Title: *In vivo* and *in vitro* effects of tunnel wash water and traffic related contaminants on aquatic organisms

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Abstract

In order to maintain the construction and safety of road tunnels, they are routinely washed. The wash water appears to be highly polluted with a plethora of contaminants in elevated concentrations. In addition, new and emerging compounds are likely to occur. The discharge water has shown acute toxic and sub-lethal effects in several organisms. In this study, ecotoxicity tests with algae (*Pseudokirchneriella subcapitata*) and *in vitro* tests with primary

rainbow trout (*Oncorhynchus mykiss*) hepatocytes were used to characterize the effect of TWW from three different tunnels. In addition, selected N- and Cl-PAHs were tested for cytotoxicity, EROD activity and CYP1A protein production. TWW samples and/or extracts from two tunnels reduced the algal growth and induced cytotoxicity, EROD activity and CYP1A protein production *in vitro*. Four of the eight tested Cl- and N-substituted PAHs induced EROD activity and CYP1A protein production at micro-molar concentrations. N-PAHs were detected in samples from the tunnel wash, highlighting substituted PAHs as potentially important traffic-related contaminants.

Key words: Tunnel wash water; algae; primary fish hepatocytes; CYP1A; toxicity; chloro- and nitro-PAHs.

1. Introduction

The growing communication and modernisation of human societies has led to increased environmental impact related to human made infrastructure and activities. A well-functioning infrastructure for transportation is fundamental in order to maintain settlements in rural areas, ensure proper safety for road users, and facilitate a safe and reliable flow of goods and services (Meland et al., 2011b). Challenging landscapes as well as increased focus on protection of humans from air pollution in urban areas has led to building of a vast number of tunnels in several European countries such as Austria, Italy, Norway and Switzerland (Meland, 2016).

The tunnel environment is harsh, and dirt and dust are deposited and accumulated on the road pavement, walls, ceiling and technical gear. In order to maintain the construction and safety of

road tunnels, they are routinely washed. The frequency of tunnel washes depends on the specific tunnel's size and traffic load, and in Norway tunnels are usually washed 2-12 times per year (Roseth and Meland, 2006). Of these, the majority is so-called "technical wash" where technical gear and traffic signs are washed and "half-wash" which includes washing of the tunnel walls and road pavement. One-two times a year a "full wash" is performed which includes washing of the entire tunnel surface including technical gear/infrastructure and traffic signs. During a washing event, a road sweeper removes dust, debris and other coarse material from the road surface. A detergent is normally applied and the tunnel washed with high pressure cleaning before the road sweeper removes dirt and un-drained wash water (Roseth and Meland, 2006).

Water consumption during tunnel wash varies with respect to the equipment used and the type of wash routine executed. Typical water consumption can be from 60L (according to contractors) to 140 L (Roseth and Meland, 2006) for each meter of tunnel washed, potentially generating around 60-140 m^3 polluted water during cleaning of 1km tunnel. Tunnel wash water (TWW) from a full wash has a larger volume and is normally more polluted than TWW from a half-wash. Technical wash involves relatively low volumes of TWW compared to the two latter. Although tunnels represent a small amount of the total road network, these represent hot-spots in terms of polluted runoff water because the pollutants accumulate over longer periods (time between washing events may span over weeks, months or even years) and are not very affected by weather conditions like wind and precipitation (Torp og Meland 2015).

The TWW appears to be highly polluted with a plethora of contaminants, including metals and polycyclic aromatic hydrocarbons (PAHs) (Meland et al., 2010a), in concentrations that can be orders of magnitude higher than concentrations measured in ordinary road runoff (Amundsen and Roseth, 2004; Andersen and Vethe, 1994; Barbosa et al., 2007; Meland, 2010). Several contaminants (e.g. Cu, Pb, Zn, benzo[*a*]pyrene, fluoranthene, pyrene) have also been detected at concentrations exceeding their corresponding environmental quality standards (Meland et al., 2010a; Paruch and Roseth, 2008a, 2008b). In addition, new and emerging chemicals such as organophosphorus compounds (OPs) are also present in TWW (Meland and Roseth, 2011). Other groups of compounds potentially occurring in TWW are the nitro- (N-) and chloro- (Cl-) substituted PAHs. Such compounds have recently been detected in environmental samples (Huang et al., 2014; Niederer, 1998; Sankoda et al., 2012; Uno et al., 2011), and in particulate matter from tunnels (Grung et al., 2016a). N-, sulfur- and oxygenated PAHs are believed to occur simultaneously with their un-substituted PAH analogues (Hinger et al., 2011), and can thus be expected to occur in TWW.

TWW and traffic related contaminants have the potential for being acute toxic as observed for amphibian larvae living in a treatment pond for road runoff water (Johansen, 2013). In addition, a wide range of sub-lethal effects in fish (Gjessing et al., 1984; Grung et al., 2016b; Meland et al., 2010a, 2010c, 2011a), including reduced growth of sea trout (*Salmo trutta*) (Meland et al., 2010a), increased activity of antioxidant defense system, problems with the regulation of plasma ions as well as increased levels of glucose and pCO_2 and affected metabolism (Meland et al., 2010c), and molecular changes in the liver of exposed fish (Grung et al., 2016; Meland et al., 2011a) have been observed. Although few effect studies with Cl- and N- substituted PAHs have been performed, it has been shown that N-PAHs can have

stronger carcinogenic and mutagenic activity than the non-substituted analogues (Tokiwa et al., 1987), and Cl-PAHs have been shown to activate the aryl hydrocarbon receptor (AhR) (Ohura et al., 2007).

Due to the toxic potential of TWW, regular chemical and/or effect screening might be necessary for tunnels with limited treatment of the discharge water to protect organisms in the recipient. In this study, ecotoxicity tests with the algae *Pseudokirchneriella subcapitata* and *in vitro* studies using primary hepatocytes from rainbow trout (*Oncorhynchus mykiss*) were used to assess the toxicity of TWW from three different tunnels after washing events. Samples from the tunnel wash were characterized by chemical analysis. Selected N- and Cl-PAHs were tested for acute toxic and dioxin-like effects in primary rainbow trout hepatocytes to investigate the potential environmental hazard of these compounds.

2. Materials and Methods

2.1 Sampling and sample preparation

Sampling

Samples of water, suspended particulate matter (SPM) and coarse grained material were collected in connection with regular detergent-free half-washes of the Nordby tunnel (sampled at two different wash events; 1 and 2) on highway E6 (Akershus county), the Oslofjord tunnel on highway Rv. 23 (Akershus county) and the Granfoss tunnel Rv. 190 (City of Oslo) (Table 1). Sampled tunnel wash water (TWW) for ecotoxicity tests was brought to the freezer (-20°C) within 4 hours after sampling and kept frozen until further preparations. Water for

chemical analyses were kept at 8°C and delivered to the laboratory directly after the tunnel wash. See supplementary for more details on sampling.

Preparation of samples for algae tests

Collected TWW samples contained large amounts of particles that could affect algae through mechanical stress and obstruction of light needed for growth. The potential for mechanical stress was reduced by filtration (0.22µm, sterivex, Merck Millipore, Billerica, MA, USA) of the TWW prior to the algae tests.

In addition to the filtrated water samples, TWW from Granfoss was extracted with liquid-liquid phase extraction to obtain the total organic fraction. 100mL dichloromethane was added to 300mL TWW and placed on a shaker for 48h. The water and dichloromethane phase were separated with a separating funnel and a solvent change from dichloromethane to DMSO was performed. Due to challenges with evaporating DMSO to the desired volume, the extracts were solved in 1L water and extracted again on Oasis® HLB cartridges (Waters S.A.S., Saint-Quentin, En Yvelines Cedex, France), eluted with methanol, evaporated and transferred to the right amount of DMSO. A maximum DMSO concentration of 0.01% was used in the algae tests.

Preparation of extracts and stock solutions for *in vitro* tests

Preparation of concentrated TWW samples was performed to allow for a 100 times dilution in assay medium. The TWW samples were filtered (0.45µm) before extraction on Oasis® HLB cartridges in order to remove a large part of the particle bound contaminants. The cartridges were eluted with dichloromethane and methanol, evaporated and transferred to DMSO in a

142 volume corresponding to a concentration factor (CF) of 2000 (2L water sample equals 1mL
143 extract), giving a maximum testing CF of 20 with a DMSO concentration of 1%.
144
145 The chemicals copper sulphate (CuSO4*5H2O, cas 7758-99-8) and 2,3,7,8-
146 tetrachlorodibenzo-*p*-dioxin (TCDD, cas 1746-01-6) were purchased from Sigma-Aldrich (St.
147 Lois, MI, US). Selected Cl- and N-PAHs, 6-chloro-benzo[a]pyrene (21248-01-1, ≥98%), 3-
148 chloro-fluoranthene (25911-51-7, ≥89.5%), 9-chloro-phenanthrene (cas 947-72-8, ≥98%), 1-
149 chloro-pyrene (34244-14-9, ≥98%), 6-nitro-chrysene (7496-02-8, ≥98%), 6-nitro-
150 benzo[a]pyrene (63041-90-7, ≥98%), 3-nitro-phenanthrene (17024-19-0, ≥98%), 1-nitro-
151 pyrene 5522-43-0, ≥98%), were purchased from Chiron (Trondheim, Norway) and transferred
152 to DMSO. The stock solutions were stored in the dark at 4°C when not in use.
153
154 **2.2 Chemical analysis**

155 Chemical analysis of water samples

156 All water samples were analyzed by the laboratory at the Norwegian Institute for Water
157 Research (NIVA, accredited according to ISO NS-EN ISO/IEC 17025) or a subcontractor. In
158 addition to the chemical analysis of silver (Ag), aluminum (Al), arsenic (As), boron (B),
159 barium (Ba), beryllium (Be), bismuth (Bi), calcium (Ca), cadmium (Cd), cobalt (Co),
160 chromium (Cr), cupper (Cu), iron (Fe), mercury (Hg), potassium (K), lithium (Li),
161 magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), nickel (Ni), phosphorus
162 (P), lead (Pb), sulfur (S), antimony (Sb), selenium (Se), silicon (Si), tin (Sn), strontium (Sr),
163 thorium (Th), titanium (Ti), thallium (Tl), uranium (U), vanadium (V), and zinc (Zn), the pH,
164 turbidity and SPM concentration were determined (described in supplementary). Water

samples for metal analyses were conserved in a 0.5% HNO_3 solution and analysed by ICP-MS.

Chemical analysis of SPM and coarse grained material

SPM from TWW and coarse grained material from road sweepers from the Nordby and Granfoss tunnels were analyzed for PAH 16 EPA, the sum of 9 groups of methylated PAHs (C_{1-3}-naphthalenes, -phenanthrenes, -dibenzothiophenes), 6 Cl-PAHs (9-Cl-9H-fluorene, 2-Cl-anthracene, 9-Cl-phenanthrene, 6-Cl-benzo[*a*]pyrene, 1-Cl-pyrene, 3-Cl-fluoranthene) and 10 nitro PAHs (1-N-naphthalene, 2-N-biphenyl, 4-N-biphenyl, 2-N-fluorene, 9-N-anthracene, 3-N-phenanthrene, 1-N-pyrene, 2-N-pyrene, 7-N-benzo[*a*]anthracene, 6-N-chrysene). Samples were extracted and detection and quantification was done using GC-EI-MS and GC-NCI-MS (for Nitro-PAHs only), detailed description in supplementary. All samples were analysed with a blank sample and spiked samples and just spiked solvents. Good recoveries for N- and Cl-PAHs (70-120%) were obtained, and limit of detection (LOD) for PAH16 ranged from 0.5-20ng/g dw.

2.3 Algae tests

Assessment of the ecotoxicity of the TWW was performed with a 72h algal growth inhibition test with P*seudokirchneriella subcapitata* according to ISO 8692 (ISO, 2012) and OECD Guideline for Testing of Chemicals No. 201: Freshwater alga and cyanobacteria, growth inhibition (OECD, 2011).

2.4 Primary rainbow trout hepatocytes

Isolation and exposure of hepatocytes

189 Juvenile rainbow trout (*Oncorhynchus mykiss*, size 200-500g) purchased from Valdres
190 Ørretoppdett (Valdres, Norway) or obtained from the Norwegian University of life sciences
191 (NMBU), were kept at the Institute of Biology at the University of Oslo (Norway) at 6±2˚C,
192 100 % oxygen saturation, pH 6.6 and 12h light/12h dark cycle. The fish were fed daily with
193 pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5% of total body
194 mass. Fish was killed with a blow to the head and a 2-step liver perfusion was performed as
195 described in Tollefsen et al. (2003). The resulting cell suspensions were diluted to 500000
196 cells/ml and seeded in 96-well primaria™ plates (Falcon, Becton Dickinson Labware,
197 Oxnard, CA, USA, 200µl/well). Only cell isolation with viability above 80% determined by
198 the trypan blue exclusion method was used. After 24h acclimatization, cells were exposed to
199 extracts, Cl- and N-PAHs, and positive controls (TCDD for EROD and CYP1A analysis, and
200 $CuSO_4$ for cytotoxicity). After 48h of exposure, cell plates determined for EROD and CYP1A
201 analyses were emptied of exposure media and stored at -80°C for subsequent analysis. Cell
202 plates determined for cytotoxicity assays were re-exposed after 48h and cytotoxicity measured
203 after a total exposure time of 96h.
204
205 Cytotoxicity assay
206 Metabolic activity and membrane integrity were assessed essentially as described by Schreer
207 et al. (2005) by use of the two probes alamar blue (AB) and carboxyfluorescein diacetate
208 acetoxymethyl ester (CFDA-AM), respectively. Cells were incubated in Tris buffer
209 containing 5% AB and 4µM CFDA-AM for 30 min before fluorescence was read using
210 excitation and emission wavelength pairs of 530-590 (AB) and 485-530 (CFDA-AM). The
211 results were normalized to the DMSO control (100% viability) and 0.01mol/L $CuSO_4$ (0%

viability). Both probes provided similar results, and only results for metabolic activity are shown.

EROD-activity and CYP1A protein production

The EROD activity was measured by incubating cells with ethoxyresorufin (ER) which is enzymatically converted by cyp1a isoenzymes to resorufin (RR). The cell plates were thawed on ice and incubated 15 min with 50mM Tris buffer containing 0.1M NaCl, 20µM dicumarol, 2µM ER, 100µM β-NADPH (200µl per well) before fluorescence was measured using excitation and emission wavelength pairs of 530nm and 595nm. The results were normalised against protein content measured with the standardised Bradford method. Results were expressed as percentage of a positive control exposed to 0.3nM TCDD.

After EROD analysis the plates were frozen at -80°C for subsequent analysis of CYP1A protein by capture ELISA. The plates were thawed and 40µl from each well was diluted in 160µl coating buffer (Sodium bicarbonate buffer), transferred (100µl) to maxisorp nunc-immonoplates (Nunc, Roskilde, Denmark), sealed and incubated overnight in the dark at 4°C. The plates were washed three times with washing buffer (PBS added 0.05% tween 20) and incubated 1h in the dark with 200µl blocking buffer (PBS with 2% BSA). After three washes, cells were incubated for 2h at 37°C with 100µl of the primary antibody rabbit-anti-fish CYP1A (CP-226, biosense laboratories, Bergen, Norway) diluted 1:1000 in PBS buffer with 1% BSA. After three washes, 100µl secondary antibody goat-anti rabbit IgG conjugated with horse radish peroxidase (HRP) was added and the plates were incubated at 37 °C for 2h. The plates were washed five times and 100µl of the substrate for HRP (TMB plus) were added. Plates were incubated for 12min and the reaction stopped by adding 50µl H_2SO_4 (1M). The

absorbance was measured at 450nm and the results were expressed as percentage of a positive control exposed to 0.3nM of TCDD.

2.5 Data analysis

Significant differences from the media and/or solvent control were investigated with a non-parametric one-way anova Kruskal-Wallis test and Dunn's multiple comparison test with a significance level of $p < 0.05$.

Results were modelled with a non-linear regression curve fit in graphpad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) with top and bottom values constrained to 100 and 0 for fitting of normalised data. Concentrations where a change in the direction of the response occurred were omitted from the curve-fitting, and the fitted concentration response curves (CRCs) were thus only valid within the concentration range included in the model fits.

3. Results

3.1 Chemicals in TWW, SPM and coarse grained material

The measured concentrations of metals (Table 2) varied between the different TWW samples. The concentration of the heavy metals As, Cd, Cr, Cu, Ni, Pb, and Tl were generally highest in the Granfoss and Nordby tunnel and lowest in the Oslofjord tunnel. The concentration of Hg was highest in the Oslofjord tunnel. There was also a difference between the two samples from the Nordby tunnel with higher concentrations generally found in the Nordby 1 sample.

The concentration of PAH16 in the SPM and in coarse grained material collected from the sweepers in the tunnels Granfoss and Nordby ranged from 790 to 4800ng/g d.w. (Table 2). No Cl-PAHs were detected above LOD in these samples, but several N-PAHs were detected. The highest concentrations were observed for 9-N-anthracene (2.6-13ng/g d.w.), 1-N-naphthalene (<0.5-1.9ng/g d.w.), 1-N-pyrene (<0.5-1.5ng/g d.w.) and 3-N-phenanthrene (<0.5-1.0ng/g d.w.).

3.2 Effects of TWW on algal growth

The filtered TWW samples had generally low effect on the algal growth rate (figure 1). Significant growth reduction (67% of control) was observed for the highest tested concentration of the Nordby 1 sample (i.e. undiluted, CF=1). The filtered samples from Nordby 2, Oslofjord tunnel and Granfoss did not reduce the algal growth rate below 90% of the control. The organic fraction from Granfoss reduced (although not significantly) the algal growht rate to 70% of control at a CF of 0.6.

3.3 *In vitro* effects of TWW extracts

Three of the four extracts (Nordby 1, Nordby 2 and Granfoss) showed cytotoxic effects on the cells with 50% reduction in metabolic activity occurring at a CF of 11 in the Granfoss extract, 4.3 in the Nordby and Nordby 2 extract (figure 1, table 3).

The same three extracts also induced the concentration of CYP1A and EROD activity compared to the procedural blank and Oslofjord control water (figure 1). A non-significant increase in EROD activity was observed at a CF of 0.3 for all extracts except Oslofjord, whereas significant increases occurred at a CF of 3. The calculated EC_{10} and EC_{50} from the

fitted CRCs for EROD activity and CYP1A protein production are given in table 3. The calculated EC_{10} and EC_{50} for EROD activity and CYP1A protein induction after exposure to the extract from the Oslofjord tunnel was outside the tested concentration range and no significant difference from the procedural blank and Oslofjord control water was observed. The extracts from Nordby 1, Nordby 2 and Granfoss significantly increased the EROD activity and CYP1A production. The EC_{10} for EROD induction was below environmental concentrations indicated by a CF below 1, and the EC_{50s} was in the CF range of 3.1-4.8. The EC_{10} for CYP1A was also below a CF of 1 and the EC_{50}s ranged from a CF of 1.9 to 7.1. The EC_{50} for CYP1A after exposure to the Granfoss extract was outside the tested concentration range and above the EC_{50} for cytotoxicity.

A non-significant increase in the level of CYP1A was observed at a CF of 0.3 for Nordby 2. Significant increase in the CYP1A level compared to controls occurred at a CF of 1 for the extract form Granfoss and at a CF of 0.3 for the extract from Nordby 1. Although a clear induction was seen for the samples from Nordby 2, no significant difference was observed probably due to higher variation and low number of replicates (n=3).

3.4 *In vitro* effects of chloro- and nitro- PAHs

Four Cl- and four N-PAHs were selected for *in vitro* effect studies. None of the tested compounds exhibited strong cytotoxic effects at the tested concentrations (figure 2). Four of the tested compounds (6-Cl-benzo[*a*]pyrene, 3-Cl-fluoranthene, 6-N-chrysene and 6-N-benzo[*a*]pyrene induced both the EROD activity and the CYP1A protein production at the tested concentrations (figure 2, table 4). Only 3-Cl-phenanthrene had an EC_{50} value (0.89µM) for induction of EROD activity within the valid concentration range for the CRC. The order

of potency based on the estimated EC_{10} values for EROD activity was 6-Cl-benzo[*a*]pyrene = 6-N-chrysene > 6-N-benzo[*a*]pyrene > 3-Cl-phenanthrene, with values ranging from 0.16-0.29µM.

Both 3-Cl-phenanthrene and 6-N-benzo[*a*]pyrene had EC_{50} values for CYP1A protein production within the valid concentration range for the CRC with 3-Cl-fluoranthene being the most potent (EC_{50} = 1.3µM). The order of potency for CYP1A induction based on the EC_{10} values was 3-Cl-fluoranthene > 6-Cl-benzo[*a*]pyrene > 6-N-benzo[*a*]pyrene > 6-N-chrysene, with values ranging from 0.24-0.31µM. The order of potency varied between the endpoints and effect levels. However, the effect levels for the four compounds differed by no more than a factor of 4, indicating a similar potency for induction of dioxin-like effects at low micro-molar concentrations.

4. Discussion

Several metals, PAHs and substituted PAHs were detected in the tunnel wash samples. The lower concentrations of certain metals in the Oslofjord TWW than the other TWW samples could be due to technical problems in the tunnel prior to the sampling event, after which heavy vehicles were not permitted through, in addition to lower AADT in this tunnel than the other two. The measured concentrations of pollutants in the tunnel wash samples were generally similar to or slightly higher than previously reported levels (Aasum, 2013; Allan et al., 2016; Meland et al., 2010a, 2010b; Paruch and Roseth, 2008a, 2008b; Roseth and Meland, 2006), showing that tunnels are a hot spot for pollution and are a source for various metals, PAHs, and substituted PAHs that could potentially affect organisms in the recipient water bodies.

330

331 **4.1 *In vivo* and *in vitro* effects of TWW**

332 Low algae toxicity of the filtered TWW samples was observed despite high concentrations of
333 metals measured in the TWW from the Nordby and Granfoss tunnels. However, the measured
334 concentrations represent the total concentrations in the unfiltered water sample and a large
335 reduction in metal concentrations between total and filtered water samples has been observed
336 (Aasum, 2013). The metal concentrations in the filtered TWW samples used in the algae tests
337 are likely lower than the measured concentrations as metals associated with suspended
338 particulate material >0.22µM was filtered out. A significant effect on the algal growth was
339 only observed for the TWW sample from the Nordby tunnel (Nordby1). Except from the
340 Oslofjord TWW sample, Nordby 1 TWW contained the lowest amount of SPM (Table 2). As
341 a high amount of the TWW pollutants can be associated with particulate matter (Aasum,
342 2013; Meland et al., 2010a), it can be hypothesized that the lack of effect on algal growth of
343 the Granfoss and Nordby 2 TWW could be linked to the higher content of SPM in these
344 samples. A higher amount of SPM could lead to more particulate matter associated pollutants
345 being filtered out before testing. Generally lower concentrations of toxic metals were found in
346 the Oslofjord TWW, potentially explaining the lack of effect on algal growth of this sample.

347

348 The organic fraction extracted from Granfoss TWW had higher effect on the algal growth
349 than the filtered TWW sample, suggesting that the majority of compounds affecting algal
350 growth were bound to particles larger than 0.22µm. The CFC collected SPM from Granfoss
351 had the highest concentrations of PAH16 of the analysed samples (table 2) and shows that
352 PAHs were bound to SPM in the TWW. This is in accordance with previous studies of TWW

where PAHs and metals like Al, Cd, Cr, Cu, Fe and Pb were shown to be highly associated with particles and colloids (Meland et al., 2010a).

Although the TWW samples showed low toxicity in the algal test, cytotoxic effects on primary hepatocytes was observed, indicating presence of compounds with potential for inducing toxic effects. Effects on EROD activity and CYP1A levels were observed at CFs corresponding to environmental concentrations, which is in agreement with effect studies of fish exposed *in situ* (Meland et al., 2010b, 2011). The potency for inducing EROD activity and CYP1A protein production was fairly similar for all extracts except from the Oslofjord sample. As the concentrations of certain metals were lower in the TWW from this tunnel, it can be assumed that the level of pollutants responsible for cytotoxicity, EROD induction and CYP1A protein production might also be lower. In addition, the level of SPM in the TWW from the Oslofjord was much lower than in the other samples, potentially leading to a lower level of particle associated pollutants (<45µm) available for extraction in this sample.

Dioxin-like effects (e.g. induction of EROD activity and CYP1A protein) are mediated through the AhR. A reason for concern of compounds with this mode of action is related to the adverse effects in terms of mortality, embryotoxicity, immunotoxicity, and carcinogenicity mediated through the AhR (Ma, 2008; Mandal, 2005; Poland and Knutson, 1982; Safe, 2001). In addition, oxidative stress has been observed in fish exposed to traffic related contaminants (Meland et al., 2011a). Oxidative stress may ultimately lead to DNA damage, and a higher level of DNA damage has been observed in fish (*Phoxinus phoxinus*) from a sedimentation pond receiving highway runoff compared to fish in an up-stream river (Grung et al., 2016b). Based on previously reported results and results obtained in this study, TWW might pose a

problem to organisms living in the recipient water bodies as most of the Norwegian tunnels do not have any form for treatment of TWW.

4.2 Effects of N- and Cl- PAHs

All tested N-PAHs except 6-N-BAP were detected in the samples from the Granfoss and Nordby tunnel (Table 2). However, only 3-N-phenanthrene and 1-N-pyrene were detected in quantifiable concentrations. These two N-PAHs showed no effects on the cytotoxicity, EROD activity or CYP1A production in the primary hepatocytes at the tested concentrations.

The effect of the tested Cl- and N-PAHs was compared to reported effects of their corresponding PAH analogues. 6-Cl-benzo[*a*]pyrene and 6-N-benzo[*a*]pyrene induced EROD activity and CYP1A protein production in this study. EROD activity was also induced by benzo[*a*]pyrene in a co-culture of primary hepatocytes and the cell line RTG-2 (Scholz and Segner, 1999). Thus benzo[a]pyrene and the two substituted benzo[a]pyrenes; 6-Cl-benzo[*a*]pyrene and 6-N-benzo[*a*]pyrene all induce AhR mediated effects.

Fluoranthene have previously been shown to reduce the EROD activity, induce DNA damages (COMET) (Wessel et al., 2012), and to reduce the EROD activity induced by benzo[*a*]pyrene in the killifish mummichog, *Fundulus heteroclitus* (Willett et al., 2001). Inconsistently, induction of EROD activity with increasing concentrations of fluoranthene was observed in goldfish (*Carassius auratus*) (Lu et al., 2008), whereas no induction of EROD activity was observed in primary hepatocytes from rainbow trout (Behrens et al., 2001) and nile tilapia (*Oreochromis niloticus)* (Pathiratne and Hemachandra, 2010). Inconsistent results of AhR mediated effects of fluoranthene have been observed. However, the 3-Cl-

fluoranthene tested in this study induced the AhR mediated EROD activity in a concentration-dependent manner.

Chrysene has previously been shown to induce the EROD activity in primary rainbow trout hepatocytes (Behrens et al., 2001). EROD activity was also induced by the substituted 6-N-chrysene tested in this study, indicating that both chrysene and 6-N-chrysene act by similar mode of action.

No induction of EROD and CYP1A from phenanthrene and pyrene substituted analogues were observed in the present study. The lack of induction by the phenanthrene analogues are coherent with a study by Pathiratne and Hemachandra (2010) where 9-Cl-phenanthrene and 3-N-phenanthrene did not induce the EROD activity or CYP1A protein production in primary hepatocytes from Nile tilapia. In contrast, pyrene is known to induce the EROD activity in fish (Pathiratne and Hemachandra, 2010; Zapata-Pérez et al., 2002), showing that substituted PAHs may not always exhibit similar effects as their non-substituted analogues. In summary, certain N- and Cl-substituted PAHs can induce EROD activity and CYP1A protein production and is thus a group of environmental concern as substituted PAH-analogues were also detected in samples from the tunnel wash.

5. Conclusion

TWW samples from the Oslofjord tunnel had no effect on algal growth, or the cell viability, EROD activity and CYP1A protein production in primary rainbow trout hepatocytes. This was probably due to lower AADT in this tunnel and lower level of contaminants and SPM in the TWW. TWW samples and/or extracts from the Granfoss and Nordby tunnels reduced the

algal growth, and reduced the cell viability and induced the EROD activity and CYP1A protein production in primary rainbow trout hepatocytes. Thus, TWW might pose an environmental hazard for organisms in recipient water bodies. Some Cl- and N-substituted PAHs were shown to induce dioxin-like effects at micro-molar concentrations. Several N-PAHs were also detected in SPM and coarse grained material from the tunnel wash, highlighting the need for further assessment of substituted PAHs as potentially important traffic-related contaminants.

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Highlights

- Tunnel wash water (TWW) were analyzed by chemical analysis and effect studies
- Metals, PAHs and N-PAHs were detected in samples from the tunnel washes
- TWW had a low effect on algal growth
- TWW extracts induced EROD activity and CYP1A production in primary fish hepatocytes
- Two Cl- and two N-PAHs induced EROD activity and CYP1A level in primary fish hepatocytes

Supplementary material to *In vivo* and *in vitro* effects of tunnel wash water and traffic related contaminants on aquatic organisms

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1. Sampling and chemical analysis

1.1 Sampling

Prior to the tunnel wash, a broom type sweeper collected coarse grained material from the road surface. Vacuumed material was pumped into a collection bin, sampled in baked glass jars and kept at -20°C until analysis. After sweeping, the walls were washed by a high-pressure washer. TWW used for ecotoxicity tests and general chemical analyses was sampled by grab sampling approximately halfway through the wash by use of submergible electric pump immersed into the ending manhole discharging TWW to the recipient. The pump and bottles were conditioned with TWW prior to sampling. Water for ecotoxicity tests and general water quality parameters was sampled in polyethylene bottles and water for metal analyses in Nalgene bottles. Water used by the contractor during the wash was collected from the washing unit (Oslofjord tunnel) and directly from the tap (Nordby tunnel), and used as control samples. Water for chemical analyses

were kept at 8°C and delivered to the laboratory directly after the tunnel wash. Water for ecotoxicity tests was brought to the freezer (-20°C) within 4 hours after sampling, and kept frozen until further preparations.

Suspended particle matter (SPM) from the TWW was collected by use of a continuous flow centrifuge (CFC), connected to the submergible electric pump used for water sampling. In brief, TWW was pumped into the spinning centrifuge (6000rpm) at approximately 1-3L/h. After 1-2h, starting halfway through the tunnel wash, sufficient material for chemical analyses had been collected. SPM attached to the centrifuge bowl wall (washed in acetone prior to sampling) was easily removed, placed in baked glass jars and kept at -20°C until analysis. SPM from the Oslofjord tunnel was only present in low levels and was not collected. The low levels of SPM might be a result of technical problems in the tunnel prior to the sampling event, after which heavy vehicles were not permitted through.

1.2 Analyses of water samples

The pH was measured by use of a combined pH sensitive electrode and a reference electrode, equipped with an automatic temperature compensation system. The turbidity was measured by a turbidity meter at 860nm, using formazin turbidity standards that provide results in formazin nephelometric units (FNU). Suspended particulate matter (SPM) in the TWW was determined by filtration through a glass fiber filter, and gain in mass on the filter (after drying) per unit volume of water filtered was defined as SPM concentration.

1.3 Chemical analysis of suspended particle material and coarse grained material

For PAH analysis, the samples were extracted with dichloromethane for 4 hours using ultrasonic bath (2 times 30 min) and intensive shaking. The extracts were dried with Na_2SO_4 before clean up with the use of gel permeation chromatography (GPC) as described by Harman et al. (Harman et al., 2008). Internal standard was added to the samples prior to the extraction. Seven deuterated PAHs (d8-naphthalene, d10-biphenyl, d8-acenaphthylene, d10-dibenzothiophene, d10-pyrene, d12-benzo[*a*]anthracene and d12-perylene) and 3 PCBs (PCB- 30-53,204) were used as internal standards. A certified reference material, SRM-1944 (NIST) were also analysed along with the samples.

For detection of nitro-PAHs, extracts were analysed using a Hewlett Packard 6890Plus GC coupled to a Hewlett Packard 5973 MS detector operated in SIM mode and negative chemical ionization (with methane). A pulsed splitless injection (2μL, injector temperature of 280°C and a pulse pressure of 50psi held for 2 min) was used to transfer analytes into a 15m-long DB-5MS (0.25mm i.d., 0.1μm film thickness) with a helium flow of 1mL/min. The GC temperature was held for 2 min at 60°C, then ramp of 10°C/min until 300°C, then 25°C/min until 345°C and then held for 2 min. This gave a total run time of 29.8 min. The temperatures of the transfer line, quadrupole and ion source were 300, 150 and 250°C respectively. Quantification of individual compounds was performed by using the relative response of surrogate internal standards.

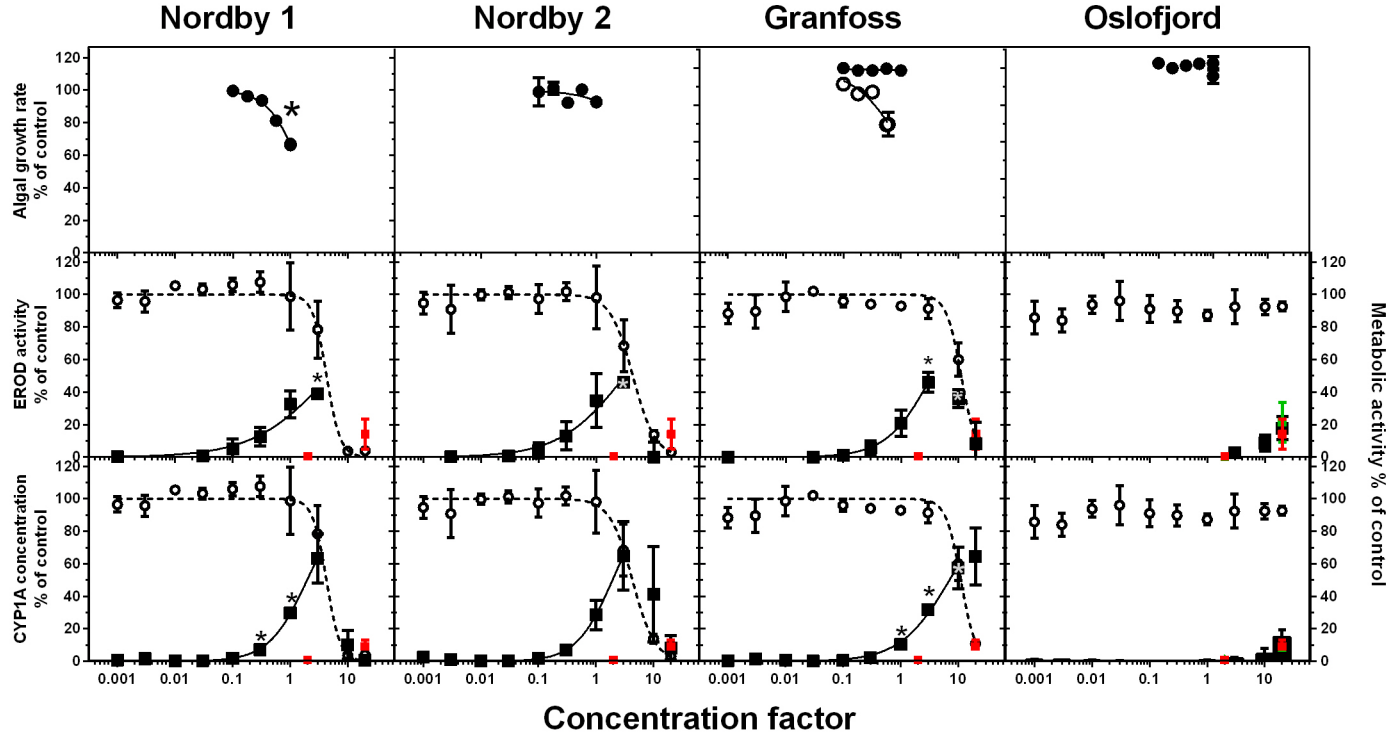
For detection of PAH16, methylated PAHs and chloro-PAHs, extracts were analysed on a HP-6890 Plus gas chromatograph equipped with a HP 5973 mass selective detector, operated in single ion monitoring mode (SIM) with electron impact ionisation (70 eV). Analytes were

separated on a 30 m DB-5 column (0.25mm i.d. and 0.25μm film thickness, Agilent JW Scientific, Santa Clara, USA) and with a helium flow of 1 mL/min. The injection was splitless and the injection volume was 1μL. The GC oven temperature was held for 2 min at 60°C before increasing to 250°C at a rate of 7°C min^{-1}. The final step was an increase to 310°C at a rate of 15°C/min (held for 5 min). Injector, transfer line, ion source and quadruple temperatures were set to 300, 280, 230 and 150°C, respectively. Quantification of individual compounds was performed by using the relative response of surrogate internal standards.

Figure legends

Figure 1. Growth rate of P*seudokirchneriella subcapitata* (top row) exposed to the filtered (0.22 µM) samples (●) from the Nordby 1, Nordby 2, Granfoss and Oslofjord tunnel wash water (TWW) and the organic fraction (○) from the Granfoss TWW. Growth rate significantly different from control ($P < 0.05$) are indicated by *, n = 3 (technical replicates). EROD induction (■, middel row) and CYP1A production (■, bottom row) as percentage of a positive control exposed to 0.3 nM TCDD and metabolic activity (○) are expressed as percentage of solvent control in rainbow trout primary hepatocytes exposed to TWW extracts. EROD induction and CYP1A levels significantly different from procedural blank and Oslofjord control (green and red squares respectively) are indicated with *. The data represent mean (± standard deviation) of 3 individual exposure experiments.

Figure 2. Induction of EROD activity (■) and CYP1A production (■) as percentage of a positive control exposed to 0.3 nM TCDD, and metabolic activity (○) expressed as percentage of solvent control in cells exposed to Cl- and N-PAHs. The data represent mean (± standard deviation) of 3 individual exposure experiments.


Nordby 1
Nordby 2
Granfoss
Oslofjord
Algal growth rate
% of control
EROD activity
% of control
CYP1A concentration
% of control
Metabolic activity % of control
Concentration factor
0.001
0.01
0.1
1
10
0
20
40
60
80
100
120

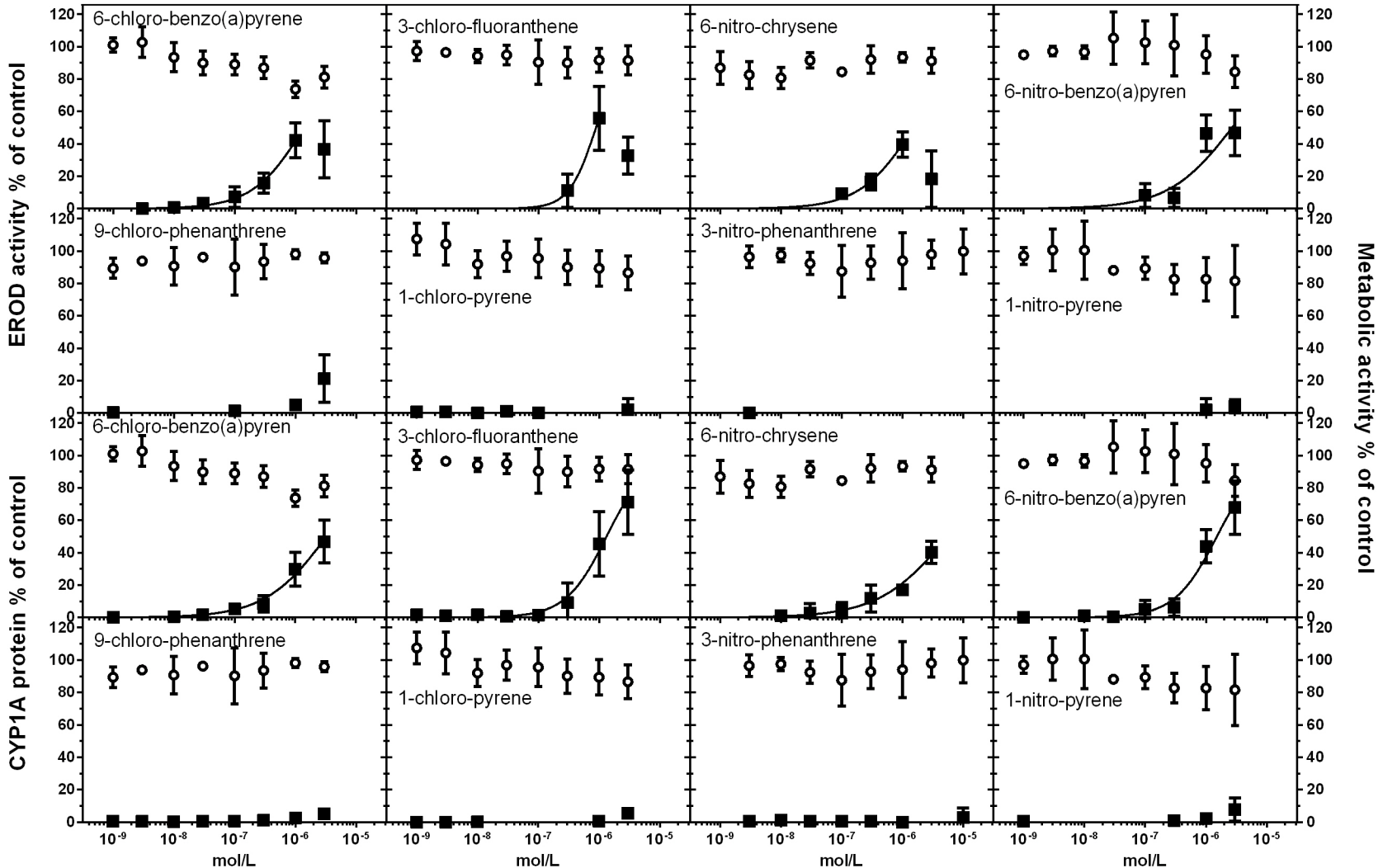

EROD activity % of control
CYP1A protein % of control
Metabolic activity % of control
6-chloro-benzo(a)pyrene
3-chloro-fluoranthene
6-nitro-chrysene
6-nitro-benzo(a)pyren
9-chloro-phenanthrene
1-chloro-pyrene
3-nitro-phenanthrene
1-nitro-pyrene
6-chloro-benzo(a)pyren
3-chloro-fluoranthene
6-nitro-chrysene
6-nitro-benzo(a)pyren
9-chloro-phenanthrene
1-chloro-pyrene
3-nitro-phenanthrene
1-nitro-pyrene
0
20
40
60
80
100
120
10-9
10-8
10-7
10-6
10-5
mol/L

Table 1. Overview of tunnel characteristics and collected samples

Tunnel, length	**Annual average daily traffic (AADT), vehicles per day**[a]	**Recipient (treatment)**	**Type of samples**	**Point of sampling**	**Sampling date**
Nordby, 3.8 km	32 600 (2013)	The river Årungselva			
Sample event 1		(sedimentation pond)	Water	Pump house	20.06.2013
Sample event 2			Water	Pump house	18.11.2013
			SPM		18.11.2013
			Coarse grained material		18.11.2013
Granfoss, 1 km	30 800 (2010)	The River Lysakerelva	Water	Last manhole in	28.02.2014
		(no treatment)	SPM	pipeline system for	28.02.2014
			Coarse grained material	discharges to	28.02.2014
				Lysakerelva	
Oslofjord, 7.3 km	6 827 (2013)[b]	The Oslofjord	Water	In pipeline system	18.11.2013
		(no treatment)		connected to	
				sedimentation basin in	
				the tunnel	

[a]From Torp and Meland (2013), [b]Considerable lower AADT is expected, due to technical problems in the tunnel. Heavy vehicles were not permitted access to the tunnel prior to the sampling event.

Table 2. Measured water parameters and concentrations of metals (µg/L) and PAHs in the total tunnel wash water samples (including suspended particulate material) from the tunnels Oslofjord, Nordby and Granfoss. Values are based on 1 grab sample.

	Nordby 1 TWW	**Nordby 2 TWW**	**Granfoss TWW**	**Oslofjord TWW**
Water parameters				
pH	7.42	7.59	7.55	7.88
Turb860 (formazin nephelometric units)	1769	1420	2706	8.77
Suspended particulate matter (mg/l)	1510	2180	1850	20.3
Metals (µg/L)				
Ag	3	<0.25	<1	<1
Al	36100	<30	38100	<30
As	4.7	<0.25	13	<1
B	110	110	103	780
Ba	313	130	553	10
Be	1.3	<0.05	1.8	<0.2
Bi	5	<0.5	5	<2
Ca	72700	61000	110000	234000
Cd	0.41	0.22	1.01	0.1
Co	33.5	0.88	43.4	0.2
Cr	133	5.6	110	<2
Cu	316	27.2	448	7.50
Fe	67000	0.040	62000	120
Hg	<0.001	<0.001		0.001
K	23000	21000	22200	68200
Li	48	12	45	42
Mg	25500	9700	43400	286000
Mn	1050	348	2350	<0.4

Mo	36	7.9	68	6.9
Na	322000	1480000	117000	2220000
Ni	70.1	4.9	103	<1
P	2580	<200	2380	<200
Pb	37.4	0.05	66.5	0.1
S	18700	25000	38100	208000
Sb	27	5.9	28	<1
Se	<20	<5	<20	60
Si	34500	4140	36300	6070
Sn	37	<0.5	48	<2
Sr	251		1220	3610
Th	11	<0.5	10.5	<2
Ti	5.07	<2	6940	8.3
Tl	<1	<0.25	<1	<1
U	3.8	0.94	5.36	21.3
V	112	3.84	158	<0.2
Zn	3290	501	2300	9.0

PAHs (ng/g d.w.)	**Nordby 2 CFC**	**Granfoss CFC**	**Nordby 2 Sweeper**	**Granfoss Sweeper**
$C_{1\text{-}3}$Dibenzothiophenes	3 500	3 700	970	740
$C_{1\text{-}3}$Phenanthrenes	2 900	4 700	1 400	810
$C_{1\text{-}3}$Naphthalenes	2 000	1 300	200	190
PAH_{16}	3 000	4 800	1 400	790
Nitro-PAHs (ng/g d.w.)				
1-N-naphthalene	1.3	1.9	0.9	<0.5
2-N-biphenyl	<5	<5	<3	<3
4-N-biphenyl	<5	<5	<3	<3
2-N-fluorene	<1	<1	<0.5	<0.5
9-N-anthracene	13	9.2	5.8	2.6
3-N-phenanthrene	0.9	1.0	0.6	<0.5

1-N-pyrene	<1	1.5	0.7	<0.5
2-N-pyrene	<5	<5	<3	<3
7-N-Benzo[*a*]anthracene	<5	<5	<3	<3
6-N-chrysene	<5	<5	<3	<3

Table 3. Summary of effects on primary hepatocytes after exposure to extracts from tunnel wash water. The EC_{10} and EC_{50} were obtained from the fitted concentration response curves. Concentrations correspond to the concentration factor where 1 corresponds to the concentration in the original water sample.

Extract	**Cytotoxicity**			**EROD activity**			**CYP1A protein production**		
	EC_{10}	EC_{50}	R^2	EC_{10}	EC_{50}	R^2	EC_{10}	EC_{50}	R^2
Nordby 1	2.3	4.3	0.95	0.18	4.8	0.91	0.37	2.0	0.96
Nordby 2	1.6	4.3	0.94	0.18	3.1	0.88	0.40	1.9	0.91
Granfoss	5.8	11	0.91	0.48	3.4	0.95	0.81	7.1	0.96
Oslofjord	-	-	-	-	-	-	-	-	-

Effect of control samples at concentration factor (CF) of 2 and 20

Extract	Cytotoxicity (% of control)		EROD activity (% of control)		CYP1A production (% of control)	
	CF 2	CF 20	CF 2	CF 20	CF 2	CF 20
Oslofjord control	110	110	0.37	22	1.9	8.6
Procedural blanc	100	97	0.67	14	1.0	9.3

Table 4. Effects in primary rainbow trout hepatocytes after exposure to the positive control TCDD and selected N- and Cl-PAHs on cytotoxicity, EROD activity and CYP1A production. EC_{10} and EC_{50} were obtained from the fitted concentration-response curves. Only results obtained from CRCs with R^2 values > 0.7 were considered reliable and are shown in the table. Estimated EC_{50} values outside the valid concentration range for the model are shown in italics.

Compound	**EROD activity**			**CYP1A production**		
	EC_{10} (µM)	EC_{50} (µM)	R^2	EC_{10} (µM)	EC_{50} (µM)	R^2
TCDD	$6.26E^{-6}$	$2.48E^{-5}$	0.96	$1.09E^{-5}$	$4.12E^{-5}$	0.97
9-Cl-phenanthrene	-	-	-	-	-	-
6-Cl-benzo(a)pyrene	0.16	*1.4*	0.91	0.25	*3.26*	0.90
1-Cl-pyrene	-	-	-	-	-	
3-Cl-fluoranthene	0.29	0.89	0.87	0.24	1.3	0.88
3-N-phenanthrene	-	-	-	-	-	-
1-N-pyrene	-	-	-	-	-	-
6-N-chrysene	0.16	*1.5*	0.87	0.31	*5.51*	0.88
6-N-benzo(a)pyrene	0.19	2.7	0.80	0.26	1.5	0.93