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Monitoring the biological effects of Nyhamna discharge water using caged Atlantic cod (*Gadus morhua*)

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REPORT

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Abstract

The potential biological effects of the process water (PW) from the Nyhamna gas processing plant on the West coast of Norway was assessed using biological responses in the Atlantic cod, *Gadus morhua*. Farmed cod were placed in large mesh cages at strategic locations from the Nyhamna PW discharge outlet including: 1) <50 m from the outlet; 2) 300-400 m downstream from the outlet; and 3) reference location (4-5 km from the outlet). A suite of biomarkers were measured in the cod following a relatively short (10 day) and a long (9 week) exposure duration. The biomarkers measured after 10 days were micronuclei formation (MN), vitellogenin (VTG) and ethoxyresorufin-O-deethylase (EROD), whilst those measured after 9 weeks included gill and gonad histopathology, peroxisomal proliferation, and neutral lipid content of the liver. Supporting parameters including condition index (CI), liver somatic index (LSI) and plasma chemistry were measured in fish from both short and long exposures. The exposure of the fish to chemicals thought to be present within the PW following treatment was determined using bile metabolites of exposed cod, together with passive sampling devices. The passive sampling devices included polar organic chemical integrated samplers (POCIS) and diffusion gradients in thin films (DGTs), which were used to target alkylphenols (APs) and metals respectively.

POCIS and DGTs were able to detect APs and metals within the receiving waters at Nyhamna but were only found to be present in low concentrations. There were no significant increases in biomarkers with proximity to the discharge outlet. Fish held within cages less than 50 m and 300-400 m from the discharge outlet had no significant effect on any of the biomarkers measured when compared to the reference and pre-exposure groups. The CI, LSI, and plasma chemistry clearly showed that fish caged for 9 weeks had suffered from starvation, which also resulted in fish mortalities in one of the fish groups.

Overall, based on the chemical assessment of APs and metals in the receiving waters at Nyhamna using POCIS, DGTs and bile metabolites, the PW discharge posed little to no environmental risk. The exposed cod appeared to be unaffected by the PW discharge with no significant biomarker responses despite the close proximity (<50 m) to the discharge outlet.

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Monitoring the biological effects of Nyhamna discharge water using caged Atlantic cod (*Gadus morhua*)

Preface

The report is a collaborative study between Det Norske Veritas (DNV) and the Norwegian Institute for Water Research (NIVA) with the principal aim to determine the potential biological effects of Nyhamna process water on fish living in the receiving waters. Atlantic cod (*G. morhua*) were deployed in cages at varying distances from the Nyhamna discharge outlet for up to 9 weeks. Chemical concentrations and biological effects endpoints were measured in the caged fish after approximately 10 days and again after 9 weeks of exposure.

The field work was performed by personnel from DNV and NIVA. The biomarker analysis was performed by NIVA, Centre for Environment Fisheries and Aquaculture Science (Cefas) UK, Institute of Marine Research (IMR) Norway, University of the Basque Country Spain, and Fürst medical laboratory, Norway.

Oslo, May, 2012

Steven Brooks

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1. Summary

The potential biological effects of the process water (PW) from the Nyhamna gas processing plant on the West coast of Norway was assessed using biological responses in the Atlantic cod, *Gadus morhua*. Farmed cod were placed in large mesh cages at strategic locations from the Nyhamna PW discharge outlet including: 1) <50 m from the outlet; 2) 300-400 m downstream from the outlet; and 3) reference location (4-5km from the outlet). A suite of biological endpoints were measured in the cod following a relatively short (10 day) and a long (9 week) exposure duration. The biomarkers measured after 10 days were micronuclei formation (MN), vitellogenin (VTG) and ethoxyresorufin-O-deethylase (EROD), whilst those measured after 9 weeks included gill and gonad histopathology, peroxisomal proliferation, and neutral lipid content of the liver. Supporting parameters including condition index (CI), liver somatic index (LSI) and plasma chemistry were measured in fish from both short and long exposures. The exposure of the fish to chemicals thought to be present within the PW following treatment was determined using bile metabolites of exposed cod, together with passive sampling devices. The passive sampling devices included polar organic integrated chemical samplers (POCIS) and diffusion gradients in thin films (DGTs), which were used to target alkylphenols (APs) and metals respectively.

POCIS and DGTs were able to detect APs and metals within the receiving waters at Nyhamna but were only found to be present in low concentrations. There were no significant increases in biomarkers with proximity to the discharge outlet. Fish held within cages less than 50 m and 300-400 m from the discharge outlet had no significant effect on any of the biomarkers measured when compared to the reference and pre-exposure groups. The CI, LSI, and plasma chemistry clearly showed that fish caged for 9 weeks had suffered from starvation, which also resulted in fish mortalities in one of the fish groups.

Overall, based on the chemical assessment of APs and metals in the receiving waters at Nyhamna using POCIS, DGTs and bile metabolites, the PW discharge posed little to no environmental risk. The exposed cod appeared to be unaffected by the PW discharge with no significant biomarker responses despite the close proximity (<50 m) to the discharge outlet.

2. Sammendrag

Potensielle biologiske effekter av prosess vann (PV) fra prosessanlegget på Nyhamna ble undersøkt ved analyse av biologiske responser hos eksponert Atlanterhavstorsk (Gadus morhua). Oppdrettstorsk ble plassert i store nettingbur satt ut ved utvalgte stasjoner nær utslippspunktet for PV: 1) <50 m fra utslippspunktet; 2) 300-400 m nedstrøms utslippspunktet; samt en referansestasjon 4-5 km unna utslippspunktet. Ulike biologiske endepunktsanalyser ble så målt i fisken etter 10 dager og 9 ukers eksponering. Biomarkøranalyser valgt for korttidseksponeringen var mikronukleusdannelse (MD), vitellogenin (VTG) og etoksyresorufin-O-deetylase (EROD), mens endepunktene målt etter langtidseksponering var histopatologisk analyse av gjelle og gonade, peroksisomal proliferering og nøytral lipid innhold i lever. I tillegg ble det målt støtteparametere som kondisjonsfaktor (KF), lever-somatisk indeks (LSI) og plasmakjemi i fisk etter både kort og langtidseksponering. Kjemisk eksponering i form av alkylfenoler og metaller ble målt i løpet av forsøket ved hjelp av passive prøvetakere som henholdsvis POCIS (Polar Organic Integrated Chemical Sampler) og DGT (Diffusion Gradients in Thin films).

Både alkylfenoler og metaller ble målt i lave konsentrasjoner i resipienten Nyhamna, men det ble ikke funnet signifikant økning biomarkøranalysene som resultat av eksponeringen. Fisk holdt i bur mindre enn 50 m og 300-400 meter fra utslippspunktet viste ingen signifikante endringer sammenlignet med referansegruppe og preeksponeringskontroll. Resultater for kondisjonsfaktor, LSI og plasmakjemi viste imidlertid at fisk holdt i fangenskap i 9 uker sultet. Dette kan trolig forklare mortalitet som ble funnet i en av forsøksgruppene.

På bakgrunn av kjemidata for alkylfenoler og metaller i resipienten Nyhamna, samt konsentrasjon av disse stoffenes metabolitter i fiskegalle, vurderes PV utslippet å utgjøre en liten eller ingen miljørisiko. På tross av nærheten til utslippspunktet (<50 m), viste eksponert torsk ingen signifikant endring i målte biomarkørnivåer.

3. Introduction

3.1 Background on Nyhamna

The plant at Nyhamna is located on the island of Gossa situated on the West coast of Norway where it processes gas, water and condensate received by pipeline from the Ormen Lange gas field approximately 120 km offshore in the Norwegian Sea. The Ormen Lange gas field was first discovered in 1997 and is one of the largest natural gas fields on the Norwegian continental shelf. Nyhamna is currently operated by Norske Shell and has been in production since 2007.

The stream of gas, condensate, water and other well fluids are transported to the Nyhamna land base by pipeline where they undergo a series of separations and clean up. The first process is the slug catcher, which reduces the velocity of the incoming materials. Gas, condensate, water and antifreeze (MEG- mono-ethylene glycol) are then separated by different processes. The gas is dried before compression and export by pipeline to the UK. The condensate is stabilised and stored in a large underground cavern prior to export by tanker, whilst the antifreeze is separated and recycled. The remaining produced/process water (PW) undergoes both biological treatment and macro porous polymer extraction (MPPE) technology. Together they remove most of the residual mono ethylene glycol (MEG) and tri-ethylene glycol (TEG, used to remove water from the gas stream), aliphatic hydrocarbons, benzene, toluene, ethylbenzene, and xylenes (BTEX), polycyclic aromatic hydrocarbons (PAHs) and naphthalene, phenanthrene and dibenzothiophenes (NPDs), and some polar compounds including alkylphenols (APs) that are typically found in PW (Aker Kværner, 2005). Before being discharged into the marine environment the PW is diluted in a ratio of approximately 1:700 with cooling water. The PW enters the receiving waters through a single discharge outlet positioned on the sea floor at a depth of approximately 40 m.

The chemical bioaccumulation and biological effects of the discharge water from Ormen Lange has previously been investigated using a combination of caged mussels and passive sampling devices (Brooks et al., 2010; 2011). These studies revealed low and/ or undetected concentrations of PAHs, metals and APs in either mussels and/ or passive samplers positioned in the receiving waters at the mouth of the discharge. However, despite these low or undetected concentrations of PW related chemicals, biological effects have been observed in exposed mussels compared to the reference mussels. Furthermore, in a separate study, APs have been found in the concentrated discharge water (prior to discharge), which were measured at concentrations considered to be potentially harmful to aquatic life (Weideborg and Vik, 2010). Due to the potential environmental risk of APs measured in the effluent (prior to discharge), Polar Organic Chemical Integrated Samplers (POCIS) devices, previously identified to be a suitable method of accumulating APs in the environment (Harman et al., 2011), were used to measure APs in the receiving waters. In addition, caged fish were used to measure the potential biological effects of exposure to the discharge effluent.

3.2 Biomarkers

A suite of biological effects endpoints (biomarkers) were measured in caged fish following both short term (10 days) and long term (9 weeks) exposure to the discharge water at Nyhamna. The biological effects measured included both general health parameters and specific effect endpoints. The biological effects methods included condition index (CI), plasma chemistry, micronuclei formation (MN), ethoxyresorufin-O-deethylase (EROD), vitellogenin (VTG), neutral lipid accumulation (NL), Acyl-CoA oxidase (AOX) activity, and gill and gonad histopathology. A short description of these methods is provided below.

3.2.1 Plasma chemistry

Fish blood samples were analysed for 12 plasma chemistry parameters, including parameters of lipid metabolism (total cholesterol, triglycerides), cations (calcium, potassium, sodium, magnesium, iron), anions (chloride), proteins (albumin, total protein), as well as lactate dehydrogenase activity (LD) and inorganic phosphate. In general, there are no established reference values for plasma metabolites in fish unlike that observed in mammals. Plasma metabolites are also known to vary considerably between different fish species. The only way to evaluate effects related to exposure to e.g. pollutants is by comparing different treatment groups with an untreated reference group. A short introduction to some of the analysed plasma parameters (above instrument threshold) are given below.

- **LD** is an indirect measure of lactate build-up in fish plasma. LD converts pyruvate, the final product of glycolysis, to lactate when oxygen is absent or in short supply. Moderately increased lactate levels indicate fatigue as a result of high muscular activity, which is common in fish during periods of stress. Abnormally high levels point at acute cellular damage in blood or liver, often provoking anaemic conditions.
- **Inorganic phosphate** levels are induced e.g. as a result of haemolysis, underperformance of the thyroid gland, renal failure, respiratory acidosis (build-up of blood CO_2 levels due to hypoventilation), metabolic acidosis (increased production of metabolic acids or disturbances in the ability to excrete acid via the kidneys), assimilation of acidic compounds from the seawater. Low plasma phosphate levels are seen as a result of vitamin D deficiency, over performance of the thyroid gland, phosphate malsorption and alkalosis (due to hyperventilation).
- **Free calcium** quantifies all calcium which is not associated with albumin. Low levels of free calcium are e.g. associated with liver diseases, but also vitamin D deficiency, magnesium deficiency, malsorption.
- **Free magnesium** quantifies all magnesium which is not associated with albumin. In fish subnormal magnesium levels are typically related to starvation/malnutrition syndromes, whereas supernormal values indicate a renal problem.
- Abnormally increased levels of plasma **iron** in fish plasma are typically associated with haemolysis or acute hepatitis. Subnormal iron levels are associated with infections.
- **Cholesterol** (total) consists of a family of lipoproteins (mainly) synthesized in the liver that together form the most important class of lipids transported in fish plasma. Low levels are typically associated with starvation-like syndromes including malsorption since lipids are being oxidised in cellular respiration once carbohydrates become depleted. Supernormal cholesterol levels are not common in (wild) fish.
- **Triglycerides** form the second most important class of lipids that are transported in fish plasma and levels usually follow the same pattern as cholesterol, however not necessarily with the same magnitude or at the same point of a starvation syndrome. Theoretically triglyceride levels, being more closely associated with energy production, are more responsive than cholesterol.

3.2.2 Micronuclei formation (MN)

As an index of chromosomal damage, the micronuclei (MN) assay is an indication of DNA damage and provides a time-integrated response to complex mixtures of pollutants. MN are chromatin-containing structures deriving from the nucleus and have no detectable link to the cell nucleus. The frequency of MN is regarded as an important tool for *in situ* monitoring of genotoxicity. This assay has been used for various organic and inorganic pollutants in laboratory studies as well as in field studies (Baršienė et al. 2006; Brooks et al., 2011) and recommended for coastal and offshore biomonitoring (Baršienė et al., 2006; Gorbi et al., 2008).

3.2.3 Ethoxyresorufin-O-deethylase (EROD)

In the cytochrome P450 (CYP P450) superfamily, CYP1A-subfamily enzymes are one of the most important concerning biotransformation/bioactivation of xenobiotics. These enzymes are induced when the cell is exposed to xenobiotics like 2,3,7,8-tetrachloro-dibenzo-dioxin (TCDD), planar

polychlorinated biphenyls (PCBs), or polycyclic aromatic hydrocarbons (PAHs) (Goksøyr & Förlin, 1992). CYP1A are heme-containing proteins, mainly located on the surface of the smooth endoplasmatic reticulum within cells. The enzymes are isolated in the so-called microsomal fraction by differential ultracentrifugation following homogenisation of the cell. Most of the enzyme activity is retained using this procedure and CYP1A activity is measured in the microsomal samples in the ethoxyresorufin-O-deethylase (EROD) assay. Here, 7-ethoxyresorufin is used as an artificial substrate for CYP1A and fluorescence of the product resorufin is measured as an indication of the CYP1A-activity.

3.2.4 Vitellogenin (VTG)

The synthesis of the yolk protein vitellogenin (VTG) takes place in the liver of oviparous female fish under the stimulation of endogenous estradiol (Tata and Smith, 1979). Male and juvenile fish of most species, which normally have extremely low levels of circulating estrogens, do not produce appreciable levels of VTG. However, these fish have numerous hepatic oestrogen receptors and are capable of producing high concentrations of VTG when exposed to exogenous estrogens. Induction of this female typical protein in male and juvenile fish has therefore been widely used as a sensitive biomarker for exposure to xenoestrogens (Sumpter and Jobling, 1995). The use of VTG as a biomarker for xenoestrogens in ecologically relevant fish species has been employed for coastal and freshwater environmental monitoring (Hylland et al., 2008; Brooks et al., 2011) and for monitoring of areas that are effected by discharge from oil production activities (Scott et al., 2006).

3.2.5 Neutral lipid accumulation

The effects of pollutants are often associated with unbalanced fatty acid metabolisms and the accumulation of neutral lipids in the lysosomal vacuolar system. The lysosomal storage of neutral lipids in fish liver has been found to be a useful marker of change in the physiology of the cells (Köhler, 1991). Neutral lipid accumulation appears to be more strictly linked to organic chemical pollution (Lowe and Clarke, 1989, Cajaraville et al., 1992). Lipophilic xenobiotics may alter the metabolism of neutral lipids leading to abnormal accumulation of that lipid class inside lysosomes (Moore, 1988).

3.2.6 Peroxisomal proliferation (measured as Acyl CoA-oxidase (AOX) activity)

Peroxisomal proliferation: Peroxisomes are membrane-bound cytoplasmic organelles involved in lipid metabolism, oxyradical homeostasis and several other important cell functions (Cancio and Cajaraville, 2000). One of the unique features of peroxisomes is their ability to proliferate and to enhance their metabolic activity under exposure to certain organic chemical compounds, a phenomenon termed "peroxisome proliferation" (Fahimi and Cajaraville 1995). Laboratory and field studies have shown that phthalate ester plasticizers, PAHs, oil derivatives, Polychlorinated biphenyls (PCBs), certain pesticides, bleached kraft pulp and paper mill effluents, alkylphenols and estrogens all provoke peroxisome proliferation in different fish or bivalve mollusc species (Cajaraville et al., 2000). Peroxisome proliferation consists of an increase in peroxisome volume and numerical densities, which is usually accompanied by the induction of some peroxisomal enzyme activities, particularly those of the fatty acid ß-oxidation system, such as acyl-CoA oxidase or AOX (Fahimi and Cajaraville 1995). In molluscs, peroxisome proliferation, measured in terms of AOX activity induction, has been proposed as an exposure biomarker for organic pollutants (Cajaraville et al., 2000).

3.2.7 Gill and Gonad histology

Histological parameters are commonly used as markers of health status in fish. The identification of pathologies and diseases are increasingly being used as indicators of environmental stress since they provide a definite and ecologically-relevant end-point for chronic/ sub chronic contaminant exposure. The application of histological markers in fish can include measures of reproductive and metabolic condition, and allows for the detection of various pathogens that may affect population mortality. The

data generated from this type of analysis in various organs (i.e. gonads, gills) is helpful in providing complementary information to support additional cellular and biochemical based biomarkers techniques (Bignell et al., 2011). Furthermore gonad development is an important aspect of research related to the biological effects of contaminants (COMPRENDO, 2006). Histological markers assessing the status of gonads may give an indication of contaminant effect on the reproductive performance.

3.2.8 Alkylphenol metabolites

The extensive bio-transformation of APs by fish greatly prevents the accumulation of these compounds in extra-hepatic tissues. Consequently, tissue concentrations of parent APs do not usually provide an adequate assessment of the AP exposure. AP metabolites concentrate in the gall bladder of fish following bio-transformation. Analysis of AP metabolites in the fish bile therefore constitutes a sensitive method for the assessment of AP exposure. The high relevance of fish bile metabolites in relation to PW exposure has been observed in environmental monitoring campaigns in the North Sea (Brooks et al., 2011; Hylland et al., 2008), as well as in pilot laboratory studies (Sundt et al., 2009). Fish AP metabolites have been determined using gas chromatography with mass spectrometric detection (GC-MS).

3.3 Passive samplers

The principle of passive sampling is the placement of a device in the environment for a fixed period of time, where it is left unattended to accumulate contaminants by diffusive and/or sorptive processes. They offer sensitive, time-averaged sampling without confounding factors which may occur when using biomonitoring organisms. In the present study they were used as a support parameter to indicate exposure to groups of chemicals. The present passive sampling design has been chosen to focus on the APs most abundant in PW ($C_{1\text{-}5}$). Biological methods for measuring exposure to APs are either lacking or poor. The chosen passive sampling device is the POCIS (Alvarez et al., 2004), which has previously been shown to be suitable for measuring PW originating APs (Harman et al., 2009).

In addition to the POCIS, diffusive gradients in thin films (DGTs) were used to provide a time integrated measure of the labile metal fraction in the water column during the exposure durations. The labile metal fraction is comprised of both the ionic form and inorganic complexed metal, which is considered to be bioavailable and thus able to pass through biological membranes. The labile metal fraction is the most biologically relevant measure for linking of exposure with observed biological effects. DGTs have been successfully used in laboratory and field studies including monitoring programmes (Montero et al., 2012).

3.4 Objectives

The main objective of the present study was to evaluate the potential biological effects of the Ormen Lange PW using biological effects measurements in caged Atlantic cod, *Gadus morhua* located at varying distances from the Nyhamna discharge outlet. In addition, in order to attempt to link biological effects with chemical exposure, passive sampling devices were used to measure selected compounds within the receiving waters.

4. Methods

4.1 Source of fish and transport

Atlantic cod (200 ± 50 g) were obtained from a fish farm on the West Coast of Norway. Over 300 cod were transported via road from the fish farm to the survey vessel at the small boat harbour of Håsundet in a large purpose-built fish transporter with continuous aeration. Upon arrival at the harbour the fish were placed into three separate 1000 L storage containers with running seawater on the research vessel, where they were kept overnight prior to cage deployment the following morning. All fish appeared in optimal health, with no mortalities recorded prior to deployment.

4.2 Pre-exposure sampling

To determine the health of the fish prior to field deployment, twenty fish were sub-sampled and measured for the same biomarker endpoints as used for the exposure fish (**Table 2**). The pre-exposure sampling took place in a temporary field laboratory set-up at Håsundet harbour on the morning after the cage deployment. The pre-exposure sampling was used to provide baseline levels of biomarkers for the fish in the study.

4.3 Cage deployment and retrieval

Fish cages were placed in the sea at varying distances downstream from the PW discharge outlet at Nyhamna on the 8th August 2011. Site 1 was positioned less than 50 m from the discharge, Site 2 approximately 300 - 400 m from the discharge and Site 3 was used as a reference location approximately 4-5 km away (**Figure 1**). Two separate fish cages were placed at each site approximately 10 m away from each other with the aim of sampling one cage after 10 days and the other after 9 weeks. The fish cages were held in position by an anchor (~300 kg). The fish cages at all stations were at a depth of 26 ± 4 m, with the cages held upright in the water column with submerged buoys at a depth of 23 ± 4 m. Approximately 50 fish were transferred with a hand held net from the 1000 L containers onboard the vessel to the fish net cages held by the side of the boat. The location of the fish cages in the water and a visual inspection of the fish within each cage were confirmed with a camera mounted remote operated vehicle (ROV, **Figure 2**). The passive sampling cages, containing POCIS were mounted to the side of the fish cages at each site, and temperature loggers were position at 5 m intervals to determine stratification of the water column (**Figure 2**).

The first retrieval took place 10 days after deployment on the 18th August, where one cage from each site was sampled. The fish were retrieved from the cages and placed in the 1000 L containers of running seawater. The fish were brought directly to the field laboratory at Håsundet harbour within 20 min and were processed immediately upon arrival.

Fish were anaesthetised in seawater containing 100 mg/L MS222. After a few minutes the fish were removed and length and weight taken. Blood was removed from the caudal vein with a needle and syringe coated with heparin and aprotinin. The blood was centrifuged at 2000g for 5 min at 4^{o}C with a refrigerated centrifuge. The supernatant was divided into 2 separate vials for VTG analysis and plasma chemistry respectively

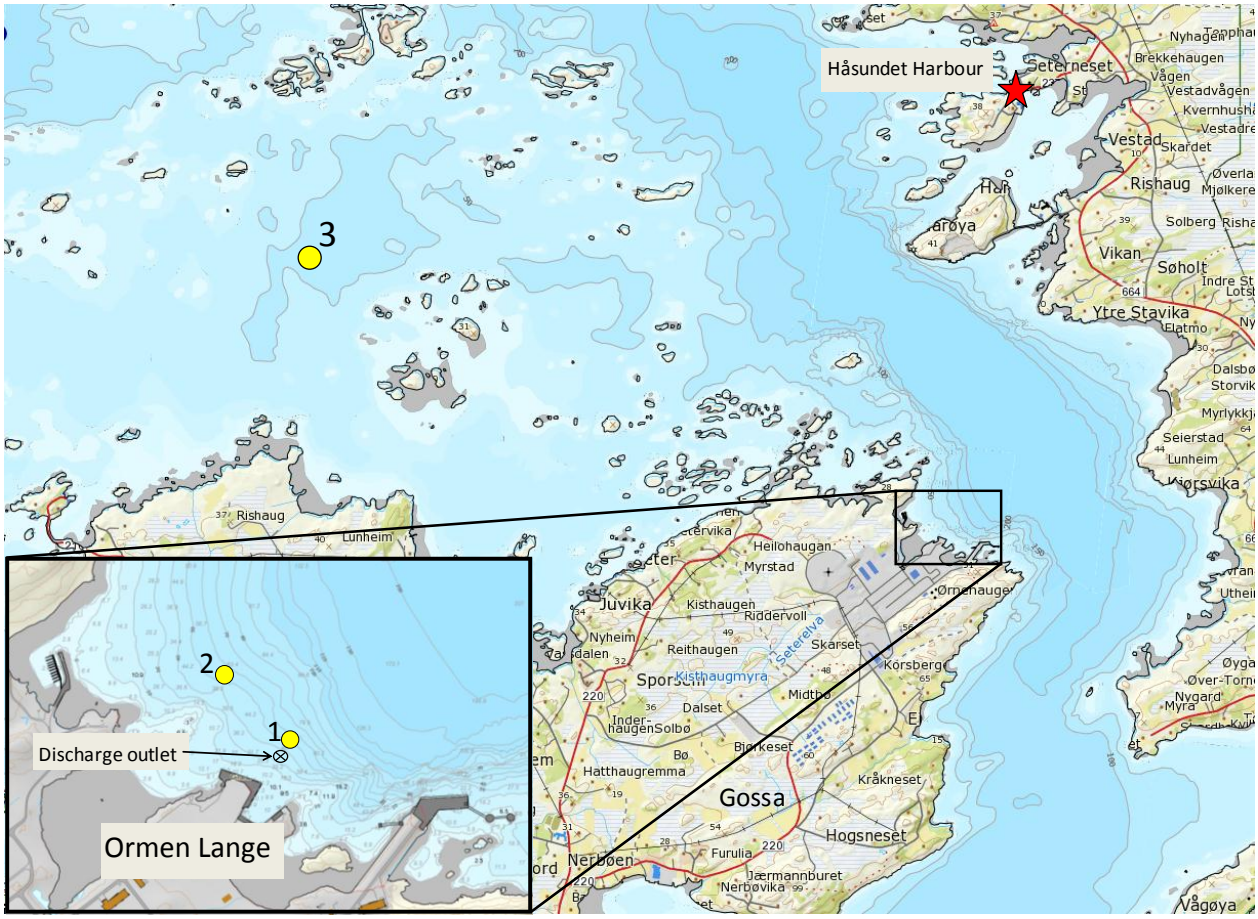

Figure 1. Locations of the large fish cages at the 3 sites. Site 1 <50 m from the discharge outlet; Site 2, 300-400 m from the discharge outlet; and 3) Reference Site, 4-5 km from the discharge.

Table 1. Position and depth information for the fish cage rigs.

Station	Position		Depth (m)		
	Latitude	Longitude	Seabed	Middle of fish net	Top buoy
1A	62 51.183	6 57.205	52	22.5	17
1B	62 51.173	6 57.198	51.5	22	17
2A	62 51.24	6 56. 986	56.5	27	21
2B	62 51.239	6 56.996	57	27	21
3A	62 51.756	6 53.715	58	28	23
3B	62 51.749	6 53.705	58.5	28.5	23.5

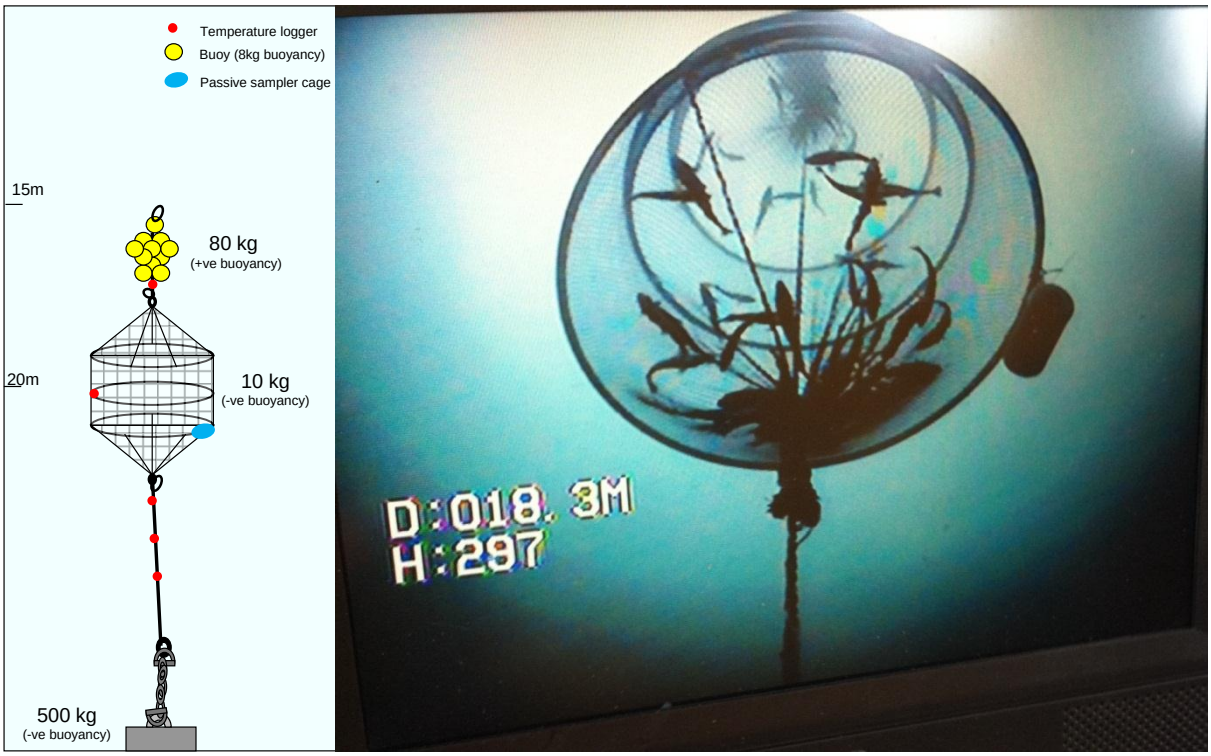

Figure 2. Image of the fish cage within the water, captured with an ROV. Inserted panel: schematic of the fish cage rig design, showing position of the temperature loggers, passive samplers and fish.

Table 2. Chemical and biological endpoints measured in the pre-exposed and exposed fish. POCIS and DGTs were used to measure alkyl phenol and metal concentrations respectively.

Exposure duration	Method	Tissue	Sampling	Information
Short (10 day)	Micronuclei	Blood	Smeared on microscope slides	Provides a measure of DNA damage.
	EROD	Liver	Fixed in liquid nitrogen and stored at -80^{o}C.	Sensitive indicator of PAH exposure.
	Vitellogenin	Blood plasma	Centrifuged and supernatant stored at -80^{o}C.	Sensitive to xenoestrogenic exposure (e.g. alkylphenols)
Long (9 weeks)	Lipid content	Liver	Fix in liquid nitrogen and stored at -80^{o}C.	Provides a general health marker.
	Peroxisomal proliferation (AOX activity)	Liver	Fix in liquid nitrogen and stored at -80^{o}C.	Exposure to organic pollutants such as petroleum products.
	Neutral Lipid accumulation	Liver	Fixed in liquid nitrogen and store at -80^{o}C.	Sensitive histochemical endpoint, providing general health marker.
	Fish gill and gonad histology	Gill/ gonad	Fixed in Bakers calcium formalin	Provides general fish health
Both Long & Short	AP metabolites	Bile	Stored at -20^{o}C.	Provides a measure of Alkyl phenol exposure (HPLC and GC-MS)
	Plasma chemistry	Blood plasma	Centrifuged and supernatant stored at -20^{o}C.	General fish health
	Condition Index/ Gonad somatic Index/ Liver somatic Index	Whole fish/ gonad/ liver	Measured in the field.	General fish health
	POCIS	Extracts	Stored frozen at -20oC until analysis.	Polar organic compounds (e.g. alkylphenols)
	DGT	Extracts	Stored in moist purpose built containers and refrigerated until analysis.	Bioavailable metals

4.4 Sea temperature

Temperature loggers (Hobo pendent Temp/light) were positioned on the rigs at each station. Five temperature loggers were positioned at known depths, with 5 m intervals to determine the temperature depth profiles at each of the stations. The main purpose of the temperature loggers was to determine the presence of a thermocline in the receiving waters as well as to detect the warmer waters of the PW discharge during the exposure.

4.5 General biological observations

Total weight and length of each cod sampled was measured in the field laboratory. Fish were sexed by visual examination of their gonad (later confirmed by histology). In addition, the weight of both the liver and the gonad from each fish was recorded.

The condition index (CI) was determined by the ratio between total weight and the cube of the fork length of the fish.

$$CI = [\text{Weight (g)}/ \text{Length (cm)}^3)] \times 100$$

The liver somatic index (LSI, liver index), which is considered to reflect fish nourishment status was calculated in pre-exposure and caged fish using the following equation:

$$LSI = [\text{Liver weight (g)} \times 100]/ \text{fish weight (g)}$$

The gonadosomatic index (GSI, gonad index), which provides an indication of the fish reproductive status was calculated using the following equation:

$$GSI = [\text{Gonad weight (g)} \times 100]/ \text{fish weight (g)}$$

4.6 Chemical measurements

4.6.1 Alkylphenol metabolites in cod bile

Bile (200 µl) was diluted in 200 µl sodium acetate buffer (0.01 M, pH 5), 36 µl *β*-glucuronidase (115600 units/ ml) was added, and samples incubated at 37°C for 2 h. A mixture of six deuterated alkylphenols as surrogate internal standards (SIS) was added to the solution, which was then further diluted with 2 ml acetic acid (0.1 %). The mixture was then loaded onto Oasis® HLB (Waters, Milford, USA) solid phase extraction (SPE) columns (4 cc volume), previously preconditioned with 1 ml methanol and 1 ml acetic acid (0.1 %), successively. Each column was then rinsed with 3 ml acetic acid (0.1 %) and dried for 30 min under vacuum. The analytes were extracted with 4 ml of methanol. Extracts were then evaporated to ca. 0.2 ml under a stream of nitrogen (40°C). The eluate was derivatizated with pentafluorobenzoyl chloride as described elsewhere (Boitsov et al., 2004) and the samples concentrated to 0.5 ml in hexane under a nitrogen stream (40°C). All samples were analysed by GC-MS (HP5890 GC; Shimadzu QP2010MS) in selected ion monitoring (SIM) mode. Over 60 AP deriviatives were analysed.

4.6.2 Alkylphenols in POCIS extracts

Standard POCIS, with a surface area per mass of sorbent ratio of ca. 180 cm^2/ g were obtained from ExposMeter (Tavelsjo, Sweden). The 'pharmaceutical' configuration was used, containing Oasis® HLB sorbent between two discs of polyethersulphone (PES) membrane. POCIS were used at all stations and deployed in commercially available stainless steel canisters (Environmental Sampling Technologies, Saint Joseph, USA,), which were attached directly to the fish cages at all stations. The

POCIS were retrieved together with the fish after 10 days and 9 weeks. After retrieval, samplers were frozen at -20^{o}C until analysis.

For extraction POCIS were carefully opened and the sorbent washed with water (Option 3, Elga™) into an empty, solvent rinsed solid phase extraction reservoir (International Sorbent Technologies, Hengoed, UK) and dried using nitrogen. The membranes were discarded. Internal standards were added before elution with 20 ml of methanol or acidified methanol. For AP analysis extracts were concentrated to 0.5 ml, derivatised with 10% pentafluorobenzoyl chloride and extracted with 4 mL hexane (Boitsov et al., 2004).

4.6.3 Metals in DGTs

DGTs for metal sampling were supplied from DGT Research LTD (Lancaster, UK). The samplers consisted of a polyacrylamide hydrogel with ion exchange Chelex resin. After removal of the gel, the resin was eluted in 1 ml conc. HNO_3 and, diluted 10 fold with ultra-pure water (Garmo et al., 2003). The trace metals (Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn) were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) using a Perkin-Elmer Sciex ELAN 6000. The mass of each metal accumulated (*M*) was measured after a known deployment time (*t*). The following equation was used to calculate the water concentration (*C*).

$$C = M\Delta g \,/\, (DtA)$$

Where *D* is the diffusion coefficient, *A* is the surface area of the sampler and Δg is the thickness of the diffusion layer (Zhang and Davison 1995). Further details concerning the determination of diffusion coefficients and correction for temperature are given by Zhang and Davison (1995).

4.7 Biomarkers in Cod

4.7.1 Plasma chemistry

Blood samples were taken from live and anesthetized fish by puncture of the caudal vein with a 2 ml syringe pre-treated with heparin to avoid blood coagulation. Blood samples were held on ice in micro tubes until being centrifuged at 2000 x g for 5 min at 4 °C. Groups of 3-5 samples were centrifuged each time. After centrifugation, plasma was transferred to a fresh tube using a Pasteur pipette and immediately snap-frozen in liquid nitrogen. Plasma was stored on dry ice during transport to the laboratory, and at -80 °C until analysed. On the day of analysis, plasma was thawed on ice and diluted 1:5 with MilliQ water in a fresh tube. Diluted plasma was held on wet ice during transport to Fűrst Medical Laboratory in Oslo, where plasma were analysed in a fully automated system using standard reagents for plasma chemistry (same as for clinical samples).

Unfortunately, and due to difficulties in sampling a sufficient blood volume (>1 ml) from each fish, a necessary dilution step of all samples resulted in five of the parameters (potassium, sodium, chloride, albumin and total protein) being below the detection limit. However, the alternative to dilution would have been pooling of samples, which would have hampered the possibility to relate the results in plasma to other biomarkers on an individual basis.

4.7.2 Micronuclei formation

A drop of blood was placed towards the end of a glass slide. A second slide was used to back into the drop of blood (at an angle of approximately 45º) and was then pushed across the slide to make the blood smear. The slides were left to dry before they were stained with bisbenzimide (1µg/ ml, 5 min) (Sigma-Aldrich, St. Louis, MO, USA), rinsed with distilled water and mounted in 1:1 glycerol-

Mcllvaine buffer containing 5 mM citric acid (Sigma-Aldrich) and 40 mM disodium hydrogen phosphate (Merck KGaA, Darmstadt, Germany).

The slides were scored under a fluorescence microscope (Olympus IX71, Olympus, Tokyo, Japan) using the Cell D imaging software (Olympus Soft imaging Solutions, Münster, Germany). A total of 2000 intact cells with distinct nuclear and cellular membranes were scored per slide. Micronuclei were identified as spherical cytoplasmic inclusions, with a sharp contour completely separated from the nucleus. The colour and texture of the micronuclei resembled the nucleus and the diameter was smaller than one-third of the nucleus.

4.7.3 Ethoxyresorufin-O-deethylase (EROD)

Microsomes were prepared on ice with pre-cooled equipment and solutions. Cryo-preserved liver samples were homogenized in a potassium phosphate buffer (0.1 M, pH 7.8) containing KCl (0.15 M), dithiothreitol (DTT) (1 mM), and glycerol (5% v/v), using a Potter-Elvehjem Teflon–glass homogenizer. The homogenate was centrifuged (10,000 × g; 30 min, 4°C) before the supernatant was recentrifuged (50,000 × g; 120 min, 4°C). The microsome fraction was obtained by resuspending the resulting pellet in potassium phosphate buffer (0.1 M, pH 7.8) containing KCl (0.15 M), DTT (1 mM), EDTA (1 mM), and glycerol (20% v/v). EROD activity was assayed fluorimetrically in a plate reader. Briefly, samples of microsomes were diluted to ~2 mg/ml in buffer and pipetted (50 µl) in 6 technical replicates onto a 96 well microplate. Pre-prepared resorufin standards (duplicates) were then added to subsequent wells. Reaction mixture (200 µl, containing 0.1 M potassium phosphate buffer, pH 8, and 3 µM 7-ethoxyresorufin) was added to the sample wells, before NADPH solution (2.4 mM in final well volume of 275 µl) was added to initiate the reaction. Transformation of 7-ethoxyresorufin to resorufin was read in 8 steps on the plate reader. Excitation was at 530 nm and fluorescence emission was measured at 590 nm. The EROD activity values were normalized to the protein content in the microsomal fraction and expressed as pmol/min/mg microsomal protein. Protein concentrations were determined according to Lowry et al. (1951), adapted to measurement by plate reader. The protein standard was bovine gamma globulin.

4.7.4 Vitellogenin

Blood samples were taken from the caudal vein of each fish with separate syringes containing heparin (10000 IU/mL, Sigma) and the protease inhibitor Aprotinin (5 TIU/mL, Sigma). The blood samples were centrifuged at 2000 g for 5 min, 4°C in a microcentrifuge before the supernatant (plasma) was removed and transferred to labelled cryovials and snap-frozen in liquid nitrogen. Plasma samples were stored at -80°C until analysis. Vitellogenin was determined in plasma from caged cod using a competitive ELISA with cod vitellogenin standard and competing antigen. The analyses were performed using a kit (V01006401) from Biosense Laboratories AS (Bergen, Norway) with anti-cod antiserum and cod vitellogenin as standard, according to the instructions of the manufacturer.

Plasma samples were diluted 50 and 5000 times in Phosphate buffer saline, pH 7.2. The plasma samples were transferred to 96 well microplates, each containing duplicates of the diluted sample, a blank and a positive control (cod sample). In addition, two VTG standard series were transferred to the microplates. The plates were sealed and incubated for 1 hour at 37 °C. The plates were washed three times in PBS buffer. Detecting antibody with dilution 1:500 was added to the wells and incubated for 1 hour at 37 °C. The plates were washed three times in PBS buffer. Secondary antibody with dilution 1:2000 was added to the wells and incubated for 1 hour at 37 °C. The plates were washed five times in PBS buffer and TMB substrate solution was added to the wells. The plates were incubated in the dark at room temperature for 30 min. The reaction was stopped with 0.3 M H_2SO_4 and the absorbance read at 450 nm. The VTG-concentration in the diluted samples was determined using the equation for the adjusted standard curve from the standard series. The VTG concentration was multiplied with the dilution factor and expressed in ng/mL.

4.7.5 Peroxisomal proliferation

Liver samples were individually homogenised on ice in a Braun-Potter homogeniser using ice cold 60 mM Tris, 0.25 M sucrose buffer (pH 8.3). After homogenisation, samples were centrifuged at 600 g for 20 minutes at 4^{o}C. Supernatants were removed and diluted appropriately to perform the assays. Actyl-CoA oxidase activity was measured as described by Small et al. (1985). Total protein of all samples was measured according to the Lowry method using a commercial protein as standard (BioRad, California). The activity is measured as mU AOX / mg protein.

4.7.6 Neutral lipid accumulation

For the determination of unsaturated neutral lipids, cryostat liver sections (10 µm) were fixed in Baker`s calcium-formol for 15 min, rinsed in distilled water and transferred into 60% triethylphosphate (v/v with distilled water) for 1 min. Sections were stained in 1% solution of Oil Red O in 60% triethylphosphate for 15 min. Then they were rinsed in 60% triethylphosphate for 30 s, washed in distilled water and mounted using aqueous mounting medium. Neutral lipid accumulation was assessed microscopically at 400x magnification.

4.7.7 Gill and gonad histology

Gill sub-samples were taken from live and anaesthetized fish, placed in pre-marked histocassettes and immediately transferred to a plastic container filled with 10% Neutral Buffered Formalin (NBF) purchased ready-to-use from Sigma Aldrich. Complete fish gonads were removed from dissected fish and treated in the same way as gill samples. Histology samples were shipped to the UK under NBF for clinical evaluation at the CEFAS laboratory in Weymouth. Samples were embedded, cut and evaluated using established CEFAS protocols.

4.7.8 Statistical treatment of the data

Analysis of variance (ANOVA) was performed on the biological data to determine whether there were differences between groups. Prior to the ANOVA, homogeneity of variances were checked using the Levene's test. Where possible, the data were log transformed to obtain homogeneity. Where this was not possible, Kruskal-Wallis non-parametric analysis was used. Where the parametric ANOVA indicated significant differences, groups were compared using Tukey's post-hoc test. The level of significance for rejection of H0: "no difference between groups" was set at 0.05.

5. Results

5.1 Temperature measurements

The temperature profiles at the three stations recorded over the 10 day exposure are presented in **Figure 3**. The temperature profiles at all stations were similar to each other with a weak thermocline present. The height of the thermocline varied between stations and was around 20-25 m, 30 m, and 35 m for stations 1, 2 and 3 respectively. At all stations the daily temperature decreased and then increased during the 10 day exposure, with all temperatures falling between 8 and 10^{o}C.

The temperature profiles at the three stations recorded over the 9 week exposure are presented in **Figure 4**. The temperature for the 3 stations ranged between 8-10^{o}C in the first 10 days rising to 13-15^{o}C after 30 days. A thermocline was present at the beginning of the exposure and up to 40 days, although appeared mixed after this time. From the temperature profiles there were no obvious signs of the warmer waters of the discharge plume at station 1 and the fish at all stations were exposed to similar water temperatures during the study.

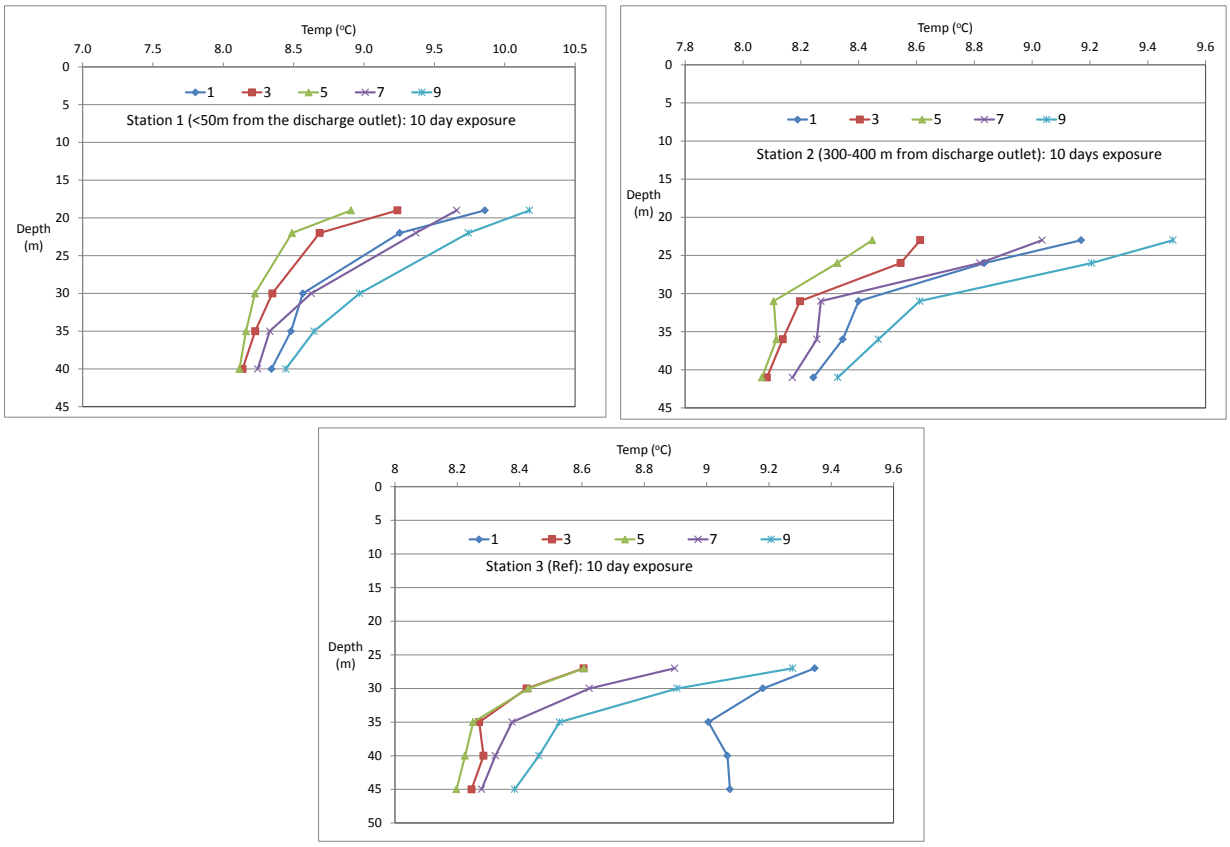

Figure 3. Temperature profiles recorded at each of the three stations during the 10 day exposure. Points denote daily mean temperatures with water temperatures recorded every 5 min.

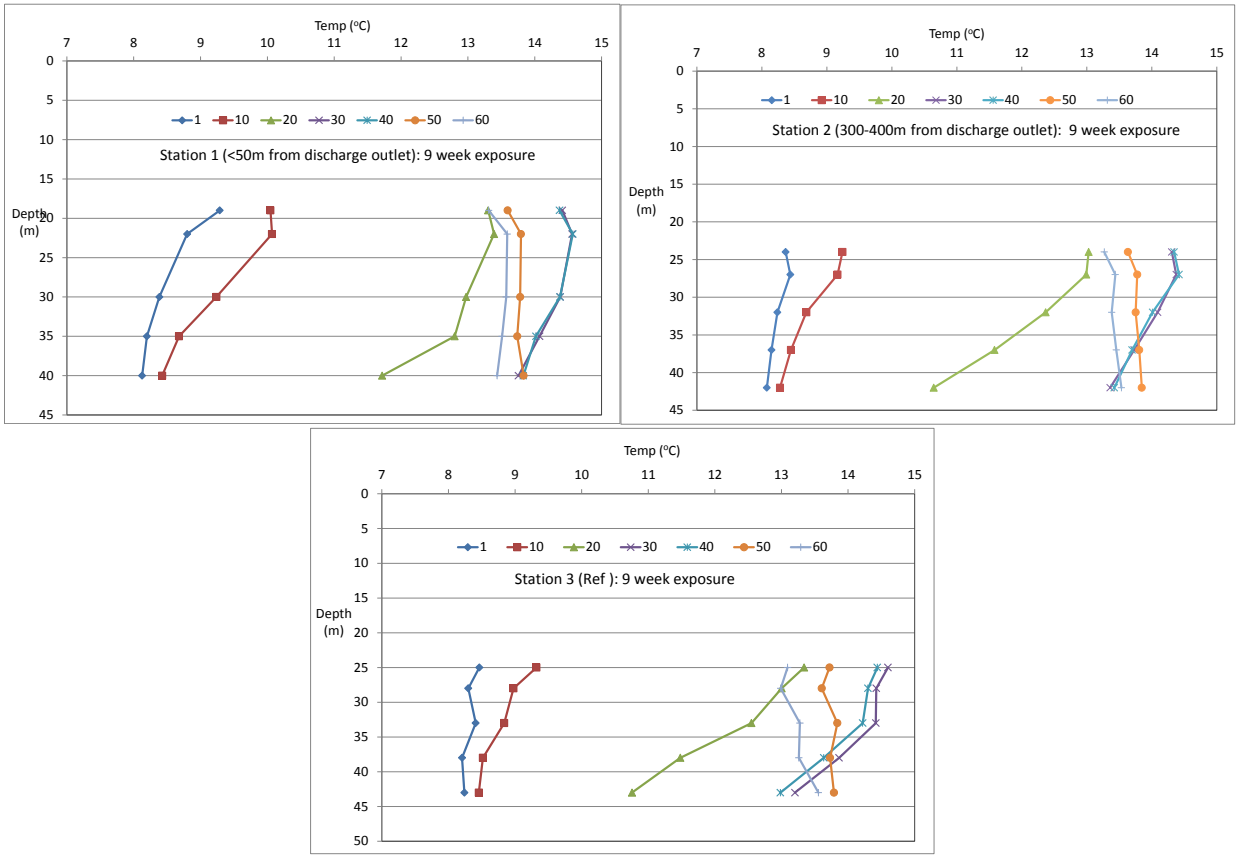

Figure 4. Temperature profiles recorded at each of the three stations during the 9 week exposure. Points denote daily mean temperatures selected every 10 days during the 9 week exposure.

5.2 Chemical concentrations

5.2.1 Metals in DGTs

The metal concentrations measured in DGT extracts after 10 day exposure are presented for each station in **Figure 5**. The data are presented as water concentrations for the ten metals that were calculated based on the duration of the exposure. The laboratory and field blanks for all ten metals were low or undetected. Of the ten metals measured only Cu and Pb showed higher concentrations at the closest station (station 1) compared to the other two stations. However, mean Cu and Pb concentrations of 0.25 µg/L and 0.013 µg/L respectively were typical of coastal water background concentrations.

Metal concentrations measured in DGT extracts following a 9 week exposure are presented for each station in **Figure 6**. The field and laboratory blanks for each metal were low or undetected. For all 10 metals measured, a clear increase in concentration was recorded at station 1 compared to the two other stations. Metal concentrations at station 1 were approximately 10 fold higher than that measured at station 2 or 3 and suggest that the DGTs from station 1 were within the path of the discharge plume.

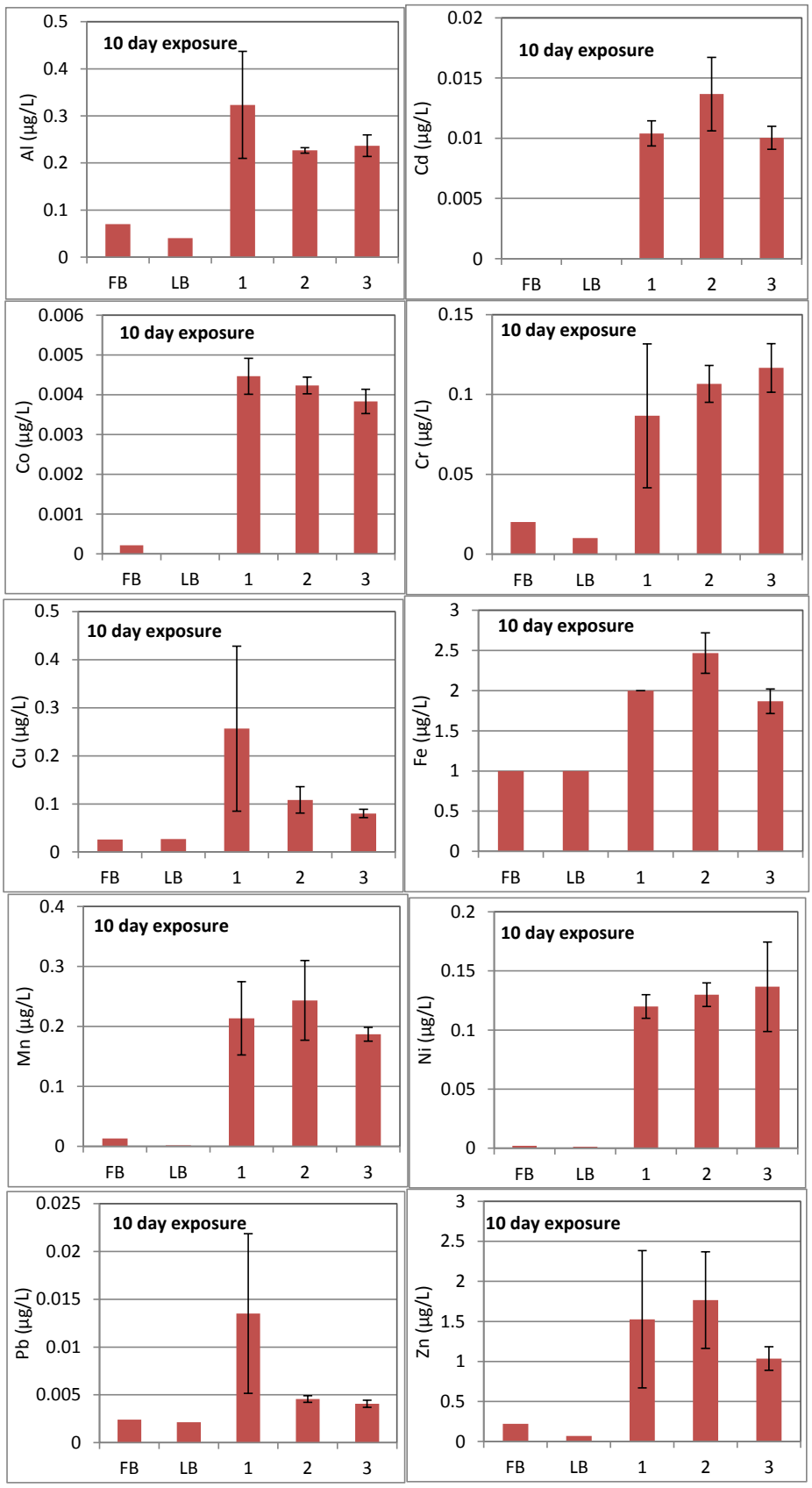

Figure 5. Metal concentrations of the receiving water at the different stations (1-3), measured in DGT samplers integrated over the 10 day exposure duration. FB - field blank; LB- laboratory blank. Mean ± SD, n=3.

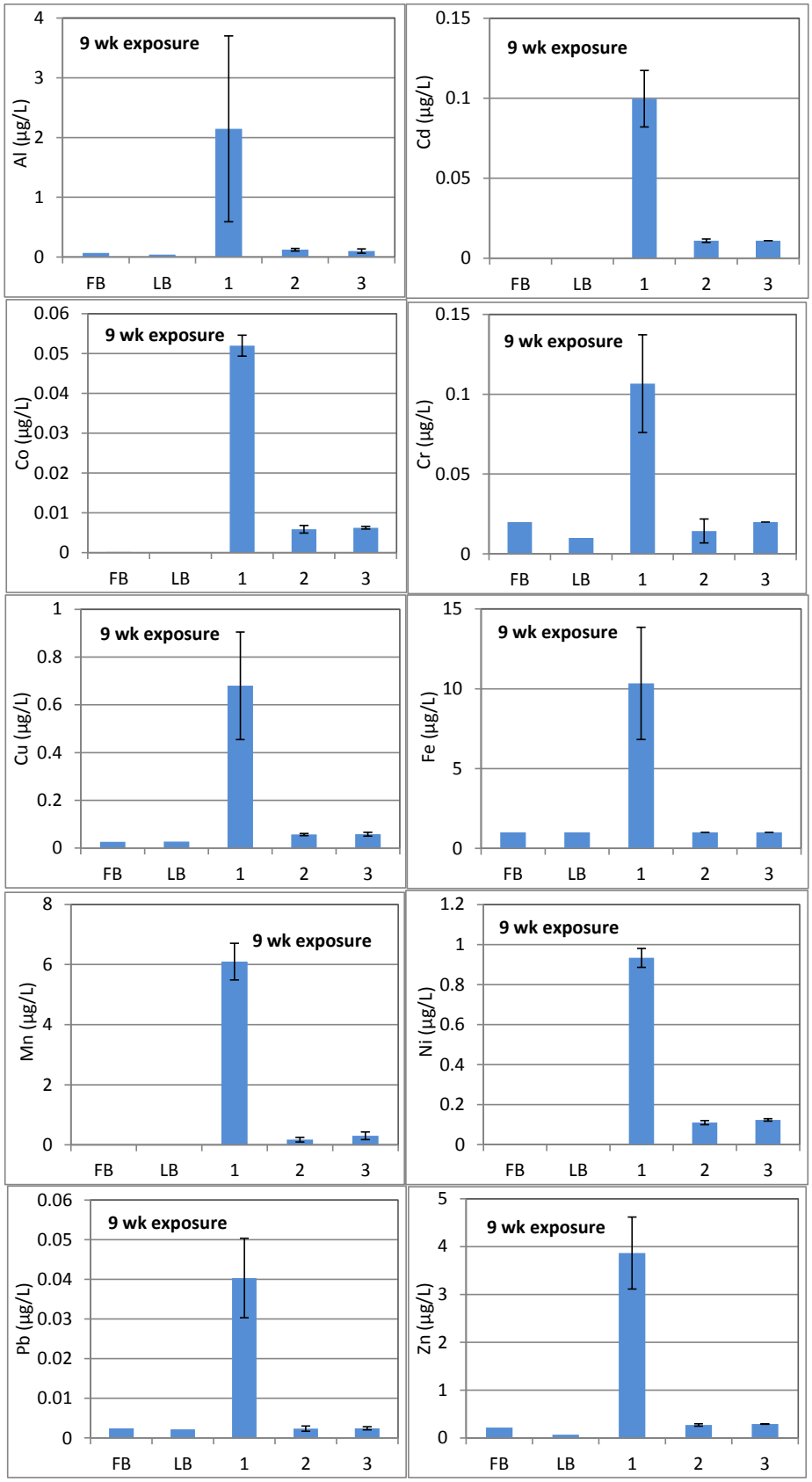

Figure 6. Metal concentrations of the receiving water at the different stations (1-3), measured in DGT samplers integrated over the 9 week exposure duration. FB - field blank; LB- laboratory blank. Mean ± SD, n=3).

5.2.2 Alkylphenols in POCIS

Table 3. Water concentrations of alkylphenols, estimated from POCIS accumulations after 10 days exposure (ng/L, SD, n = 3).

Compounds	10d							
			<50m		300-400m		REF	
	MDL	R_s	Station 1		Station 2		Station 3	
	ng/L	L/d	ng/L	SD	ng/L	SD	ng/L	SD
Phenol	39.939	N/A						
o-Cresol	12.342	0.657	<3.768		<3.768		<3.768	
m-Cresol	2.142	0.228	<2.982		<2.982		<2.982	
p-Cresol	2.894	0.228	5.203	*0.332*	4.578	*0.194*	<4.345	
Total C1 alkylphenols			**5.203**	***0.332***	**4.578**	***0.194***	**<QL**	
2-Ethylphenol	0.031	0.213	<2.985		<2.985		<2.985	
4-Ethylphenol	0.036	0.213	<54.087		<54.087		<54.087	
2,6-Dimethylphenol	0.039	0.143	<9.01		<9.01		<9.01	
2,5-Dimethylphenol	0.034	0.139	0.287	*0.024*	0.269	*0.014*	0.227	*0.010*
2,4-Dimethylphenol/ 3-Ethylphenol	0.181	0.181	<90.638		<90.638		<90.638	
2,3-Dimethylphenol	0.025	0.143	0.205	*0.011*	0.172	*0.016*	0.155	*0.009*
3,5-Dimethylphenol	0.393	0.141	<0.798		<0.798		<0.798	
3,4-Dimethylphenol	0.028	0.143	<0.614		<0.614		<0.614	
Total C2 alkylphenols			**0.492**	***0.034***	**0.441**	***0.030***	**0.382**	***0.019***
2-*n*-Propylphenol	0.009	0.101	<0.108		<0.108		<0.108	
3-*n*-Propylphenol	0.010	0.114	<0.131		<0.131		<0.131	
4-*n*-Propylphenol	0.018	0.126	<0.070		<0.070		<0.070	
2,3,5-Trimethylphenol	0.014	0.259	<0.034		<0.034		<0.034	
2,3,6-Trimethylphenol	0.004	0.256	0.133	*0.041*	0.200	*0.029*	0.144	*0.023*
2,4,6-Trimethylphenol	0.002	0.253	<0.050		<0.050		<0.050	
2-Isopropylphenol	0.057	0.185	<0.437		<0.437		<0.437	
3-Isopropylphenol	0.018	0.185	<0.266		<0.266		<0.266	
4-Isopropylphenol	0.030	0.185	<0.387		<0.387		<0.387	
Unknown C3 alkylphenol, no1	0.004	0.185	<0.027		<0.027		<0.027	
Unknown C3 alkylphenol, no2	0.001	0.185	<0.014		<0.014		<0.014	
Unknown C3 alkylphenol, no3	0.008	0.185	<0.036		<0.036		<0.036	
Unknown C3 alkylphenol, no4	0.000	0.185	0.018	*0.012*	0.055	*0.025*	0.028	*0.024*
Total unknown C3 alkylphenols			**0.018**	***0.012***	**0.055**	***0.025***	**0.028**	***0.024***
Total C3 alkylphenols			**0.150**	***0.031***	**0.255**	***0.040***	**0.172**	***0.033***
4-*n*-Butylphenol	0.004	0.048	<0.044		<0.044		<0.044	
2-*tert*-Butylphenol	0.218	0.228	<0.147		<0.147		<0.147	
3-*tert*-Butylphenol	0.003	0.228	<0.059		<0.059		<0.059	
4-*tert*-Butylphenol	0.116	0.228	<3.802		<3.802		<3.802	
4-*sec*-Butylphenol	0.015	0.228	<0.025		<0.025		<0.025	
4-Isopropyl-3-methylphenol	0.007	0.201	<0.010		<0.010		<0.010	
5-Isopropyl-2-methylphenol	0.138	0.201	0.420	*0.015*	0.357	*0.031*	0.292	*0.033*
2,3,5,6-Tetramethylphenol	0.004	0.194	<0.082		<0.082		<0.082	
Unknown C4 alkylphenol, no1	0.014	0.194	<0.019		<0.019		<0.019	
Unknown C4 alkylphenol, no2	0.002	0.194	<0.018		<0.018		<0.018	
Unknown C4 alkylphenol, no3	0.001	0.194	<0.028		<0.028		<0.028	
Unknown C4 alkylphenol, no4	0.000	0.194	<0.048		<0.048		<0.048	
Total unknown C4 alkylphenols			**<QL**		**<QL**		**<QL**	
Total C4 alkylphenols			**0.420**	***0.015***	**0.357**	***0.031***	**0.292**	***0.033***
4-*n*-Pentylphenol	0.022	0.275	0.051		0.061	*0.002*	0.054	*0.001*
2-*tert*-Butyl-5-methylphenol	2.128	0.275	0.187		0.133		0.409	*0.142*
2-*tert*-Butyl-4-methylphenol	0.178	0.292	<0.211		<0.211		<0.211	
4-*tert*-Butyl-2-methylphenol	0.032	0.256	<0.036		<0.036		<0.036	
4-(1,1-Dimethylpropyl)phenol	0.008	0.275	<0.253		<0.253		<0.253	
Total C5 alkylphenols			**0.080**	***0.097***	**0.105**	***0.079***	**0.445**	***0.169***
4-*n*-Hexylphenol	0.000	0.297	<0.083		<0.083		<0.083	
2,6-Diisopropylphenol	0.000	0.302	ND		ND		ND	
2,5-Diisopropylphenol	0.011	0.302	<0.023		<0.023		<0.023	
2-*tert*-Butyl-4-ethylphenol	0.009	0.216	<2.847		<2.847		<2.847	
4-(1-Methyl-2,2-Dimethylpropyl)phenol	0.019	0.297	<0.014		<0.014		<0.014	
Total C6 alkylphenols			**<QL**		**<QL**		**<QL**	
2,6-dimetyl-4-(1.1-dimetylpropyl)phenol	0.010	0.078	<0.042		<0.042		<0.042	
4-(1-Ethyl-1-methylpropyl)-2-methylphenol	0.000	0.078	ND		ND		ND	
4-*n*-Heptylphenol	0.054	0.078	<0.236		<0.236		<0.236	
UK7 no1	0.007	0.078	<0.025		<0.025		<0.025	
UK7 no2	0.007	0.078	<0.009		<0.009		<0.009	
UK7 no3	0.009	0.078	<0.005		<0.005		<0.005	
UK7 no4	0.012	0.078	<0.177		<0.177		<0.177	
Total unknown C7-alkylphenols			**<QL**		**<QL**		**<QL**	
Total C7 alkylphenols			**<QL**		**<QL**		**<QL**	
4-*n*-Octylphenol	0.084	0.078	<0.129		<0.129		<0.129	
2.6-Di-*tert*-butylphenol	0.000	0.078	ND		ND		ND	
2.4-Di-*tert*-butylphenol	0.016	0.078	<0.187		<0.187		<0.187	
4-*tert*-Octylphenol	0.000	0.078	<8.536		<8.536		<8.536	
2-Metyl-4-*tert*-octylphenol	0.004	0.078	<1.693		<1.693		<1.693	
Total C8 alkylphenols			**<QL**		**<QL**		**<QL**	
4-n-Nonylphenol	6.510	0.078	<3.731		<3.731		4.829	
TOTAL ALKYLPHENOLS			**6.344**		**5.737**		**6.120**	

Where an average but no SD is provided this indicates that the compound was only quantifiable in one replicate. Method detection limit (MDL) calculated as the procedural blank average (n =3) plus 3 x the SD. The quantification limit (Q.L.) of each AP in POCIS was calculated as the average POCIS blank value (n =3) plus 10 x SD. Where accumulated amounts were below the QL this value was used in water concentration estimates to provide a theoretical maximum level. Totals are for quantifiable compounds only, where no individual compound within a group was >QL, then no total is reported. Rs is the compound and exposure specific sampling rate for each compound, which is an adjusted laboratory based value as outlined in detail by Harman et al, (2009)

Table 4. Water concentrations of alkylphenols, estimated from POCIS accumulations after 9 weeks exposure (ng/L, SD, n = 3).

Compounds			9week					
			<50m		300-400m		REF	
	MDL	R_s	Station 1		Station 2		Station 3	
	ng/L	L/d	ng/L	SD	ng/L	SD	ng/L	SD
Phenol	39.939	N/A						
o-Cresol	12.342	0.657	0.747	*0.083*	0.833	*0.059*	0.821	*0.119*
m-Cresol	2.142	0.228	0.698	*0.014*	0.762	*0.050*	0.786	*0.100*
p-Cresol	2.894	0.228	1.463	*0.106*	1.411	*0.073*	1.375	*0.075*
Total C1 alkylphenols			**2.908**	***0.164***	**3.006**	***0.175***	**2.982**	***0.290***
2-Ethylphenol	0.031	0.213	<0.459		<0.459		<0.459	
4-Ethylphenol	0.036	0.213	10.995		8.483		9.008	*0.039*
2,6-Dimethylphenol	0.039	0.143	1.471	*0.489*	0.613	*0.146*	0.265	*0.039*
2,5-Dimethylphenol	0.034	0.139	0.243	*0.023*	0.277	*0.057*	0.246	*0.011*
2,4-Dimethylphenol/ 3-Ethylphenol	0.181	0.181	18.874		14.027		14.044	
2,3-Dimethylphenol	0.025	0.143	0.282	*0.044*	0.236	*0.055*	0.178	*0.008*
3,5-Dimethylphenol	0.393	0.141	0.227	*0.039*	0.230	*0.042*	0.218	*0.010*
3,4-Dimethylphenol	0.028	0.143	0.142	*0.021*	0.145	*0.029*	0.134	*0.002*
Total C2 alkylphenols			**12.322**	***17.221***	**9.005**	***13.256***	**11.728**	***11.638***
2-*n*-Propylphenol	0.009	0.101	0.022	*0.008*	0.021	*0.005*	0.022	*0.002*
3-*n*-Propylphenol	0.010	0.114	0.144	*0.031*	0.074	*0.017*	0.035	*0.001*
4-*n*-Propylphenol	0.018	0.126	0.022	*0.013*	0.016	*0.003*	0.015	*0.001*
2,3,5-Trimethylphenol	0.014	0.259	0.039	*0.010*	0.021	*0.003*	0.013	*0.002*
2,3,6-Trimethylphenol	0.004	0.256	0.059	*0.014*	0.060	*0.019*	0.053	*0.025*
2,4,6-Trimethylphenol	0.002	0.253	0.022	*0.005*	0.014	*0.003*	0.011	*0.001*
2-Isopropylphenol	0.057	0.185	<0.067		<0.067		<0.067	
3-Isopropylphenol	0.018	0.185	0.055	*0.009*	0.054	*0.006*	0.053	*0.001*
4-Isopropylphenol	0.030	0.185	0.113	*0.018*	0.117	*0.020*	0.112	*0.007*
Unknown C3 alkylphenol, no1	0.004	0.185	<0.004		<0.004		<0.004	
Unknown C3 alkylphenol, no2	0.001	0.185	<0.002		<0.002		<0.002	
Unknown C3 alkylphenol, no3	0.008	0.185	<0.006		<0.006		<0.006	
Unknown C3 alkylphenol, no4	0.000	0.185	0.003	*0.001*	0.003	*0.001*	0.003	*0.001*
Total unknown C3 alkylphenols			**0.003**	***0.001***	**0.003**	***0.001***	**0.003**	***0.001***
Total C3 alkylphenols			**0.479**	***0.096***	**0.382**	***0.072***	**0.318**	***0.021***
4-*n*-Butylphenol	0.004	0.048	0.063		<0.007		<0.007	
2-*tert*-Butylphenol	0.218	0.228	<0.023		<0.023		<0.023	
3-*tert*-Butylphenol	0.003	0.228	0.010	*0.000*	<0.009		<0.009	
4-*tert*-Butylphenol	0.116	0.228	<0.585		<0.585		<0.585	
4-*sec*-Butylphenol	0.015	0.228	0.015	*0.006*	0.008	*0.002*	0.004	
4-Isopropyl-3-methylphenol	0.007	0.201	0.015	*0.005*	0.009	*0.002*	0.004	
5-Isopropyl-2-methylphenol	0.138	0.201	0.232	*0.044*	0.306	*0.069*	0.243	*0.010*
2,3,5,6-Tetramethylphenol	0.004	0.194	0.014		<0.013		<0.013	
Unknown C4 alkylphenol, no1	0.014	0.194	<0.003		<0.003		<0.003	
Unknown C4 alkylphenol, no2	0.002	0.194	<0.003		<0.003		<0.003	
Unknown C4 alkylphenol, no3	0.001	0.194	<0.004		<0.004		<0.004	
Unknown C4 alkylphenol, no4	0.000	0.194	<0.007		<0.007		<0.007	
Total unknown C4 alkylphenols			**<QL**		**<QL**		**<QL**	
Total C4 alkylphenols			**0.294**	***0.083***	**0.323**	***0.073***	**0.252**	***0.010***
4-*n*-Pentylphenol	0.022	0.275	0.032	*0.022*	0.025	*0.012*	0.020	*0.003*
2-*tert*-Butyl-5-methylphenol	2.128	0.275	0.023	*0.007*	0.036	*0.026*	0.065	*0.002*
2-*tert*-Butyl-4-methylphenol	0.178	0.292	0.039		<0.032		<0.032	
4-*tert*-Butyl-2-methylphenol	0.032	0.256	0.027	*0.027*	0.009	*0.002*	<0.006	
4-(1,1-Dimethylpropyl)phenol	0.008	0.275	<0.039		<0.039		<0.039	
Total C5 alkylphenols			**0.087**	***0.083***	**0.057**	***0.025***	**0.063**	***0.037***
4-*n*-Hexylphenol	0.000	0.297	0.034		0.015	*0.003*	0.026	
2,6-Diisopropylphenol	0.000	0.302	ND		ND		ND	
2,5-Diisopropylphenol	0.011	0.302	0.024		<0.003		<0.003	
2-*tert*-Butyl-4-ethylphenol	0.009	0.216	<0.438		<0.438		<0.438	
4-(1-Methyl-2,2-Dimethylpropyl)phenol	0.019	0.297	<0.002		<0.002		<0.002	
Total C6 alkylphenols			**0.019**	***0.033***	**0.010**	***0.009***	**0.009**	***0.015***
2,6-dimetyl-4-(1.1-dimetylpropyl)phenol	0.010	0.078	0.072		<0.007		<0.007	
4-(1-Ethyl-1-methylpropyl)-2-methylphenol	0.000	0.078	0.068		0.004		ND	
4-*n*-Heptylphenol	0.054	0.078	0.097		<0.036		<0.036	
UK7 no1	0.007	0.078	<0.004		<0.004		<0.004	
UK7 no2	0.007	0.078	<0.001		<0.001		<0.001	
UK7 no3	0.009	0.078	<0.001		<0.001		<0.001	
UK7 no4	0.012	0.078	<0.027		<0.027		<0.027	
Total unknown C7-alkylphenols			**<QL**		**<QL**		**<QL**	
Total C7 alkylphenols			**0.079**	***0.137***	**0.001**	***0.002***	**<QL**	
4-*n*-Octylphenol	0.084	0.078	<0.020		<0.020		<0.020	
2.6-Di-*tert*-butylphenol	0.000	0.078	ND		ND		ND	
2.4-Di-*tert*-butylphenol	0.016	0.078	<0.029		<0.029		<0.029	
4-*tert*-Octylphenol	0.000	0.078	<1.313		<1.313		<1.313	
2-Metyl-4-*tert*-octylphenol	0.004	0.078	<0.260		<0.260		<0.260	
Total C8 alkylphenols			**<QL**		**<QL**		**<QL**	
4-n-Nonylphenol	6.510	0.078	14.642	*18.399*	6.109		1.586	
TOTAL ALKYLPHENOLS			**30.830**		**18.893**		**16.938**	

Where an average but no SD is provided this indicates that the compound was only quantifiable in one replicate. Method detection limit (MDL) calculated as the procedural blank average (n =3) plus 3 x the SD. The quantification limit (Q.L.) of each AP in POCIS was calculated as the average POCIS blank value (n =3) plus 10 x SD. Where accumulated amounts were below the QL this value was used in water concentration estimates to provide a theoretical maximum level. Totals are for quantifiable compounds only, where no individual compound within a group was >QL, then no total is reported. Rs is the compound and exposure specific sampling rate for each compound, which is an adjusted laboratory based value as outlined in detail by Harman et al, (2009)

In total 60 APs, (48 known and 12 unknown) were analysed in POCIS extracts. Only 8 of these were above quantification limits (Q.L), at the station closest to the discharge point after 10 days compared to 34 compounds at the same station after 9 weeks (Table 3 and Table 4). Overall, the concentrations of APs measured were low, ranging from a few pg/L to around 10 ng/L for some compounds. No clear signal from the discharge was apparent, i.e. there was no overall difference in the pattern of APs accumulated between sites (**Figure 7**). Slightly higher concentrations of some AP groups were measured at the site closest the discharge; C_6, C_7 and nonylphenol (NP), although these concentrations were only higher in one POCIS replicate and thus have large variation, for example NP 14.64 ± 18.40 ng/L.

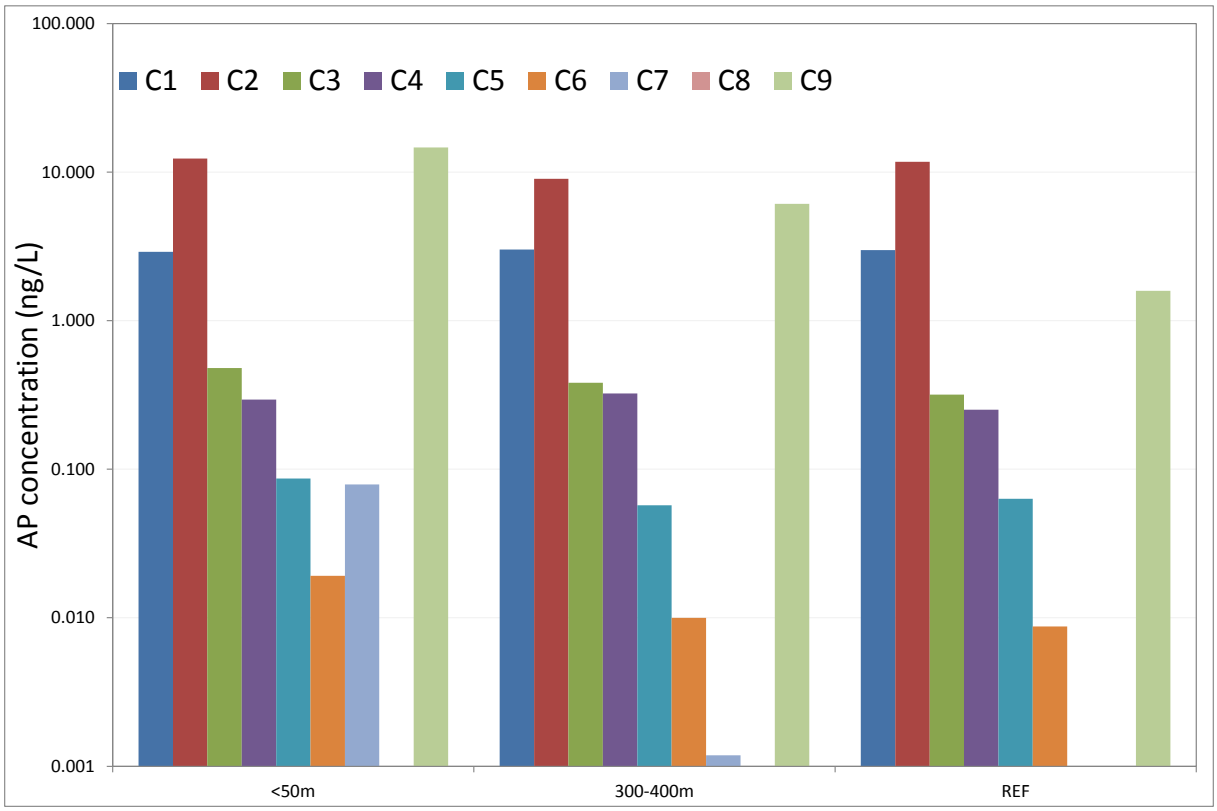

Figure 7. Water concentrations of alkylphenols as estimated from POCIS exposed for 65 days at the different stations (ng/L as totals for each group). Logarithmic scale.

5.2.3 Alyklphenol metabolites in fish bile

10 day exposure

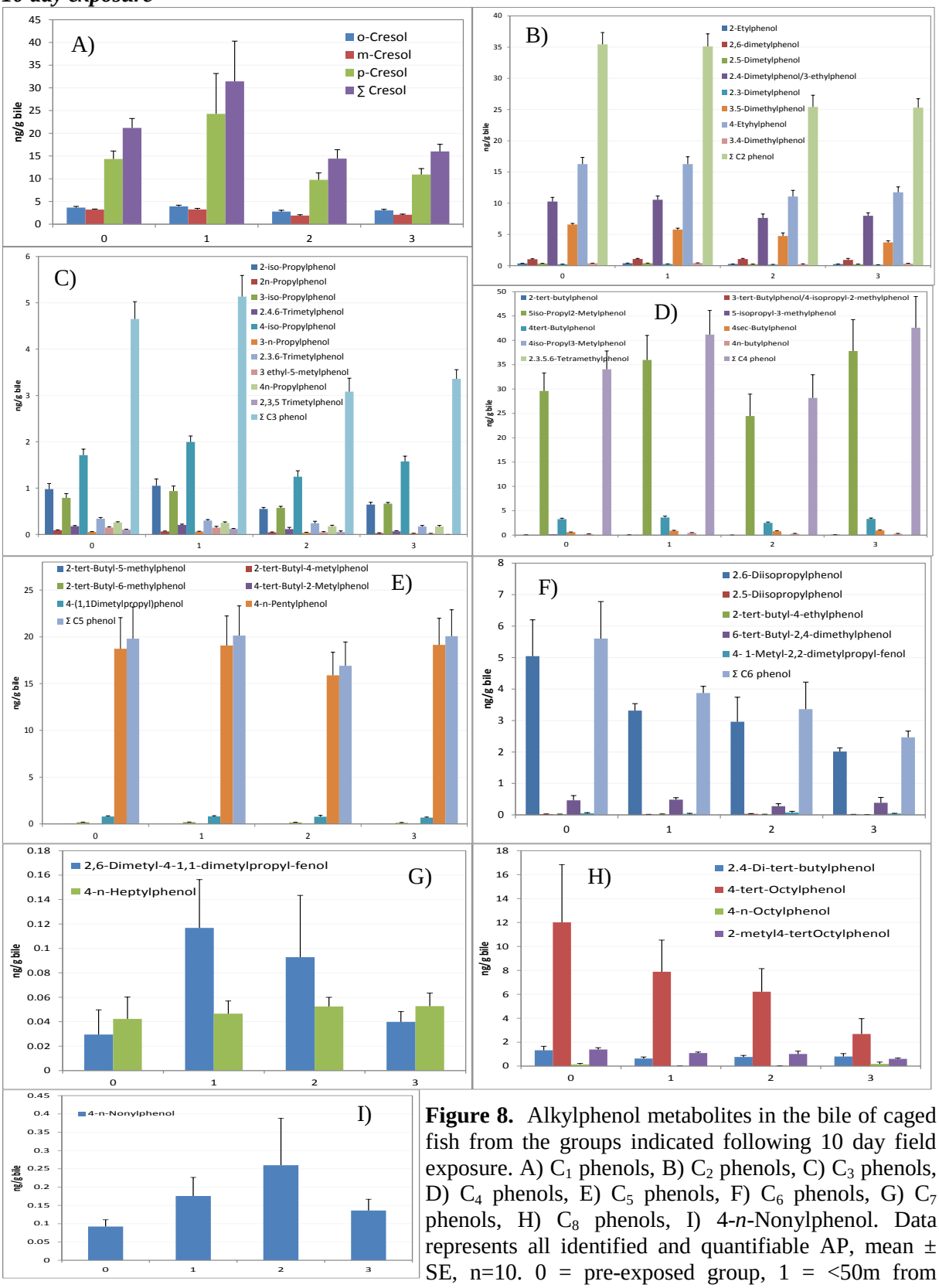

Figure 8. Alkylphenol metabolites in the bile of caged fish from the groups indicated following 10 day field exposure. A) C_1 phenols, B) C_2 phenols, C) C_3 phenols, D) C_4 phenols, E) C_5 phenols, F) C_6 phenols, G) C_7 phenols, H) C_8 phenols, I) 4-*n*-Nonylphenol. Data represents all identified and quantifiable AP, mean ± SE, n=10. 0 = pre-exposed group, 1 = <50m from discharge, 2 = 300-400m from discharge, 3= reference.

9 week exposure

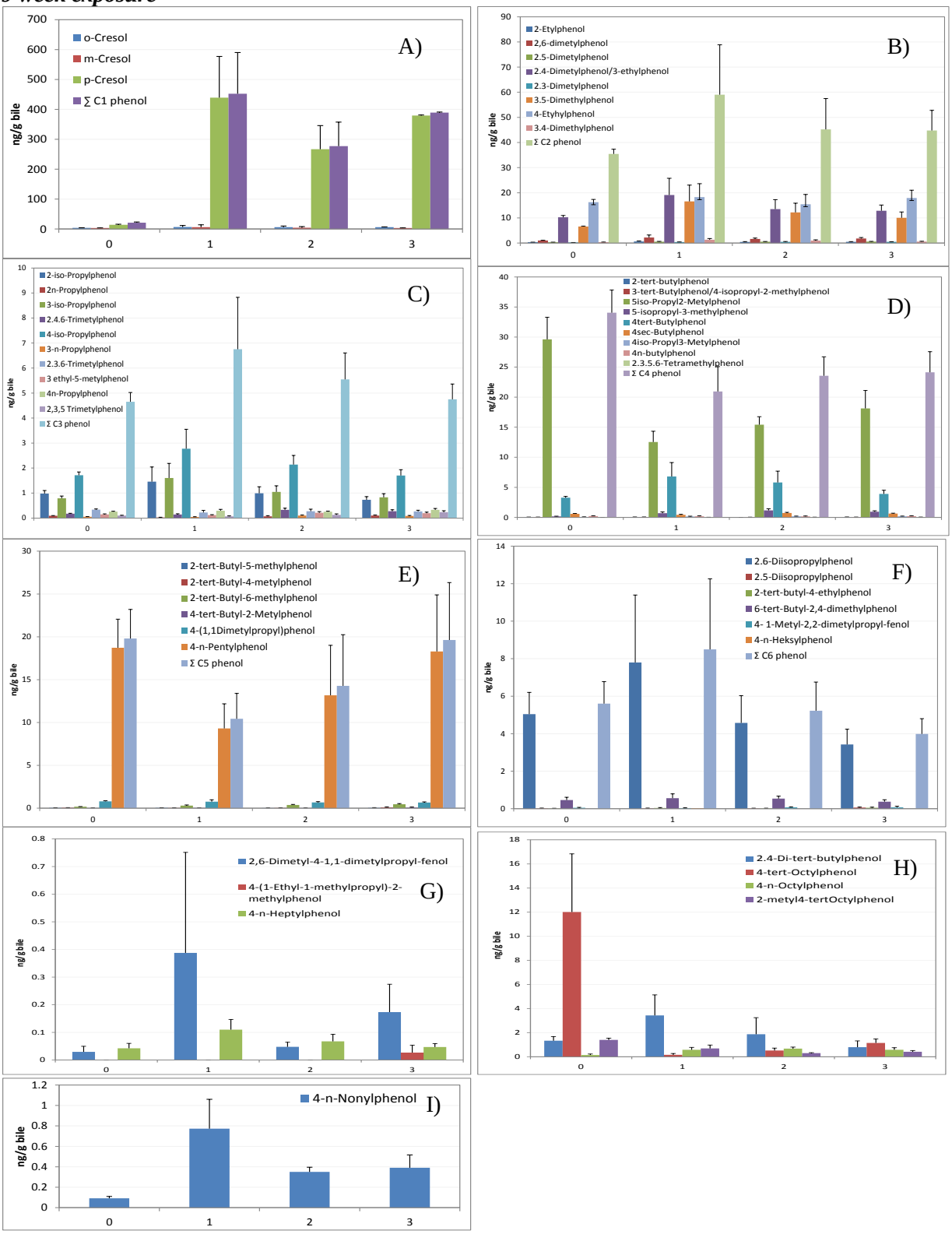

Figure 9. Alkylphenol metabolites in the bile of caged fish from the groups indicated following 9 week field exposure. A) C_1 phenols, B) C_2 phenols, C) C_3 phenols, D) C_4 phenols, E) C_5 phenols, F) C_6 phenols, G) C_7 phenols, H) C_8 phenols, I) 4-*n*-Nonylphenol. Data represents all identified and quantifiable AP, mean ± SE, n=10. 0 = pre-exposed group, 1 = <50m from discharge, 2 = 300-400m from discharge, 3= reference.

AP metabolites from C_1 to C_9, measured in the bile of cod following a 10 day exposure, are displayed in **Figure 8**. For C_1 AP, *p*-cresol was the dominate compound with highest concentrations (30 ng/L bile) measured in cod from group 1 (<50 m from the discharge, **Figure 8**A). A total of eight C_2 APs were measured of which 4-ethylphenol, 2,4-dimethylphenol and 3,5-dimethylphenol were the most abundant (**Figure 8**B). For sum C_2 as well as the sum of C_3, highest concentrations were found in fish from group 1 and day 0, with lower concentrations found in group 2 and 3. However, the highest sum of C_3 phenols was approximately 5 ng/g bile (**Figure 8**C). The dominant C_4 phenols were 5 iso-propyl2-methylphenol, which made up over 90% of the C_4 phenols in each group (**Figure 8**D). Mean concentrations of 5 iso-propyl2-methylphenol ranged from 24 to 37 ng/g bile with highest concentrations at the reference site (group 3) and <50 m from the discharge (group 1). For C_5 phenols, 4-*n*-pentylphenol accounted for over 90% of the total C_5 phenols measured (**Figure 8**E). Concentrations of 4-*n*-pentylphenol were similar between groups ranging between 16 and 19 ng/g bile. For C_6 phenols, highest concentrations were found in the pre-exposed fish (Day 0 group), with 2,6 diisopropylphenol accounting for over 90% of the total C6 phenols in all groups (**Figure 8**F). Two compounds were detected as C_7 phenols of which 2, 6-dimethyl-4-1,1-dimethylpropyl-phenol was highest in fish from group 1 and group 2 compared to reference group 3 and pre-exposure group 0 **Figure 8**G. However, the highest concentration of this compound was very low with a mean measured value below 0.12 ng/g bile for group 1. The compound 4-*tert*-octylphenol was the dominant C_8 phenol, although highest concentrations of this were found in the pre-exposure group (group 0, 12 ng/g bile, **Figure 8**H). The only quantifiable C_9 phenol was 4-*n*-nonylphenol, slightly higher concentrations were found in the groups 1 and 2, although only at very low concentrations (mean, 0.25 ng/g bile, **Figure 8**I).

AP metabolites from C_1 to C_9, measured in the bile of cod following a 9 week exposure, are displayed in **Figure 9**. For C_1 AP, *p*-cresol was the dominate compound with highest concentrations (430 ng/L bile) measured in cod bile from group 1 (<50 m from the discharge, **Figure 9**A). C_1 *p*-cresol was measured in markedly high quantities in the bile of caged fish compared to fish from the pre-exposure group (group 0). Of the eight C2 AP compounds measured, 4-ethylphenol, 2,4-dimethylphenol and 3,5-dimethylphenol were the most abundant (**Figure 9**B). For sum C_2 as well as the sum of C_3 (**Figure 9**C), highest concentrations were found in fish from group 1, at approximately 60 and 7 ng/g bile respectively. Out of nine C_4 phenols measured 5 iso-propyl2-methylphenol was the most abundant making up over 90% of the C_4 phenols in each group (**Figure 9**D). Mean concentrations of 5 iso-propyl2-methylphenol ranged from 12 to 29 ng/g bile with highest concentrations in the pre-exposure group (group 0) and lowest <50m from the discharge (group 1). For C_5 phenols, 4-*n*-pentylphenol accounted for over 90% of the total C_5 phenols measured, with highest concentrations measured in fish from the pre-exposure group (group 0, 18 ng/g bile, **Figure 9**E). For the sum C_6 phenols, highest concentrations were found in fish bile < 50 m from the discharge (group 1), with 2,6 diisopropylphenol accounting for over 90% of the total C6 phenols in all groups (**Figure 9**F). C7 phenols were detected at low concentrations in fish from all groups (**Figure 9**G). Highest concentrations of C_7 phenol 2, 6-dimethyl-4-1,1-dimehylpropyl-phenol were found in fish from group 1, although the mean concentrations were extremely low at 0.38 ng/L. C_8 phenols were measured, with markedly higher 4-tert-octylphenol found in the bile of the pre-exposure group (**Figure 9**H). The C_8 phenol 2, 4-di-tert-butylphenol was highest in fish from group 1 (3.6 ng/L). Of the C_9 phenols measured only 4-n-nonylphenol could be identified (**Figure 9**I). Highest concentrations of 4-n-nonylphenol were found in fish from group 1, with a mean concentration of 0.78 ng/L.

5.3 Fish biometry

The biometric data of the fish used in the study included length, weight, condition index, liver somatic index and gonad somatic index (**Figure 10** to **Figure 13**). The median lengths of the different fish groups were between 25 and 28 cm, only fish measured in fish group 2 following 9 weeks exposure were found to be significantly smaller than the pre-exposure group (**Figure 10**). Fish mortalities were found in fish group 2 after 9 weeks, resulting in only 13 fish available for measurement. With regard

to the weight of the fish, a wider range of fish weights were measured with median values for each fish group between 110 g and 200 g. Fish group 2, week 9 was significantly lighter than the fish from the pre exposure group as well as fish group 1 (10 day) (ANOVA, Tukey, $p<0.05$).

The condition index (CI) of the fish in all groups after 10 days exposure did not differ significantly from the pre-exposure group (**Figure 11**). However, the fish groups after 9 weeks exposure showed a reduction in their CI, which were all significantly lower than the CI of the fish groups after 10 days exposure (ANOVA, Tukey, $p<0.05$). Only fish group 2 after 9 weeks exposure had a significantly lower CI compared to the pre-exposure group.

The median liver somatic index (LSI) was highest in fish from the pre-exposure group (**Figure 12**). The LSI of the pre-exposed group was significantly different from all 9 week exposure groups, but was only significantly different from the LSI of fish from group 2 after 10 days (ANOVA, Tukey, $p<0.05$). The LSI of fish from all groups after 9 week exposures was significantly different to all LSI in fish after 10 days (ANOVA, Tukey, $p<0.05$).

The gonad somatic index (GSI) showed a large amount of variation within fish groups and no significant differences between the groups were found (**Figure 13**).

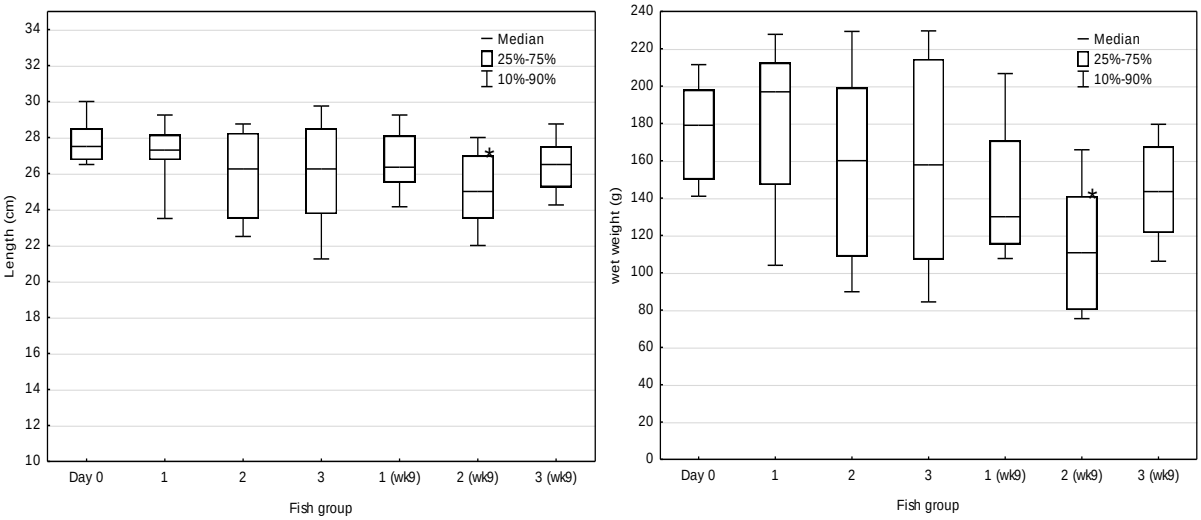

Figure 10. The length and weight of all fish sampled within each group. For length, * indicates significant difference from the day 0, whilst for weight * indicates significant difference from day 0 and fish group 1(Kruskal Wallis ANOVA, $p<0.05$, n = 20). Median, quartiles, 10/90 percentiles, n=20, note n=13 for fish group 2(wk9)).

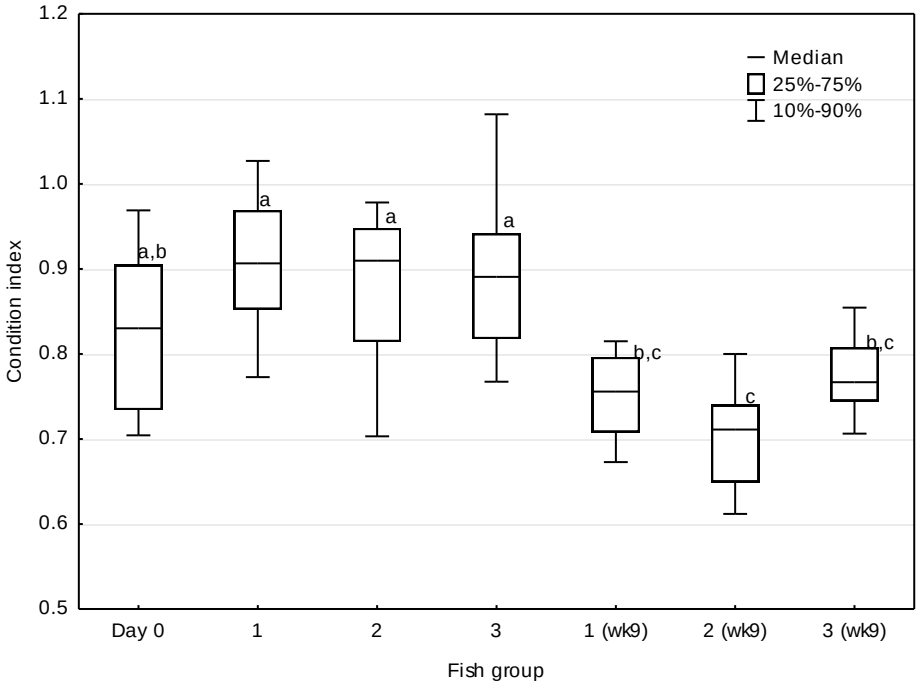

Figure 11. Condition index from all fish sampled within each group (n=20). Groups with the same letter are not significantly different from one another (ANOVA, Tukey $p<0.05$). Median, quartiles, 10/90 percentiles n=20).

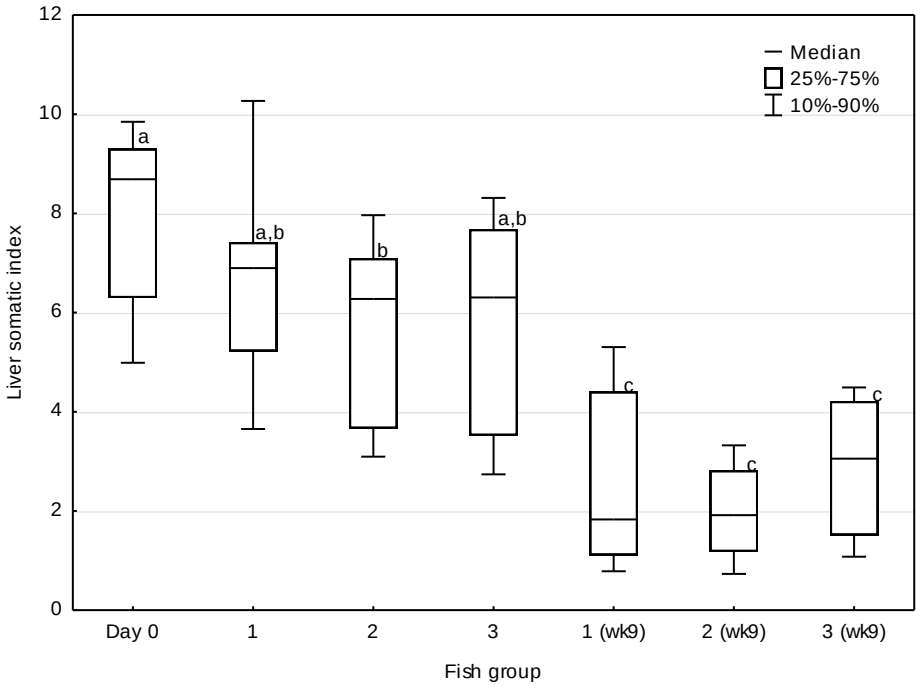

Figure 12. Liver somatic index from all fish sampled within each group (n=20). Groups with the same letter are not significantly different from each other (ANOVA, Tukey $p<0.05$). Median, quartiles, 10/90 percentiles n=20).

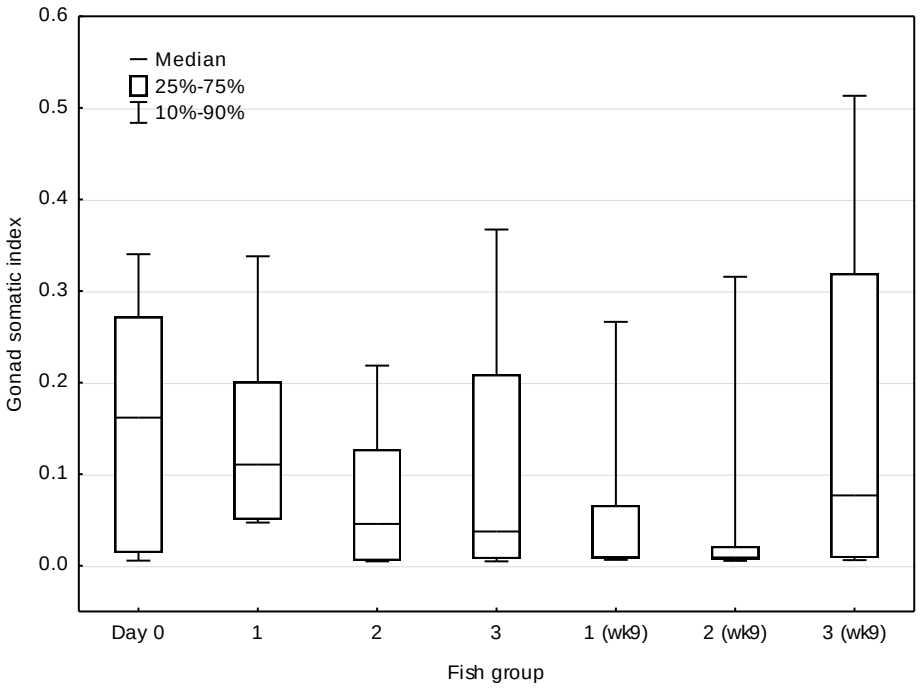

Figure 13. Gonad somatic index from all fish sampled within each group (n=20). Median, quartiles, 10/90 percentiles n=20).

5.4 Biological effects

5.4.1 Plasma chemistry

Plasma chemistry parameters with measureable results generally showed low or no effects that could be related to differences in exposure regimes. The most evident effect was a significant increase of LD in the group held closest to the effluent (station 1) after 10 days (1 week) exposure (ANOVA, Tukey, $p<0.05$), suggesting exposure-related stress and fatigue in this group (Left panel top in **Figure 14**). LD levels were generally lower after 9 weeks and differences between the groups were no longer evident (Left panel centre in **Figure 14**). A comparison of plasma LD levels in the three reference groups sampled day 0, after 10 days and 9 weeks respectively, also highlighted that zero reference fish displayed similar LD levels as fish held at Station 1 after 10 days exposure (Left panel bottom in **Figure 14**). This effect may derive from transport-induced stress.

Plasma levels of inorganic phosphate remained relatively stable from 0-9 weeks. Increasing trends relative to reference individuals were seen in fish sampled from Station 1 and Station 2 with a significant difference between the reference and station 1 (ANOVA, Tukey, $p<0.05$) after 1 week (10 days). The reference group after 1 week (10 days) was significantly higher than the day 0 fish (ANOVA, Tukey, $p<0.05$).

Plasma calcium and magnesium did not show any trends that could be related to distance to the discharge from Nyhamna (**Figure 15**). Furthermore, intra-group variations of magnesium were rather large.

Fish sampled from Station 1 after 10 days (1 week) showed an increasing trend of plasma iron levels, although not found to be statistically different (ANOVA, Tukey, p>0.05), therefore, should be interpreted as natural variation for this fish stock (**Figure 16**). There was also a downward trend in plasma iron concentrations in the reference fish as the experiment progressed but also not found to be statistically different (ANOVA, Tukey, p>0.05).

There was a clear trend for plasma levels of both cholesterol and triglycerides showing a reduction with increased length of exposure in the reference groups with a significant reduction from 1 week to 9 weeks (ANOVA, Tukey, p<0.05, **Figure 17**). However, no differences could be related to the exposure regime and the results are thus interpreted as expressing the onset of a starvation syndrome in caged fish between 1 and 9 weeks.

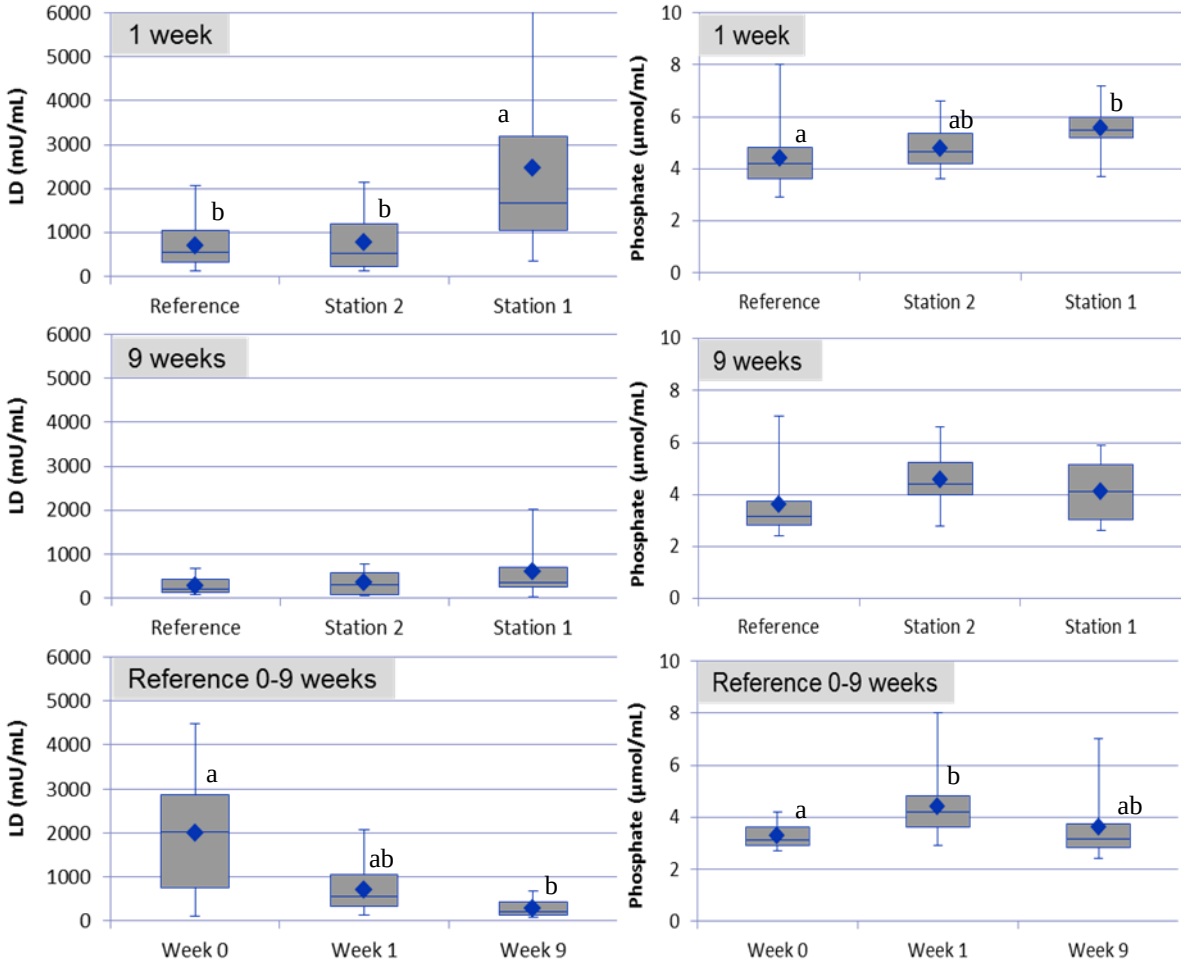

Figure 14. Plasma LD (left panel) and inorganic phosphate (right panel) concentrations in fish from the groups indicated. Week 0 refers to pre-exposure group, station 1 - <50 m from the discharge outlet, station 2 – 300-400 m from the discharge outlet. Median (line), quartile (box) highest observation (upper whisker), lowest observation (bottom whisker), mean (diamond). Data with the same letter are not significantly different from each other (ANOVA, Tukey p>0.05).

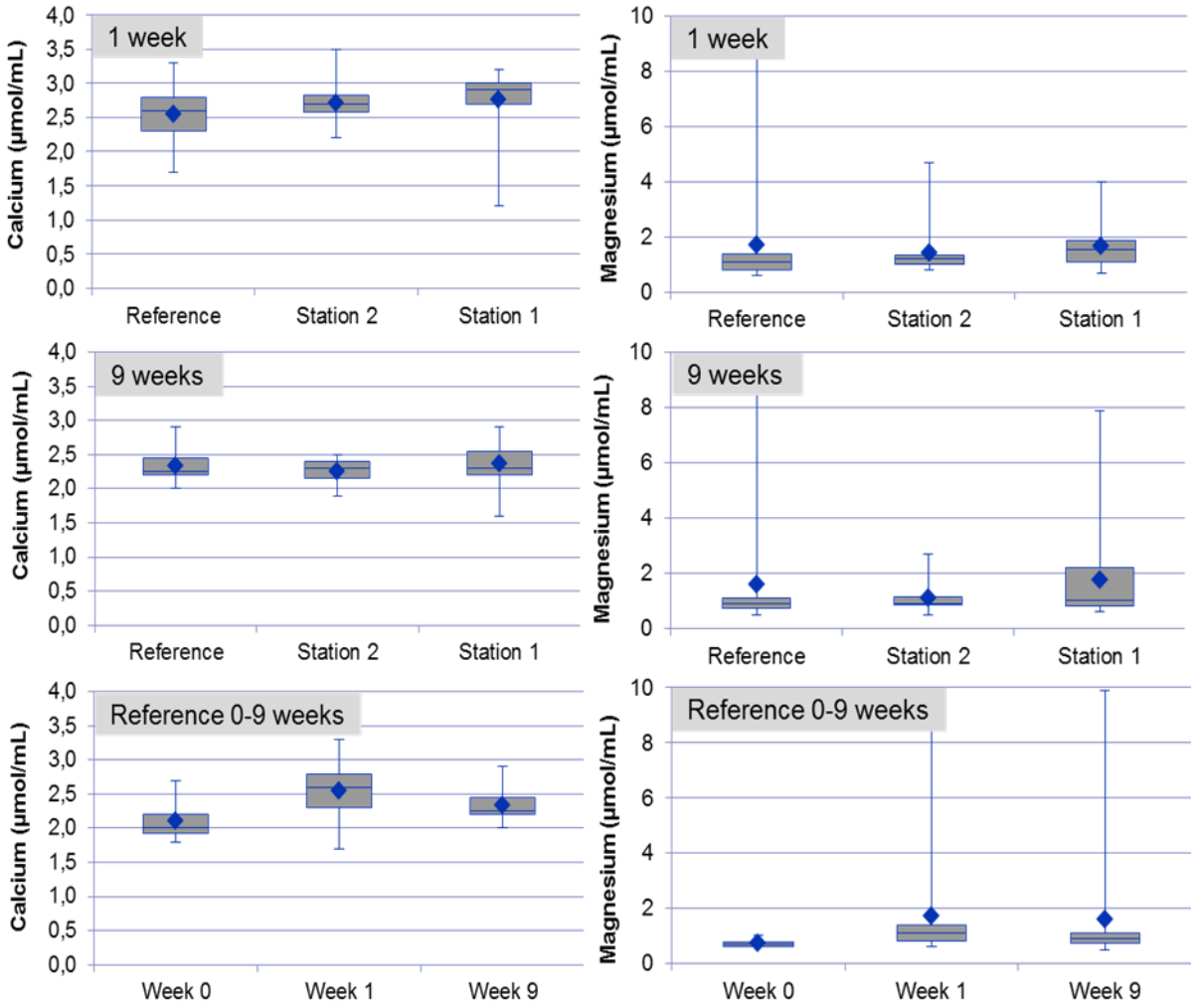

Figure 15. Plasma calcium (left panel) and magnesium (right panel) concentrations in fish from the groups indicated. Week 0 refers to pre-exposure group, station 1 - <50 m from the discharge outlet, station 2 – 300-400 m from the discharge outlet. Median (line), quartile (box) highest observation (upper whisker), lowest observation (bottom whisker), mean (diamond)..

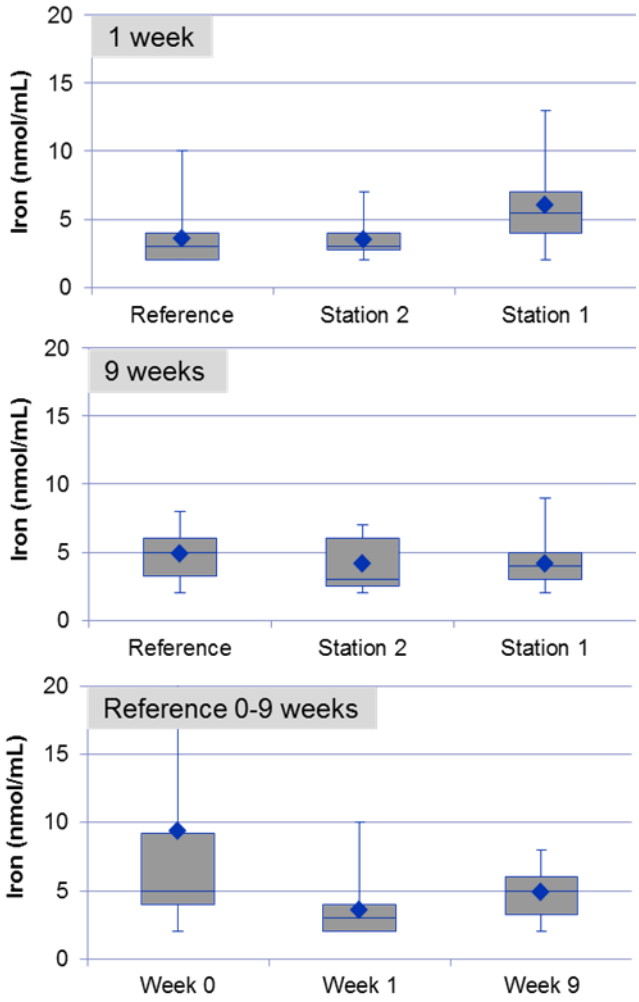

Figure 16. Plasma iron concentrations in fish from the groups indicated. Week 0 refers to pre-exposure group, station 1 - <50 m from the discharge outlet, station 2 – 300-400 m from the discharge outlet. Median (line), quartile (box) highest observation (upper whisker), lowest observation (bottom whisker), mean (diamond).

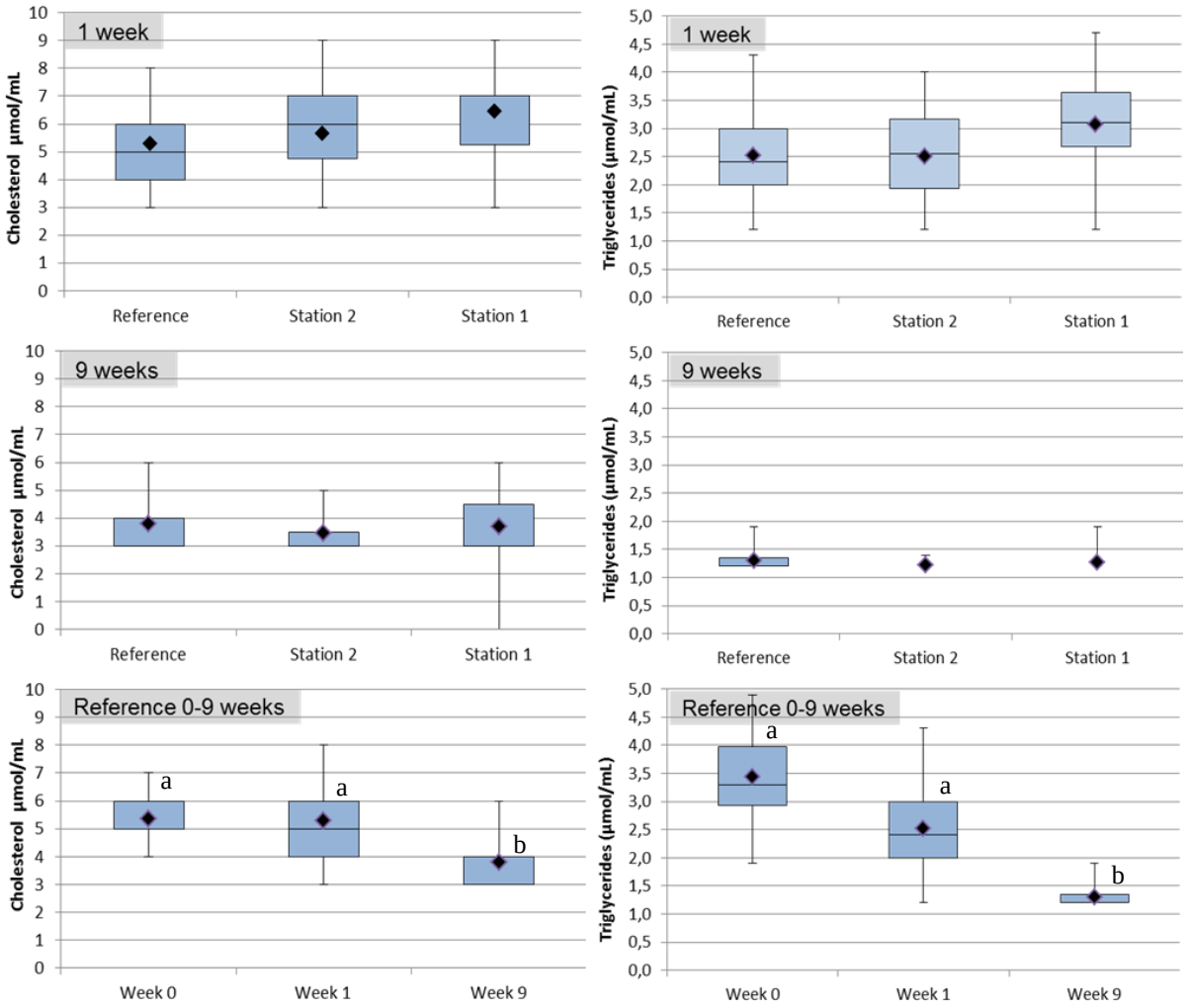

Figure 17. Plasma cholesterol and triglyceride concentrations in fish from the groups indicated. Week 0 refers to pre-exposure group, station 1 - <50 m from the discharge outlet, station 2 – 300-400 m from the discharge outlet. Median (line), quartile (box) highest observation (upper whisker), lowest observation (bottom whisker), mean (diamond). Data with the same letter are not significantly different from each other (ANOVA, Tukey $p>0.05$ for cholesterol, Kruskal-Wallis, $p>0.05$ for triglyceride).

5.4.2 Micronuclei formation

The micronuclei (MN) formation was assessed in fish exposed after 10 days as well as the pre-exposure group (**Figure 18**). Median MN frequency was the same for all fish groups (i.e. 0.5 per 1000 cells) with no significant differences recorded.

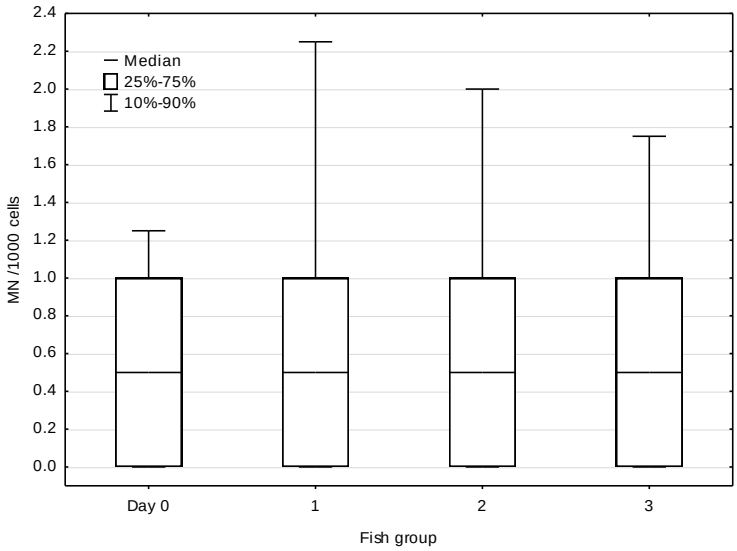

Figure 18. The frequency of micronuclei formation in blood cells of cod from the groups indicated. Data presented as median, quartiles and 10/90 percentiles, n=10.

5.4.3 Ethoxyresorufin-O-deethylase (EROD)

The EROD activity in liver samples was assessed in fish exposed after 10 days as well as the pre-exposure group and was divided by gender (**Figure 19**). Median EROD activity ranged between 20 and 40 pmol/ min/ mg protein, with no significant differences between the groups or between genders.

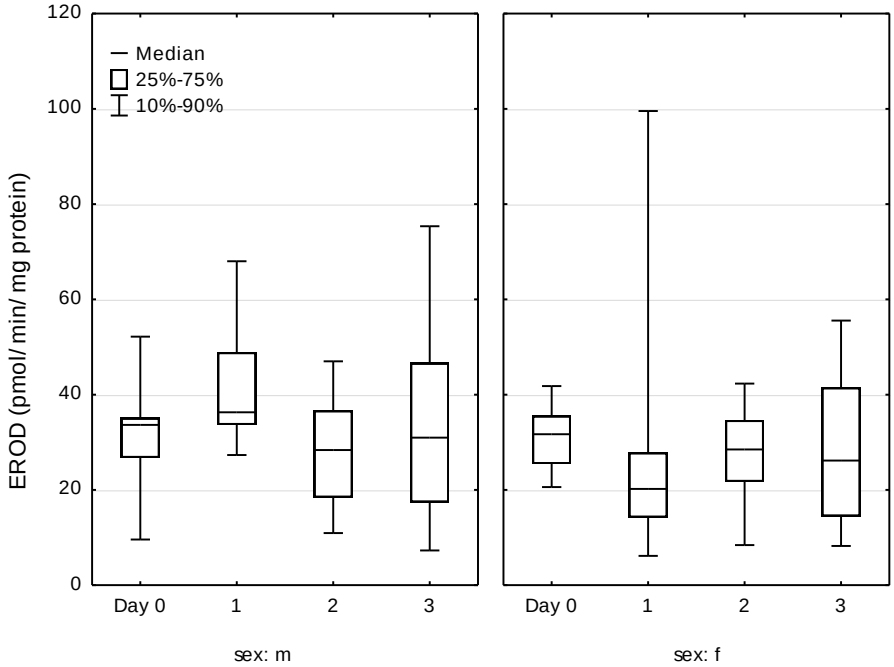

Figure 19. EROD activity in caged cod from the groups indicated following 10 day field exposure. Data presented as median, quartiles and 10/90 percentiles for males and females separately.

5.4.4 Vitellogenin (VTG)

Vitellogenin (VTG) was measured in fish from the pre-exposure group and those after the 10 day exposure, and separated by gender (**Figure 20**). A large variation in VTG was observed in many of the fish groups, although the median VTG was low in all groups with no significant differences between the groups or between genders.

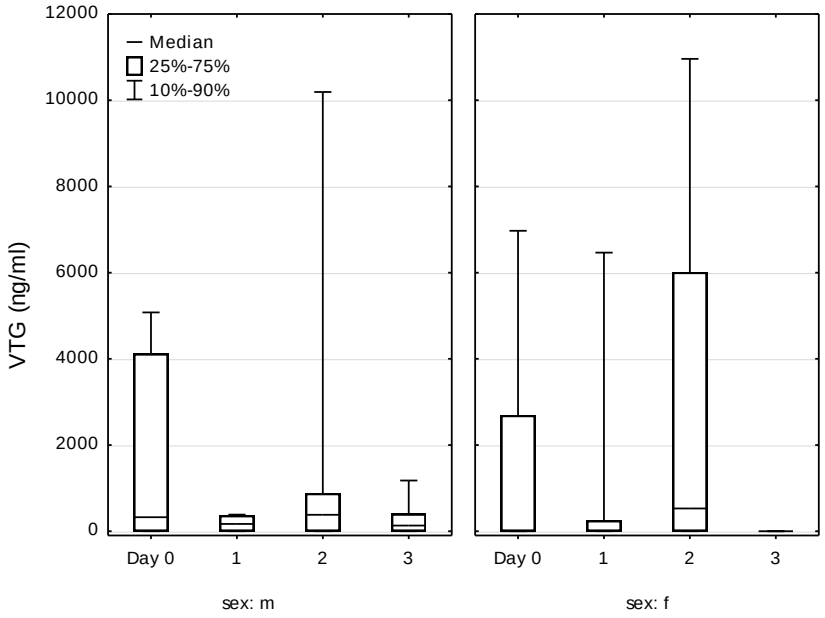

Figure 20. Vitellogenin concentrations in caged cod from the groups indicated following 10 day field exposure. Data presented as median, quartiles and 10/90 percentiles for males and females separately.

5.4.5 Peroxisomal proliferation

Peroxisomal proliferation, measured as AOX activity, was measured in fish livers from the pre-exposure group and those after the 9 weeks exposure (**Figure 21**). Median AOX activities appeared slightly elevated in fish from groups 1 and 2 compared to fish from the pre-exposure group and group 3, although no significant differences were found between the groups.

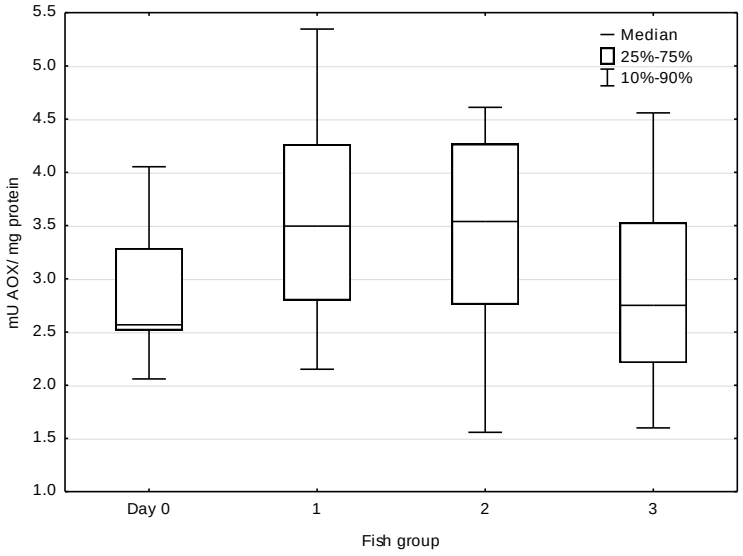

Figure 21. Peroxisomal proliferation measured as AOX activity in caged fish from the groups indicated following 9 week field exposure. Data presented as median, quartiles and 10/90 percentiles.

5.4.6 Neutral lipid

Liver sections stained with Oil Red O (ORO) for the preparation of neutral red accumulation for each fish group are represented in **Figure 22**. Due to the high lipid content, the liver proved to be extremely difficult to section and the staining revealed large variations in the quality of the sections within groups. This caused lipid generated artefacts to mask the relationship between neutral lipid and the

tissue as can be seen in the images below. For this reason the neutral lipid accumulation was not quantified but it was decided that the total lipid content of the liver would be measured.

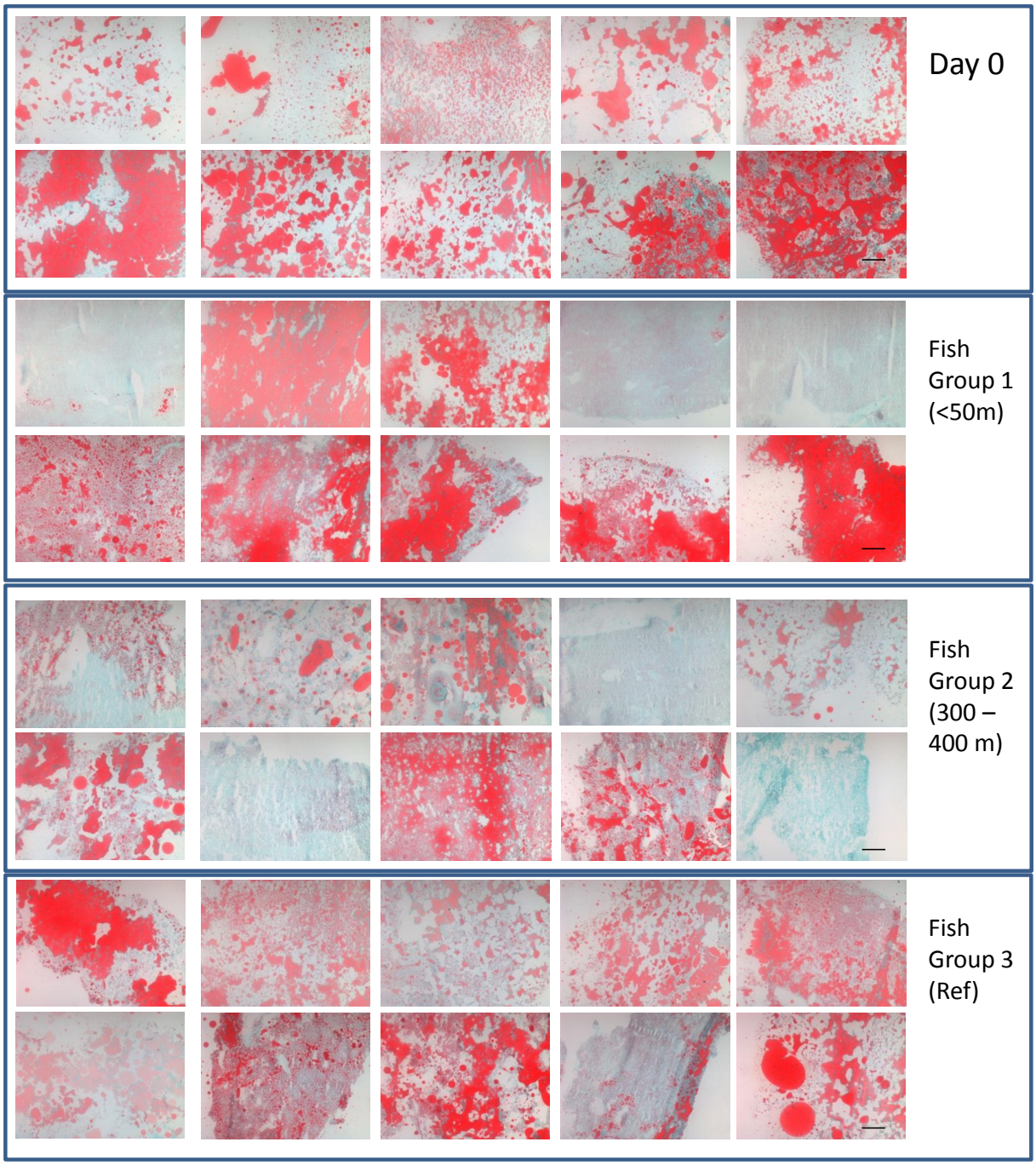

Figure 22. Cod liver sections (10 µm) stained for neutral lipid accumulation from the fish groups indicated (Scale bar = 300 µm). Images represent the livers of 10 individual fish per group.

5.4.7 Lipid content of cod liver

The lipid content of the liver from the pre-exposed and fish exposed for 9 weeks is shown in **Figure 23**. The percentage lipid content of the liver of fish from the pre-exposure group was significantly higher than all fish groups following the 9 week exposure (ANOVA, Tukey, $p<0.05$). There was no significant deference between caged fish groups (i.e. fish groups 1-3) after 9 weeks exposure.

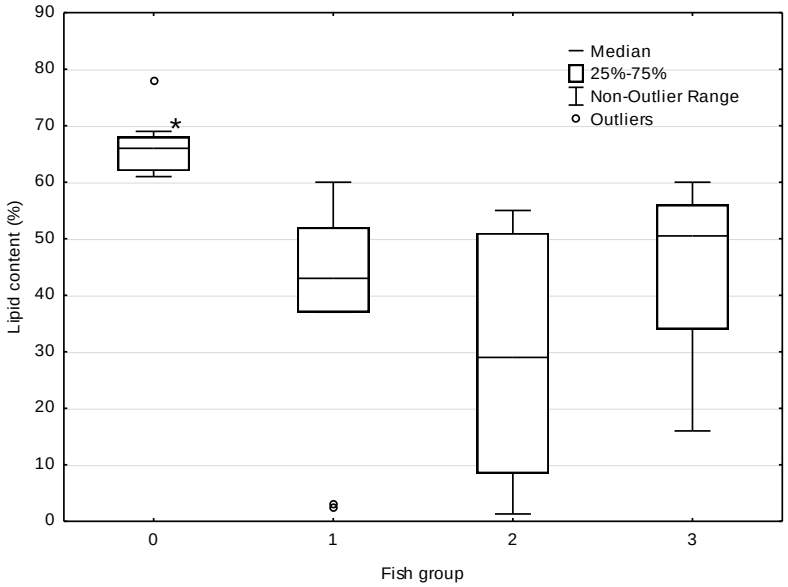

Figure 23. Lipid content of the liver of caged cod from the groups indicated following 9 week field exposure. Data presented as median, quartiles with outlier range. Fish group 0 = pre-exposure group.

5.4.8 Histopathology

Gill subsamples and entire gonads from individuals sampled after 9 weeks, as well as from day 0 reference fish, were assessed for histological abnormalities. The histological assessment of both gonads and gills was qualitative, i.e. abnormalities were either scored as being "present" or "absent" with no further subdivision of the severity of observed abnormalities.

Four different abnormalities were assessed in gills; hyperplasia (local cell proliferation), necrotic tissue, fusion of gill lamellae and aneurysms (blood vessel bulges). Except for necrosis, all four abnormalities were scored in fish. However, and although abnormalities were only scored in deployed fish and not in the day 0 reference group, the frequency of abnormalities was low (one or two individuals in each group of 15 assessed individuals) and there were no obvious differences in the general appearance of gills that could be related to the distance to the discharge from Nyhamna.

Four different abnormalities were assessed in the gonad; intersex, vacuolation, apoptotic cells and necrotic tissue. All four abnormalities were scored in fish, the two most common being apoptosis, which was scored in 7 of 58 assessed individuals, and intersex, which was scored in 3 individuals. However, none of the four abnormalities could be traced to exposure from Nyhamna, e.g. fish in group 1, that were caged in the vicinity of the discharge, showed the lowest frequency of gonad abnormalities of all groups, whereas day 0 reference fish showed the highest frequency. Of the total of four individuals expressing intersex, a very serious abnormality, two individuals were found in the day 0 reference group.

The histological assessment of gonads also showed that groups 1-2 sampled after 9 weeks had a strong bias to male fish. Except four individuals all fish displayed immature gonads, the remaining four individuals, which were all male, had maturing gonads.

The histological scoring sheets are summarised in Table 5 (gills) and Table 6 (gonads).

Table 5. Gill histopathology scoring sheet from the groups indicated. Green colour indicates normal gills whereas orange colour indicates "presence" of that particular abnormality.

Fish no.	Exposure	Station	Gills			
			Hyperplasia	Necrosis	Fusion	Aneurysms
1	0 weeks	Reference				
2	0 weeks	Reference				
3	0 weeks	Reference				
4	0 weeks	Reference				
5	0 weeks	Reference				
6	0 weeks	Reference				
7	0 weeks	Reference				
8	0 weeks	Reference				
9	0 weeks	Reference				
10	0 weeks	Reference				
11	0 weeks	Reference				
12	0 weeks	Reference				
13	0 weeks	Reference				
14	0 weeks	Reference				
15	0 weeks	Reference				
101	9 weeks	1				
102	9 weeks	1				
103	9 weeks	1				
104	9 weeks	1				
105	9 weeks	1				
106	9 weeks	1				
107	9 weeks	1				
108	9 weeks	1				
109	9 weeks	1				
110	9 weeks	1				
111	9 weeks	1				
112	9 weeks	1				
113	9 weeks	1				
114	9 weeks	1				
115	9 weeks	1				
201	9 weeks	2				
202	9 weeks	2				
203	9 weeks	2				
204	9 weeks	2				
205	9 weeks	2				
206	9 weeks	2				
207	9 weeks	2				
208	9 weeks	2				
209	9 weeks	2				
210	9 weeks	2				
211	9 weeks	2				
212	9 weeks	2				
213	9 weeks	2				
301	9 weeks	3				
302	9 weeks	3				
303	9 weeks	3				
304	9 weeks	3				
305	9 weeks	3				
306	9 weeks	3				
307	9 weeks	3				
308	9 weeks	3				
309	9 weeks	3				
310	9 weeks	3				
311	9 weeks	3				
312	9 weeks	3				
313	9 weeks	3				
314	9 weeks	3				
315	9 weeks	3				

Table 6. Gonad histopathology scoring sheet from the groups indicated. Green colour indicates normal gonads whereas orange colour indicates "presence" of that particular abnormality. Red colour indicates individuals expressing intersex, a particularly serious abnormality.

Fish no.	Exposure	Station	Gonad							
			Male	Female	Immature	Maturing	Intersex	Vacuolation	Apoptosis	Necrosis
1	0 weeks	Reference	x			x				
2	0 weeks	Reference	x		x					
3	0 weeks	Reference	x			x				
4	0 weeks	Reference		x						
5	0 weeks	Reference		x	x					
6	0 weeks	Reference	x		x					
7	0 weeks	Reference		x	x					
8	0 weeks	Reference		x	x					
9	0 weeks	Reference		x	x					
10	0 weeks	Reference	x		x					
11	0 weeks	Reference		x	x					
12	0 weeks	Reference		x	x					
13	0 weeks	Reference		x	x					
14	0 weeks	Reference		x						
15	0 weeks	Reference		x	x					
101	9 weeks	1		x	x					
102	9 weeks	1		x	x					
103	9 weeks	1	x		x					
104	9 weeks	1		x	x					
105	9 weeks	1	x		x					
106	9 weeks	1	x		x					
107	9 weeks	1	x		x					
108	9 weeks	1	x		x					
109	9 weeks	1	x		x					
110	9 weeks	1	x		x					
111	9 weeks	1	x		x					
112	9 weeks	1	x		x					
113	9 weeks	1	x		x					
114	9 weeks	1	x		x					
115	9 weeks	1	x		x					
201	9 weeks	2		x	x					
202	9 weeks	2	x							
203	9 weeks	2	x		x					
204	9 weeks	2	x		x					
205	9 weeks	2	x		x					
206	9 weeks	2	x		x					
207	9 weeks	2		x	x					
208	9 weeks	2	x		x					
209	9 weeks	2	x		x					
210	9 weeks	2	x		x					
211	9 weeks	2	x		x					
212	9 weeks	2	x		x					
213	9 weeks	2								
301	9 weeks	3	x		x					
302	9 weeks	3	x		x					
303	9 weeks	3		x	x					
304	9 weeks	3		x	x					
305	9 weeks	3		x	x					
306	9 weeks	3	x		x					
307	9 weeks	3	x			x				
308	9 weeks	3	x							
309	9 weeks	3	x		x					
310	9 weeks	3		x	x					
311	9 weeks	3		x	x					
312	9 weeks	3								
313	9 weeks	3	x			x				
314	9 weeks	3		x	x					
315	9 weeks	3		x	x					

6. Discussion

6.1 Chemical exposure

Produced water, although a highly variable and complex mixture, typically contains trace amounts of PAHs and their alkyl substituted analogues such as NPDs, as well as APs and metals. However, previous studies measuring PAH-NPD compounds in the receiving waters at Nyhamna, as well as in controlled laboratory studies with the Nyhamna PW effluent, have reported extremely low or undetected concentrations of PAH-NPD compounds in the PW using SPMD and mussel tissues (Brooks et al., 2011; Brooks et al., 2012, NIVA report, 2011). The PW treatment system in place at Nyhamna has been shown to remove up to 99% of the PAH-NPD compounds (Aker Kværner, 2005), and was considered to be the reason for such low discharges of these chemicals. Alkylphenols were targeted in the present study as a result of a recent report, which highlighted elevated concentrations of C_4-C_5 phenols in the concentrated effluent at Nyhamna prior to discharge (**Table 7**, Weideborg and Vik, 2010). The APs which contributed to the sum C_4-C_5 concentration of 144 µg/L measured in the PW prior to dilution with the cooling water included: C_4 phenols, 4-*tert*-butylphenol (110 µg/L) and 4-isopropyl-3-methylphenol (5.8 µg/L); and C_5 phenols, 2-*tert*-butyl-4-methylphenol (10 µg/L) and 4-*tert*-butyl-4-methylphenol (13 µg/L).

Table 7. Data taken from the Weideborg and Vik (2010), where elevated concentrations of alkylphenols in the PW effluent after treatment were measured, which pose a potential threat to aquatic life. Predicted environmental concentration (PEC), Predicted no effect concentration (PNEC). PEC/ PNEC > 1 would indicate the potential to cause harm.

Compound	PEC (µg/L)	PNEC (µg/L)	PEC/PNEC (without dilution with cooling water)	PEC/PNEC (dilution 1:217 with cooling water)	PEC/PNEC (10x dilution with receiving water)
Phenol	4	2	2	0.009	0.0009
Sum C0-C3	61.6	2	31	0.14	0.014
Sum C4-C5	144	0.4	360	**1.6**	0.16
Sum C6+	0.93	0.2	4.7	0.021	0.0021

Since APs are more water soluble, particularly the lighter APs, the Nyhamna treatment system is less efficient at removing them from the PW effluent (Aker Kværner, 2005). As a result, APs may be released into the receiving waters with potential biological effects on the local aquatic organisms. The exposure of the fish to the PW effluent was assessed using a combination of passive sampling devices in the water column and metabolites collected in the bile of the exposed fish. POCIS were used to provide a time-integrated and quantitiative measure of up to 50 APs in the water column. The sensitivity of this method allows measurements of AP in the low nanogram per litre range.

6.1.1 Alkylphenols in POCIS

As only a few target APs were quantifiable in POCIS after 10 days, the following discussion is limited to results after the 9 week deployment. Overall, it is likely that the low concentrations measured represent background or near background concentrations. The higher than expected concentration of 4-*n*-nonylphenol at the site closest to the discharge (14.6 ng/L) is probably due to contamination as elevated concentrations were only found in one replicate, which also showed higher concentrations of C_6 and C_7 AP. However, it cannot be ruled out that it is a real measurement. In either case such levels of NP are below relevant limits set by authorities such as the environmental quality standard (EQS) outlined in the European Water Framework Directive of <300 ng/L and <2000 ng/L (yearly average and maximum values respectively). It should be noted that these limits refer to total concentrations whereas POCIS measures only the freely dissolved fraction. This is more of an issue for highly

hydrophobic compounds which are more likely to be bound to particulates or dissolved organic carbon than the AP of interest in this study.

In an earlier study of the discharge water C_4 and C_5 AP were determined as being of potential risk (Weideborg and Vik, 2010). The PNEC value used in those calculations for C_4-C_5 AP was 0.4 µg/L (400 ng/L) whereas total POCIS derived concentrations for these groups were <1 ng/L in all cases. 4-*tert*-butylphenol, which was identified as the compound contributing most significantly to the levels of C_4 AP measured in the PW in the previous discharge water study, was not quantifiable in any POCIS sample. The maximum theoretical water concentration estimated from the Q.L. for 4-*tert*-butylphenol, was 3.8 and 0.6 ng/L (10 day and 9 week, respectively), which is more than 100 times less than the PNEC. The *actual* concentration was almost certainly much lower. Based on the current results we find very little evidence of elevated levels of AP in the recipient. This is similar to earlier results using similar passive sampling devices to sample for both PAH and APs from Nyhamna PW (Brooks et al., 2011; Brooks et al., 2012).

6.1.2 Alkylphenols in bile metabolites

Bile is considered as the best target tissue for the bioaccumulation of PAHs and APs and ideal for exposure assessment (Beyer et al., 2011). However, it has been recently reported that AP metabolites within the bile have a relatively short half life between 10 and 20 hours (Sundt et al., 2009). Therefore the concentrations of APs in the bile are an indication of the current or recent exposure within 24 hours of sampling. Overall, the concentrations of AP metabolites in the bile of the caged fish were small with most APs falling below 1 ng /g bile. Higher AP concentrations were found in individual compounds from C_1, C_3 and C_5, although maximum concentrations were below 50 ng/g bile in 10 day exposed fish. For the 10 day exposure, only two AP compounds showed a relationship with proximity to the discharge outlet with low concentrations in the pre-exposure fish, these included C_1 phenol p-cresol and C_7 phenol 2, 6-dimethyl-4-1,1-dimethylpropyl-phenol. However, the smallest APs need to be used with caution as indicators of PW exposure since they are also present as natural breakdown products both in the environment and within living organisms (Beyer et al., 2011). In particular, in excretion fluids such as bile their presence can be expected as a result of the catabolism of natural endogenous biomolecules and of natural feed substances. Therefore, the increase in *p*-cresol (4-methylphenol) is likely to be not as a result of exposure to the discharge outlet but rather natural sources. The C_7 phenol found highest in fish bile closest to the discharge outlet was less likely to originate from an endogenous source, although maximum concentrations of this C_7 phenol were <0.12 ng/g bile and unlikely to cause any environment risk.

For the 9 week exposure, C_1 to C_3 APs showed a relationship with distance from the discharge outlet with *p*-cresol up to 400 ng/g bile. However, as mentioned earlier natural and endogenous factors are considered to be the main sources of these compounds. Of the larger APs, that are considered to be more environmentally harmful, C_6 (2,6-diisopropylphenol), C_7 (2,6-dimethyl-4-1,1-dimethylpropyl-phenol), C_8 (2,4-di-*tert*-butylphenol) and 4-n-nonylphenol showed higher concentrations in the bile of fish caged closest to the discharge outlet. However, the concentrations of C_6, C_8 and C_7/C_9 where less than 10, 5 and 1 ng/g bile respectively and were not considered to represent any significant environmental risk.

The concentrations of certain small AP metabolites were markedly higher after 9 weeks than measured after 10 days. This was particularly the case for C1 phenol (*p*-cresol) and to a lesser extent C2 phenol. The reason for this is not entirely clear, although it is known that *p*-cresol can be taken up from the gut, when tyrosine in food is degraded by anaerobic bacteria (Bakke, 1969) and consequently contribute to the background level of this compound in the bile (Jonsson et al. 2008). In most other cases, AP metabolite concentrations of the individual compounds were similar between 10 days and 9 weeks exposure.

Comparison with AP metabolites measured in the bile of cod exposed for 6 weeks at approximately 300 m from an offshore PW discharge in the North Sea, show individual C_2 phenols up to 100 ng/g bile (Brooks et al., 2009). Unfortunately, only 5 APs were measured (C_2-C_3 phenols) in the offshore study, although some of those that were measured e.g. 2, 4, 6-trimethylphenol were almost 500 fold higher than that measured in the fish bile closest to the discharge at Nyhamna after 10 days and 9 weeks (~0.2 ng/g bile). The low concentrations of APs in the bile of the caged fish correspond with the similarly low concentrations of APs in the POCIS and support the view that the concentration of APs in the receiving waters posed little environmental risk.

6.1.3 Metals in DGTs

The DGT provides a measure of the bioavailable portion of the metal, which essentially concerns the free ion and the inorganic fraction, referred to as the labile fraction. This fraction is dependent on a variety of influencing factors including dissolved organic carbon, competing ion concentrations, particulate matter and water hardness (Luoma, 1983). It is this bioavailable fraction that is responsible for causing potential biological effects in target organisms since these compounds can cross biological membranes and effect cellular function. Therefore, such a measurement has high biological relevance for the assessment of environmental risk.

The labile metal concentration of Al, Cu and Pb in the receiving water based on the 10 day DGT exposures revealed a relationship with the distance from the discharge outlet, with highest concentrations at the closest station to the discharge outlet. However, the labile concentrations of these metals were typical of low coastal water background concentrations where 0.3, 0.25 and 0.013 µg/L for Al, Cu and Pb respectively was measured.

The DGT data collected for the metal accumulations over 9 weeks showed a clear signal at the closest station (<50 from the outlet), with all metals measured showing a significant increase compared to station 2 (300-400 m away) and the reference station. This confirms that the DGTs attached to the side netting of the fish cage were exposed to the PW discharge plume. The environmental quality standards (EQS) of metals have been criticised since they often consist of one value for the total metal concentrations with no regard for the factors that can affect metal bioavailability and potential synergic effects to aquatic organisms (Environment agency, 2008). However, the same report highlighted the potential of DGTs for improving the EQS of metals in aquatic systems by measuring the bioavailable fraction.

Table 8. Summary of the European Environmental Quality Standards (EQS) or maximum allowable concentrations (MAC) for the measured metals in sea water.

Metal	EQS (µg/ L)	Metal	EQS (µg/L)
Al	–	Fe	1000
Cd	2.5	Mn	–
Co	100 (MAC)	Ni	30
Cr	15	Pb	25
Cu	5	Zn	40

With exception to Al and Mn, where EQS values are not available, all the metals measured in the present study had seawater concentrations much below their respective metal EQS values (**Table 8**). The bioavailable metal determined with the DGTs was between 10 fold and 1000 fold lower than their EQS values, which may suggest that the metals were well within safe environmental limits and unlikely to be a risk to marine life.

6.2 Biological effects

6.2.1 General fish biometry

The physiological state of the fish before and after the 10 day and 9 week exposure was evaluated using a range of biometric measurements. The length and weight of the pre-exposed fish (day 0), were the same as all the other fish groups except for group 2 (300-400 m from the outlet) after 9 weeks. The fish from this group had unusually high mortalities, which resulted in only 13 fish available for sampling. These 13 fish were significantly smaller than the fish from the other groups and appeared to have been lacking sufficient food during the exposure. From the calculated condition index it was clear that the fish held in cages for 9 weeks had a significantly lower health status as those fish from the pre-exposure groups and those held in cages for 10 days. This was particularly the case for the fish from group 2 after 9 weeks, which is in agreement with the lower weight of these fish and suggests starvation.

The LSI, which can provide an indication of nourishment status, supports the results from condition index. All fish held in cages for 9 weeks had significantly lower LSI than fish from the day 0 and 10 day exposures. This indicates that the fish held in cages for 9 weeks experienced insufficient nourishment during the exposure. Although blue/green lights were placed within the cages to encourage phytoplankton and zooplankton to enter and thus act as a food source for the fish, it appeared insufficient to keep the fish in optimal health during the longer exposure duration. Since farmed fish were used in the study, which are conditioned to be fed with food pellets, the transplantation into a cage and the need to search for food independently may have increased the stress on the fish. After only 10 days exposure in the fish cages, the fish had no notable sign of stress with weight and condition index unaltered from the day 0 fish. However, the LSI for the 10 day exposed fish was slightly lower than the pre-exposed fish, although only significantly different in fish group 2, suggesting that food nourishment was lower than optimum during even the shorter exposure duration. These differences in fish nourishment during the caged exposures must be taken into consideration with respect to the biomarker data.

The gonad somatic index (GSI), showed large variation and no differences between groups were evident. This may not be surprising, since immature fish were used in the study at slightly varying degrees of maturity. The age of maturation in Atlantic cod has been shown to vary markedly starting at around 3 years old (Godø and Haug, 1999). All the cod used in the current exposure were below the age of maturation.

6.2.2 10 day exposure

The biological responses measured following the shorter exposure duration included MN, VTG and EROD. The shorter exposure was selected for these biomarkers since they have been previously found to be faster responding, particularly for the enzyme biomarkers (EROD, VTG), where initial up regulation can be followed by down-regulation of the protein product following prolonged exposure durations (Grosvik et al., 1997; Williams and Oleksiak, 2011). In the present study there was no significant difference between the fish groups for MN, VTG or EROD.

The median frequency of MN in the fish blood was found to be identical in all groups at 0.4 MN per 1000 cells. Background assessment criteria (BAC) have been established for many biomarker responses in a variety of monitoring species by the International Co-operation for the Exploration of the Seas (ICES) working group on the biological effects of contaminants (WGBEC, WGBEC, 2012). The BAC for MN is currently 0.4 MN/ 1000 cells. Therefore, the level of genotoxicity experienced by the fish in all groups was typical of background responses.

Vitellogenin expression in the blood plasma of the Atlantic cod was found to be highly variable in all groups with no differences between the fish groups or between genders. APs have been found to be

estrogenic with some classified as endocrine disrupting substances (Thomas et al., 2004). However, the estrogenic potency of APs linked to the binding affinity to the oestrogen receptors is low. For example, the binding affinity of C4-C6 AP is approximately 2 million fold lower than that of the endogenous 17β-estradiol (E2), whilst the most estrogenic AP, C8-C9 have a binding affinity 20,000 fold lower than E2 (Tollefsen & Nilsen, 2008). When considering this, it appears unlikely that differences in VTG following exposure to AP at the background concentrations reported would elicit a positive VTG expression in the caged fish. Background assessment criteria (BAC) of VTG in Atlantic cod have been set at 0.23 µg/mL (WGBEC, 2012). The VTG concentrations reported in the fish blood plasma from all groups was below or slightly above this BAC value and suggest background levels of exposure.

Although exposure to APs and other potential xenoestrogens are unlikely to illicit VTG expression, other effects relating to the feminisation of the fish have been reported at concentrations as low as 0.1 µg/ L (Mills and Chicester, 2005). Potential responses to low doses of AP include the reduction in the circulating levels of E2 hormones in females leading to slower oocyte development, lower GSI and reduced spawning time (Meier et al., 2007).

An increase in EROD activity has been linked with exposure to organic compounds in the laboratory including PAHs (Bucheli and Fent, 1995). EROD expression showed no relationship with the distance from the PW discharge outlet and showed no statistical differences between the fish groups. The BAC for EROD in the microsomal fraction of homogenated liver of the Atlantic cod has been suggested by the ICES working group to be 145 pmol/ min/ mg protein. The median EROD activity of the caged cod in the present study ranged from 20 to 40 pmol/min/ mg protein and was therefore markedly lower than the BAC.

Plasma chemistry parameters analysed after 10 days exposure showed significantly higher levels of lactate (measured indirectly as LD activity) and inorganic phosphate in fish from group 1, which was held close to the discharge point. These observations suggest a higher muscular activity in the fish from group 1 e.g. induced by stress, resulting in a build-up of plasma lactate levels, an acidic metabolite which can inhibit the phosphate in the blood (metabolic acidosis). Other plasma parameters analysed after 10 days did however not display differences between exposure groups. Plasma chemistry represents an attractive (while simple and cost-effective) and potentially non-destructive analysis platform for a general expression of fish health. However, plasma chemistry cannot distinguish between pollutant classes in the same way as e.g. hepatic EROD activity and is, therefore, to be considered complementary to established exposure biomarkers and histological assessment.

Overall, the biological responses after a 10 day exposure showed only small differences to the reference fish and appeared to be unaffected by the proximity (< 50 m) from the discharge outlet.

6.2.3 9 week exposure

The longer 9 week exposure was considered suitable to determine histochemical and histopathological responses of exposure to the discharge effluent, which are known to be exhibited following longer exposure durations. The histochemical biomarkers that were original planned proved unsuitable due to the high lipid content of the liver that prevented adequate sectioning of the tissue. Due to this reason, lysosomal membrane stability (LMS) and lipofuscin accumulation were replaced with peroxisomal proliferation and liver lipid content of the liver.

The histochemical assessments of the fish liver following the 9 week exposure showed no significant differences between the pre-exposure fish and those from the caged groups. The histopathological assessment of gills and gonads did not reveal any higher-order effects that could be linked to exposure. The gills generally appeared normal in all groups and serious abnormalities were not detected in any

of the fish. The gonad of three individuals expressed intersex, however two of these were found in the day 0 reference group, which was never employed in the area potentially exposed to discharges from Nyhamna.

Plasma chemistry analysed after 9 weeks showed significantly lower levels of circulating lipids (triglycerides) and lipoproteins (cholesterol) compared to fish sampled earlier in the experiment. These results hence support the biometry data (CI, LSI) and underline that fish deployed for 9 weeks were subjected to starvation.

There was still a trend after 9 weeks that fish held close to the discharge (group 1) expressed elevated lactate levels, which were however not significant from the other groups. In general, plasma lactate levels dropped with increasing exposure time in all groups, including fish from group 3 (reference station).

7. Conclusions

- APs were not detected at elevated concentrations in POCIS deployed for both 10 days and 9 weeks in the receiving water at Nyhamna. The AP concentrations, showed no clear relationship with the distance from the discharge outlet.
- AP metabolites were detected in the fish bile at low ng/g concentrations for both 10 day and 9 week exposures. There was no relationship between AP metabolite concentrations and distance from the discharge outlet at Nyhamna, which corresponds with the AP concentrations from POCIS.
- The DGTs accumulated metals during the 10 day and particularly during the 9 week exposure. Significantly higher concentrations of all 10 metals measured (Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn) were found at the closest station < 50 m from the discharge outlet following 9 weeks exposure. However, the concentration of these metals at the closest station were still between 10 and 1000 fold lower than the environmental quality standard (EQS) for the respective metal.
- Overall, based on the chemical assessment of APs and metals in the receiving waters at Nyhamna using POCIS, DGTs and bile metabolites, the PW discharge posed little to no environmental risk.
- Significantly lower CI and LSI were found in fish held in cages for 9 weeks compared to fish from day 0 and 10 day caged groups. This was considered to be the result of starvation of the fish during the longer exposure duration. Starvation was severe in at least one group (station 2) after 9 weeks resulting in fish mortalities.
- The biological responses measured after 10 days (MN, VTG, EROD, CI, plasma chemistry) and 9 weeks (Liver and gonad histopathology, neutral lipid, peroxisomal proliferation, plasma chemistry and CI) showed no relationship with distance from the PW discharge outlet.
- Overall, the fish appeared to be unaffected by the PW discharge despite the close proximity from the discharge outlet (<50 m).

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