimulus

Supplemental Table S6. The significantly expressed GO terms (molecular function) in liver of brown trout exposed to 4 h of traffic related contaminants at time 86 h. The two first columns on the left hand side show the GO term id and GO-term level 3 used in Fig. 2 and Fig. 3 in the paper. The last two columns show the down- and up-regulation of various GO terms within the GO-term level 3, respectively.

GO-id	GO-term (L3) – Molecular function	Down-regulated	Up-regulated
GO:0003676	nucleic acid binding	DNA binding	translation elongation factor activity
		nucleic acid binding	
		transcription factor activity	
GO:0004803	transposase activity	transposase activity	
GO:0003735	structural constituent of ribosome	structural constituent of ribosome	
GO:0016787	hydrolase activity		hydrolase activity, hydrolyzing O-glycosyl compounds
GO:0016491	oxidoreductase activity		oxidoreductase activity
GO:0016853	isomerase activity		pseudouridine synthase activity
GO:0005515	protein binding	lipoprotein binding	
GO:0022892	substrate-specific transporter activity	protein transporter activity	

Supplemental Table S7. The significantly expressed GO terms (Cellular component) in liver of brown trout exposed to 4 h of traffic related contaminants at time 86 h. The two first columns on the left hand side show the GO term id and GO-term level 3 used in Fig. 2 and Fig. 3 in the paper. The last two columns show the down- and up-regulation of various GO terms within the GO-term level 3, respectively.

GO-id	GO-term (L3) - Molecular function	Down-regulated	Up-regulated
GO:0044464	cell part	eukaryotic translation initiation factor 4F complex	cytoplasm
		nucleus	cytoplasmic part
			eukaryotic translation elongation factor 1 complex
			lysosome
			lytic vacuole
			mitochondrion
			vacuole
GO:0043227	membrane-bounded organelle	nucleus	lysosome
			lytic vacuole
			mitochondrion
			vacuole
GO:0043234	protein complex	eukaryotic translation initiation factor 4F complex	eukaryotic translation elongation factor 1 complex