

Effects of mechanically and chemically dispersed oil on the osmo- and ion-regulatory capacity of juvenile lump sucker (*Cyclopterus lumpus*)

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*"If you torture the data long enough,
it will confess".*

- Ronald Coase

Abstract

The effects of 48 hours exposure to mechanically and chemically dispersed oil on juvenile lumpsuckers, as well as the use of gill EROD activity and bile PAH metabolites as potential biomarkers were assessed in this study. This was done by measuring the gill EROD activity, bile PAH metabolites, plasma osmolality, plasma chloride concentration, and gill $\text{Na}^+/\text{K}^+\text{ATPase}$ activity. The mechanical and chemical dispersion did not differ significantly in terms of gill EROD induction, PAHs metabolism, plasma osmolality and chloride concentration, and gill $\text{Na}^+/\text{K}^+\text{ATPase}$ activity. The oil exposure and metabolism of PAHs were confirmed by the presence of PAH metabolites in the bile. Thus, this study can recommend the use of juvenile lumpsucker's bile metabolites as a biomarker in detecting oil exposure. The gill EROD activity showed a lack of response upon exposure to oil. Further investigation is recommended to be able to determine the suitability of its use as a biomarker. A significant relationship was seen between crude oil concentration and plasma osmolality and plasma chloride concentration, i.e. as the oil concentration increases, the plasma osmolality and plasma chloride concentration also increases. The mechanistic link between oil exposure and gill $\text{Na}^+/\text{K}^+\text{ATPase}$ activity was not clear, thus warrants further investigation. This study has shown that the acute exposure to crude oil was not fatal to the fish but caused impairment on hypo-osmoregulation mechanism.

Abbreviation

ADP	Adenosine diphosphate
AhR	Aryl hydrocarbon receptor
AM	Assay mixture
AM-O	Assay mixture with ouabain
AMAP	Arctic Monitoring and Assessment Programme
ANOVA	Analysis of variance
ARNT	Aryl hydrocarbon nuclear translocator
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
β	Beta
CD	Dispersant control
CDL	Chemical dispersion- low oil concentration
CDH	Chemical dispersion-high oil concentration
Cl	Chloride
CYP	Cytochrome P450–dependent monooxygenase
CYP1a	Cytochrome P4501a
δ	Delta
DNA	Deoxyribonucleic acid
FF	Fixed wavelength fluorescence
EROD	Ethoxyresorufin-O-deethylase
K ₂ HPO ₄	Dipotassium phosphate

KCL	Potassium chloride
KH ₂ PO ₄	Monopotassium phosphate
IB	Imidazole buffer
λ	Lambda
L	Liter
L/hr	Liter per hour
MDL	Mechanical dispersion- low oil concentration
MDH	Mechanical dispersion- high oil concentration
Mg ²⁺	Magnesium ion
mmol/L	millimole per liter
mOsm	milliosmole
MRC	Mitochondria-rich cells
Na	Sodium
NAD ⁺	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydride
NADPH	Nicotinamide adenine dinucleotide phosphate
Na ⁺ /K ⁺ -ATPase	Sodium potassium adenosine triphosphatase
nmol	nanomole
NKCC	Sodium potassium chloride
PAH	Polycyclic Aromatic Hydrocarbon
PHH	Planar hydrogenated hydrocarbon
Pi	Phosphate
pmol	picomole

ppm	parts per million
rpm	rotation per minute
SFS	Synchronous fluorescence scan
SO ₄ ²⁻	Sulphate

Acknowledgment

I have started this Master's oppgave without knowing how to stir a chemical using a magnetic stirrer. But after many hours of labwork, I not only knew how this magnetic stirrer works, but I got to learn a lot of new things too, like running assays such as EROD, osmolality, $\text{Na}^+\text{K}^+\text{ATPase}$, etc.

I was (or I still am) a crappy writer. Writing was very difficult for me- with so many things I wanted to say, it takes a clear mind to be able to filter out which ones are important and which ones are not (my advisers can attest to that). But writing this manuscript has taught me some valuable lessons (and techniques), especially when writing a physiological work. So, with this, I am happy for.

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1. INTRODUCTION

1.1 General Background

The decreasing ice cover in the arctic in the recent years has led to increased oil and gas exploration and maritime shipping activities. Although these can be beneficial within an economical context, they can also pose environmental risks through discharges and accidental oil spills. Although accidental oil spills are not considered a major source of pollution (UNEP/IOC/IAEA, 1992), the consequences of such potential accidents can be disastrous to the local marine flora and fauna (Dauvin, 1998; Cadiou et al., 2004; Claireaux et al., 2004).

The major constituents of petroleum are the polycyclic aromatic hydrocarbons (PAHs). They are lipophilic, organic environmental pollutants that contain 2- and more fused aromatic rings that are arranged in a planar structure (AMAP, 2010). They can enter into the marine environment through maritime activities, natural oil seeps, petroleum extraction activities, etc., but most concern is related to accidental oil spills.

In the environment, PAHs exist as complex mixtures of many individual compounds that differ primarily by number and position of aromatic rings (Di Giulio and Hinton, 2008). Most of them exhibit low water solubility, can accumulate to high levels in sediment, and are readily taken up by aquatic organisms (Di Giulio and Hinton, 2008). Low molecular weight PAHs, e.g. naphthalenes, are slightly water soluble and can be bioaccumulated in

organisms from the 'dissolved' phase while the high molecular weight PAHs, e.g. benzo(a)pyrene, have very low water solubility (AMAP, 2010).

Degradation of oil through natural processes takes some time. This is why chemical dispersants are applied in case of oil spills to accelerate its degradation. Chemical dispersants are mixtures of "surface-active" chemicals (surfactants) and solvents. Surfactant molecules have one end that stick to the oil and another end that stick to the water. This will allow the oil to be attached to the water, causing it to be "dispersed" into tiny droplets that will remain suspended in the water column. The solvents, on the other hand, will just evaporate to the atmosphere (Boyd et al., 2001). The advantage of using a dispersant is that it accelerates the natural dispersion process by converting the slick into small oil droplets and thereby increasing the potential for biodegradation (Thiem, 1994; Churchill et al., 1995).

The elimination of oil slicks on the surface may benefit the marine mammals and birds, however, the dissolution of the oil into the water column increases its bioavailability to the nekton species, as well as to the benthic species once it settles down to the sediments. At present, the information available on the effects of dispersants on Arctic and sub-Arctic aquatic organisms are sparse. Chemical dispersion may be a quick way to remove the slick from the surface but its effects on the aquatic organisms in the water column as well as in the benthic area must be investigated to come up with a sound basis in evaluating whether its use in response to oil spill create more good than harm.

1.2 The test organism, lumpsucker (*Cyclopterus lumpus*)

The lumpsucker, *Cyclopterus lumpus* is a unique species, with no close relatives. It belongs to Class Actinopterygii, Order Scorpaeniformes, and Family Cyclopteridae. It is the only species of genus *Cyclopterus* (Davenport, 1985). It is widely distributed in the boreal region of both sides of the North Atlantic, between 70°N and 37°N. Often, growing larvae and juveniles can be found in intertidal pools throughout the summer after spring spawning, while older juveniles are semi-pelagic (Davenport, 1985). Adults are substantially pelagic (*contrary to the belief that they were benthic dwellers*), but switch to become demersal from winter until they spawn in spring time (Blacker, 1983). Adult male can grow up to 55 cm while females up to 63 cm (Pethon, 1989). They reach sexual maturity between the ages of five to seven years old (Cox, 1920). Economically, lumpsuckers are an important resource for the northern Norwegian coastal fisheries, mainly utilized for caviar production.

Lumpsucker was chosen in this study to represent a benthic/semi-pelagic fish species in the Arctic and sub-Arctic coastal areas. It is a relevant species to use in investigating the effects of dispersed oil as the juveniles are poor swimmers, with limited possibility to escape the oil spill. Further, there is still insufficient information on the effect of dispersed oil on this species and a need to develop biomarkers of oil exposure which can be applied on this species.

1.3 Background summary of “Effects of oil spills in coastal waters” project

As part of the Joint Industry Project entitled, “Effects of oil spill in coastal waters” we have conducted a study that assessed the acute responses and long-term effects of acute exposure to dispersed oil on juvenile lumpsuckers (Frantzen et al., 2015).

In that study, juvenile lumpsucker, *Cyclopterus lumpus* were exposed for 48 hours to North sea crude oil only, i.e. M treatment (mechanical dispersion) and to North sea crude oil + Dasic NS dispersant, i.e. C treatment (chemical dispersion). The nominal oil concentrations used were 30, 67, 147, 323 and 710 mg oil/L sea water, which corresponds to treatment groups M1 and C1, M2 and C2, M3 and C3, M4 and C4 and M5 and C5, respectively. A control (clean sea water only) and a dispersant control (CD), i.e. no oil + 28.4 mg dispersant/L sea water, a concentration equivalent to the highest concentration used for chemically dispersed oil treatment were also included (Table 1). After the exposure, post-exposure growth and survival were monitored for 1.5 months to evaluate the long-term consequence of the acute exposure. The following factors, i.e. PAH uptake in muscle tissue, gill histopathology, liver EROD activity and acute toxicity were investigated as indicators of acute responses.

In the water chemistry analysis, it showed that the C treatment contained higher PAH levels, both initially and after 48 hours of exposure than the M treatment. This was probably due to the hydrocarbon content of the solvent in the Dasic NS dispersant mixture which contain 30-60% petroleum distillates, as stated in the Dasic NS manufacturing sheet (Frantzen, et al. 2015) (Table 1).

Table 1. Chemical characterization of exposure media from experiments utilizing the same exposure set-up and protocol as in the present study. Water samples were collected at the start (T0) and at the end (T48) of the exposure period. (N=2, mean \pm standard deviation except from M5 and C5 where N=1). Ctrl; control, CtrlD; dispersant control, M; mechanically dispersed oil, C; chemically dispersed oil. All data included in the table were extracted from Frantzen et al., 2015. Treatments highlighted in gray correspond to treatments included in the present study.

Treatment	Nominal [oil] (mg/L)	Nominal [Dasic NS] (mg/L)	Start of exposure (T0)		End of exposure (T48)	
			THC (mg/L)	SUM 26 PAH (mg/L)	THC (mg/L)	SUM 26 PAH (mg/L)
Ctrl	0	0	0.1 \pm 0.0	2.9 \pm 1.3	0.1 \pm 0.1	1.3 \pm 0.4
CtrlD	0	0	5.3 \pm 0.4	6.5 \pm 1.4	2.0 \pm 0.8	3.5 \pm 0.8
M1	30	0	1.4 \pm 1.6	75 \pm 37	0.6 \pm 0.4	23 \pm 7
C1	30	1	6.3 \pm 2.2	200 \pm 93	1.7 \pm 0.2	36 \pm 11
M2	67	0	9.9 \pm 3.0	313 \pm 117	3.8 \pm 3.3	98 \pm 86
C2	67	3	15 \pm 6	425 \pm 71	3.7 \pm 0.1	83 \pm 4
M3	147	0	20 \pm 11	681 \pm 308	5.5 \pm 3.9	242 \pm 117
C3	147	6	31 \pm 3	965 \pm 17	5.7 \pm 2.4	179 \pm 0
M4	323	0	52 \pm 44	1526 \pm 955	11 \pm 10	314 \pm 225
C4	323	13	81 \pm 1	2172 \pm 224	15 \pm 11	417 \pm 164
M5	710	0	174	3838	41	1109
C5	710	28	257	4724	20	693

The PAH in the muscle tissues showed a clear exposure concentration-dependent increase in body burden (Frantzen et al., 2015). The PAH type were dominated by naphthalene and substituted naphthalene homologues, followed by fluorine and phenanthrene. The hepatic EROD activity was significantly higher in all concentrations (except in the highest concentration) than the control. A positive correlation between hepatic EROD activity and muscle PAH concentration was seen at the lower oil concentrations, while inhibition of enzyme activity was seen at the higher concentrations (Frantzen et al., 2015). In gill histopathological analysis, the significant trait observed was an increase in mucocyte numbers at CD than the control. There were also occurrences of epithelial hyperplasia, epithelial lifting and epithelial hypertrophy, however, in general,

these traits did not have any clear correlation to dispersion method nor oil concentrations (Frantzen et al., 2015).

Mortality only occurred at M5 group, i.e. one fish, and C5 group, i.e. all fish. All the fish that died had a swollen abdomen filled with water (Frantzen et al., 2015).

1.4 The present study

1.4.1 Development of biomarkers

Biomarkers are important in environmental monitoring since they give an “early warning” signal. Peakall (1994) has proposed a definition for biomarker, which is a *“biological response to a chemical or chemicals that gives a measure of exposure, and sometimes, also, of toxic effect”*.

In this study, gill EROD activity was used as a biomarker to determine the induction of CYP1A, while the bile PAH metabolites was used as a biomarker and end point in the exposure and uptake of PAHs.

1.4.1.1 EROD Activity

One of the most commonly used biomarkers in determining PAHs exposure in fish is the gill and liver Ethoxyresorufin-O-deethylase (EROD) activity, which is a laboratory assay that quantify the *in situ* activity of gill and liver cytochrome P450 (CYP) 1A activity. EROD

activity is considered as a highly sensitive indicator of contaminants in many fish species since PAH exposure cause a receptor-mediated induction of the CYP1A enzyme activity (Payne et al., 1987). The CYP1A enzyme catalyses the substrate 7-ethoxyresorufin and metabolized it into resorufin.

The enzyme CYP1A is a monooxygenase subfamily that belongs to heme-containing Cytochrome P450. The induction of CYP1A begins by the binding of PAHs to cytosolic transcriptional factor Aryl hydrocarbon Receptor (AhR). After the ligand binding, the AhR will form a heterodimer and bind to an aryl hydrocarbon nuclear translocation protein (ARNT) on the nucleus. The AhR will bind to a specific regions of DNA that will initiate the transcription of CYP1A (Whyte et al., 2000). This is the Phase I of biotransformation, i.e. oxidation and functionalization (Williams, R., 1959). The role of CYP1A is to hydrolyse the PAH by introducing a polar group, e.g. a hydroxyl group into the molecule (Di Giulio and Hinton, 2008). This compound that acquires a polar group will then proceed to Phase II of biotransformation, i.e. conjugation and detoxication (Williams, R., 1959). Phase II enzymes, e.g. Glutathione S-transferase, will use the polar group to link the compound to various polar endogenous, e.g. sugar derivatives, amino acids, peptides, and sulfate. This will reduce the toxicity and enhance the water solubility and elimination of the toxic compound (Di Giulio and Hinton, 2008).

Although the CYP enzymes are expressed in different tissues such as kidney, alimentary canal, heart, gills, olfactory system, gonads, brain and endocrine tissues, EROD activity is traditionally measured in the liver since it is the major organ of PAH biotransformation in

fish (Sarasquete and Segner, 2000; Frantzen et al., 2015). However, the exposure of fish to PAHs is not always accurately reflected in the hepatic EROD activity, as some of these are biotransformed in the gills (Andersson and Pärt, 1989; Jönsson et al. 2006), which is directly exposed to water-born PAHs (Jönsson et al., 2004; Griffitt et al., 2007). Previous experiments also showed the CYP1A enzymes in the gills to be more sensitive to contaminants than the liver (Abrahamson et al., 2007).

1.4.1.2 Bile PAH Metabolites

Bile PAH metabolites are the by-products of Phase II PAH metabolism in the liver. After biotransformations, these compounds are excreted to the bile, thus PAH bile metabolites are commonly used as a biomarker for PAH exposure for both the pyrogenic- and petrogenic-type of PAHs (Krahn et al., 1987; Ariese et al., 1993; Lin et al., 1996).

When fish are exposed to PAHs, only traces of these amounts can be found in the tissues (Varanasi et al., 1989) because these compounds are rapidly being transformed and excreted to the bile. Thus, analysis of bile metabolites can give an impression of the actual exposure to PAHs compounds that are not reflected in the other tissues (Vuontisjärvi and Vuorinen, 2004). Moreover, it is proven to be simple to use and a sensitive method for screening PAH contamination in fish (Aas et al., 2000).

1.4.2 Osmo- and ion-regulation of marine teleosts

The osmolality of seawater is 1050 mOsm.kg⁻¹, while that of the marine teleosts is *ca* 452 mOsm.kg⁻¹. This means that the fish must constantly regulate its ionic and water flow to be able to maintain their body fluids to stay hypo-osmotic to seawater. The gill is a major tissue in ion regulation (Evans et al., 2005) and, at the same time, directly exposed to water-born PAHs. Hence, the gills must be considered a vulnerable tissue for PAH-related effects.

1.4.2.1 Ion- and osmoregulation mechanisms

Marine teleosts maintain a much lower ion concentration in their body fluid than their surrounding medium, i.e. they are hypo-osmotic and need to be able to replace water loss by drinking seawater and excrete accompanying ions such as sodium (Na) and chloride (Cl). The general mechanisms of net salt excretion were first described by Smith (1930). Their model suggested that the branchial (rather than renal) is the major site of salt excretion, and the main driving force responsible for maintaining the osmotic gradient is Na⁺/K⁺-ATPase pump, a model that has proven to be valid (Kirschner, 2004). The Na⁺/K⁺-ATPase pump is a transport protein made up of a polypeptide chain with α subunit which is phosphorylated during ATP hydrolysis (where ouabain attaches) and a β subunit which is heavily glycosylated and function in anchoring the pump to the membrane (Renzis and Bornancin, 1984). In general, since the Na⁺/K⁺-ATPase pump is the driving force of ion excretion in fish in seawater, its activity increases with increasing external salinity (McCormick, 1995). Gill Na⁺/K⁺-ATPase pumps are commonly found at

the afferent edge and inter-lamellar region of the filaments, specifically in mitochondria-rich cells (MRC), generally termed “chloride cells”, together with other assisting pumps. Regulation of the water balance is a matter of maximum water saving, which is achieved through the production of very concentrated urine (McCormick, 1995).

1.5 Aim of the present study

The present study is a continuation of the abovementioned study and focuses on the physiological effects of dispersed oil, with emphasis on the ion- and osmo-regulation. Specifically, it will look into the effects of acute oil exposure to gill Na^+/K^+ -ATPase, plasma chloride concentration, and plasma osmolality. These acute responses will be evaluated in conjunction with the findings of the first study. Moreover, this study will also assess the suitability of juvenile lumpsuckers as a model species in detecting oil contamination by looking at the effects of dispersed oil on gill EROD activity and bile PAH metabolites.

1.6 Objectives of the study:

1. To investigate the acute effects of exposure to different concentrations of crude oil on gill CYP1A enzyme induction, bile PAH metabolites, plasma osmolality, plasma chloride, and gill Na^+/K^+ -ATPase activity;
2. To compare the acute responses, i.e., gill CYP1A enzyme induction, bile PAH metabolites, plasma osmolality, plasma chloride, and gill Na^+/K^+ -ATPase activity between mechanically and chemically dispersed oil; and
3. To determine the suitability of juvenile lumpsuckers as model species in detecting oil contamination by measuring the gill EROD activity and bile PAH metabolites as biomarkers of oil exposure.

2 MATERIALS AND METHODS

2.1 Experimental animals

The study was carried out at the Akvaplan-niva Marine Research Facility, Tromsø, Norway (69°N) in July 2013. Approximately one year old juvenile lumpsuckers, (*C. lumpus*) used in the experiment had a (mean \pm S.D.) fork length of 7.53 ± 0.71 cm and weight of 17.38 ± 4.84 g (Table 22). They were the offspring of wild-caught fish collected in June 2012. They spawned naturally in rearing tanks and the eggs were hatched in August 2012. Three weeks after hatching, they were fed with *Artemia nauplii* (BioMar, Brande, Denmark), and during weaning, Anglo Norse high phospholipid formulated feed was offered for two weeks. The juveniles were fed with dry pellets (Skretting, Norway) by automatic disk feeders. The feed size was adjusted according to fish size.

Table 2. Summary table of the minimum (min), mean (\pm standard deviation; SD), maximum (max) length and weight, and number (N) of juvenile lumpsucker used in the experiment

	Length (cm)	Weight (g)
Min	6.10	8.80
Mean	7.53 (\pm 0.71)	17.38 (\pm 4.84)
Max	8.70	26.54
N = 84		

2.2 Acclimatization period

Prior to oil exposure, 84 juveniles were randomly placed into 12 fish cages (n=7 per cage) that were placed into 120L acclimatization tank that was similar to exposure tanks. The fish cages were made of stainless steel and equipped with a cover and a resting wall made of plexiglass (Fig. 1). The animals were acclimatized for 48 hours without feeding. The flow rate was maintained at 100 L/hr. Temperature, pH, and oxygen saturation were $6.6 \pm 0.07^\circ\text{C}$, 8.4 ± 0.01 , $94.3 \pm 5.0\%$, respectively. These values are the mean (\pm SE) of daily measurements].

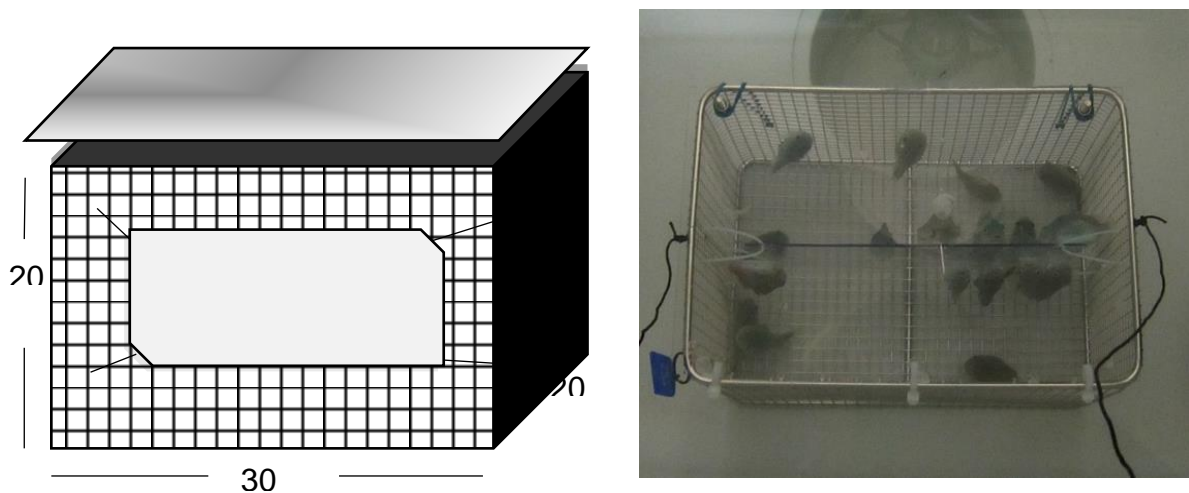


Figure 1. Schematic sketch (left) and picture (right) of specially designed fish cages utilized for the experiment. Each cage was equipped with a transparent plexiglas wall in the central half of the cage (with free swimming passageways above and below the wall) for lumpsuckers to suck onto and rest on throughout the acclimation and exposure periods, and a transparent plexiglas cover to be able to view the inside of the cage.

2.3 Experimental design and set-up

The set-up was composed of 12 120L exposure tanks, where one cage was placed into each tank (Fig. 2). It had a funnel on the surface that was linked to a 12V water pump which was attached at the bottom.



Figure 2. Experimental set-up showing the treatment tanks where the juvenile lumpsuckers were exposed for 48 hours to North sea crude oil (mechanical dispersion treatment, i.e. MDL and MDH) and North sea crude oil + chemical dispersant Dasic NS (chemical dispersion treatment, i.e. CDL and CDH), Dasic NS only, i.e. Control Dispersant (CD) and Control (C).

This study has used the North Sea Troll, a naphthenic crude oil, and Dasic NS (Dasic International Limited, UK), a chemical dispersant. The two methods that were used to disperse oil were mechanical dispersion and chemical dispersion. Mechanical dispersion refers to the dispersion of oil through the use of pumps, i.e. to mimic what happens when wave action disperses the oil in the natural environment. Chemical dispersion, on the other hand, refers to dispersion of oil by mechanical means plus premixing of 4% Dasic NS to the North Sea Troll crude oil. The method for dispersion was adapted from

the protocol developed by Cedre, France for the DISCOBIOL project (Milinkovitch et al., 2011). The crude oil (for mechanical dispersion treatment) and pre-mixed crude oil with Dasic NS (for chemical dispersion treatment) were poured into the funnel. The pump that was attached at the bottom of the tank ensured that the oil and water were continuously and homogeneously mixed throughout the water column.

The mechanical dispersion treatment includes Mechanical Dispersion-Low oil concentration (MDL) and Mechanical Dispersion-High oil concentration (MDH), while the chemical dispersion treatment includes Dispersant control (CD), i.e. no oil was added, only a dispersant with the same concentration as CDH, Chemical dispersion-Low oil concentration (CDL), and Chemical dispersion-High oil concentration (CDH). There were two replicate tanks for each treatment. The nominal oil concentrations for mechanical dispersion treatment and chemical dispersion treatment used in the present study were equivalent to M2, M4 (mechanical dispersion treatment) and CD, C2, C4 (chemical dispersion treatment) of the first study. Table 3 shows the amount of North Sea Troll crude oil and Dasic NS that was used in the respective tanks.

Table 3. The amount and concentration of North Sea Troll crude oil and chemical dispersant (Dasic NS) in the treatments used in the study

Treatment	North Sea Troll Crude Oil Amount (g)	Dasic NS Amount (g)	Nominal Crude Oil Concentration in 110L seawater (ppm)
Control	0	0	0
Low Mechanical Dispersion (MDL)	7.34	0	66.7
High Mechanical Dispersion (MDH)	36.66	0	333
Control Dispersant (CD)	0	1.47	0
Low Chemical Dispersion (CDL)	7.34	0.30	66.7
High Mechanical Dispersion (CDH)	36.66	1.47	333

2.4 Fish exposure

Oil was poured to the treatment tanks and allowed to weather for 24 hours before the fish was introduced. Thereafter, the fish cages, containing 7 juveniles each, were transferred from the acclimation tanks and randomly distributed into the exposure tanks (n=1 cage per exposure tank). This was designated time 0 (T0). The exposure experiment was conducted for 48 hours. During this time, the water system remained static but with constant oxygenation throughout the exposure period, ensuring an O₂ saturation of <90%. The behavior of the fish, e.g. swimming behavior, ability to suck/rest on a surface, loss of equilibrium, and mortality were monitored at time 1, 2, 4, 8, 12, 24, 32, 36, and 48 h.

The temperature, pH and dissolved oxygen in the exposure tanks were $5.3 \pm 0.1^{\circ}\text{C}$, 7.8 ± 0.03 , and $107.9\% \pm 1.5 \text{ O}_2$ saturation, respectively (mean \pm SE of daily measurements).

2.5 Sample treatment

After 48 hours of exposure, cages were randomly taken out of the exposure tanks. The animals were anaesthetized with Metakain (0.60 g/10 L). The fork length (cm), wet weight (g), and sex were noted down prior to tissue/organ extraction.

2.5.1 Gill preparation for EROD activity analysis

Gill tissues were taken for gill EROD analysis. Gill tissues taken from the left side of the operculum was labelled A, while the ones taken from the right side was labelled B. The tissues were put in cryotubes and freeze immediately in liquid nitrogen. They were stored at -80°C until analysis.

2.5.2 Bile preparation

Bile was extracted from the fish and transferred to cryotubes. They were freeze immediately in liquid nitrogen and stored at -80°C until analysis.

2.5.3 Blood preparation

For the analysis of plasma osmolality and plasma chloride, blood samples were extracted from the caudal vein using lithium-heparin vacutainers. The blood was centrifuged and the blood plasma (supernatant) was pipetted into eppendorf tubes, and stored at -80°C.

2.5.4 Gill preparation for Na⁺/K⁺-ATPase activity analysis

For the analysis of Na⁺/K⁺-ATPase activity, three to five gill filaments were removed from both sides of the fish using forceps. These were labelled gill A, i.e. filaments taken from the left side of the fish and gill B, i.e. filaments taken from the right side. Gill filaments were transferred to cryotubes containing 100 µl ice-cold SEI buffer (150 mM sucrose, 10 mM Na₂EDTA, and 50 mM Imidazol) and immediately freeze in liquid nitrogen. They were stored at -80°C until analysis.

2.6 Chemical and biochemical analyses

2.6.1 Gill EROD activity

Gill EROD activity describes the rate of CYP1A-mediated deethylation of the substrate 7-ethoxyresorufin to form resorufin. The amount of resorufin produced was measured fluorometrically using a spectrometer plate reader (Perkin Elmer 1420 Multilabel Counter Victor), related to the amount of protein in the gill sample used for the analyses and expressed as pmol resorufin min⁻¹ mg⁻¹ protein. The method used in this analysis was

based on the 7-ethoxyresorufin O-deethylase (EROD) described by Stagg and McIntosh (1998).

After the samples were thawed, they were transferred to CR28 Precellys tubes with potassium phosphate buffer (100mM, pH 7.8) containing KH_2PO_4 (100 mM), K_2HPO_4 (100 mM), KCl (150 mM), DTT (1 mM) and 5% glycerol. The tissue weight:buffer ratio was 1:9. The samples were homogenized using Precellys 24-Dual (Bertini Technologies, France) at 5000 x g, 2x 20s, 5s pause.

The homogenates were centrifuged at 10,000 x g, at 4°C for 30 min. The supernatants were centrifuged at 50,000 x g at 4°C for 2 h. The microsomal fraction (the pellet) was resuspended in 1 ml potassium phosphate buffer and 20% glycerol, and homogenized using Potter-Elvehjem homogenizer at 900 rpm for 10 s. The homogenates were aliquoted into 0.5 ml eppendorf tubes and stored at -80°C until further analysis.

Resorufin standard curve was prepared with final concentrations in wells of 0 μM , 0.001 μM , 0.005 μM , 0.01 μM , and 0.027 μM . The reaction mixture in well-plate is presented in Table 4.

Table 4. Reaction mixture in 96-well plate

Final concentration of reagents	Standard curve (μl)	Sample (μl)
Phosphate buffer, pH 8	210	200
Sample		10
Ethoxyresorufine WS (2 μM)		10
Resorufine Standard	10	
NADPH (0.25 μM)	10	10
Total volume	230	230

The excitation/emission wavelengths were set at 544/584 nm respectively. The production of resorufin was measured in four replicates for 20 min at 20°C.

The total amount of protein in the sample was calculated through colorimetry method which was based on Bradford (1976). Bovine serum albumin (BSA) was used as the standard reference. The standard curve consisted of 0, 0.001, 0.002, 0.003, 0.004, 0.005, 0.006, and 0.008 mg BSA/L. Coomassie blue was prepared by diluting 250 µl coomassie blue with 20 ml distilled water (1:5 dilution). All samples were diluted 100x in distilled water.

The wells were filled in triplicates, containing 100 µl standard or sample + 250 µl coomassie blue. Using Perkin Elmer 1420 Multilabel Counter Victor, the plate was analysed for 2 minutes and absorbance measured at 595 nm.

2.6.2 Bile PAH metabolites

The presence of PAH metabolites in the bile was detected by fluorescence spectroscopy method using FL Winlab. The PAHs are strong fluorophores, thus this method is able to discriminate other compounds present in the bile that show little or no fluorescence.

A volume of 5 µl bile and 195 µl milli Q water (1:40) were added to eppendorf tubes and mixed using a vortex. The sample was further diluted to 1:1600 by pipetting out 75 µl of the 40x diluted bile and adding 2925 µl milli Q water. Quartz cuvette was used for the

fluorescence scan. A blank (distilled water) was run at the start of analysis, and after every 20 samples analysed.

The synchronous fluorescence scan (SFS) was used to identify the type of PAHs present in the sample, within the 200 to 500 nm excitation, slid width set to 2.5 nm and $\delta\lambda$ 42 nm. The structural properties of PAHs has different wavelength optima for light excitation and fluorescence emission, thus creating a possibility for distinction among the types of PAHs. The fixed wavelength fluorescence (FF) was used to calculate the intensity of the PAH. It was set to determine the intensity of the 2-, 4- and 5-ring PAH metabolites by using the wavelength pairs 290/335 (naphthalene-type), 341/383 (pyrene-derived), and 380/430 (benzo(a)pyrene), respectively.

The mean of the control and each treatment were calculated. The average value of the blank was subtracted from the averages to get the final result.

2.6.3 Plasma osmolality

Plasma osmolality was determined using the Fiske One-Ten Osmometer. This method is based on the freezing point depression principle, wherein the presence of a solute decreases the freezing point of the solvent. The thermistor probe sense the temperature of the sample, and based on this, the osmolality is calculated.

A 15 μ l volume of blood plasma sample was used for the analysis. The osmolality of the sample was calculated as the average of the two readings and expressed as mOsm/kg. In cases where the two readings had a marked difference, a third reading (and sometimes a fourth reading) was done. After each reading, the thermister probe was cleaned using a filter. A standard (290 mOsm \pm 2mOsm) was analysed before the start of the analysis and after every 10 samples. All samples were thawed and kept on ice throughout the analysis.

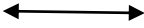
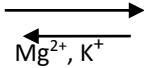
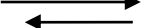
2.6.4 Plasma chloride

The plasma chloride concentration was measured using Corning 925 chloride titrator (CIBA Corning Diagnostics, Essex, England). A standard solution (100 mmol/L) was analysed prior to the first sample and after every 10 samples. A 20 μ l blood plasma sample was used for the analysis. Each analysis was done in replicates and the average was computed. In cases where the two readings had a marked difference, a third reading (and sometimes a fourth reading) was done. All samples were thawed and kept on ice during the analysis.

2.6.5 Gill Na⁺/K⁺-ATPase activity

The activity of the gill Na⁺/K⁺-ATPase was determined based on the amount of ATP that is hydrolyzed. The plate reader calculates the linear rate of oxidation of NADH to NAD⁺. For every mole of ATP hydrolyzed, one mole of NADH will be oxidized to NAD⁺.

Principle:

ATP + H ₂ O	ATPase 	ADP + Pi (I)
ADP + Phosphoenolpyruvate	Pyruvate kinase 	Pyruvate + ATP (II)
Pyruvate + NADH + H ⁺	Lactate hydrogenase 	Lactate + NAD ⁺ (III)

The microassay method used to determine the Na⁺/K⁺-ATPase activity on gill was based on McCormick (1993) and Schrock *et al.* (1994). The preparation of different solutions was adapted from Zaugg (1982), McCormick (1993) and Schrock *et al.* (1994) (Appendix I).

After the samples were thawed, 100 µl ice-cold SEI buffer and 25 µl ice-cold 0.5% SEID (0.01 M Sodium deoxycholate added to SEI buffer) were added. They were homogenized for a few seconds using *Pellet pestle motor* (Kontes, New Jersey, USA). After the homogenization, samples were centrifuged until the rpm reached 4630 rpm (around less than a minute), at 4°C.

A standard solution is a mixture of Imidazole buffer (IB) (50 mM Imidazole, pH 7.5) and ADP standards. Four standard solutions were prepared with a concentration of 0, 5, 10, and 20 nmol/10µl (Table 5).

Table 5. Preparation of ADP standard solutions

Concentration (nmol/10 μ l)	IB (μ l)	ADP standard (μ l)
0	200	0
5	175	25
10	150	50
20	100	100

Standard solutions, samples, and assay mixtures, i.e. AM mixture and AM-O mixture (See Appendix I) were vortexed and pipetted into a 96-well microplate. Table 6 shows the amount of each that was added into the well:

Table 6. Reaction mixture in each well

	Standard curve (μ l)	Sample (μ l)
Standards	10	
Sample		10
Assay Mixture	200	200
Total volume	210	210

In the microplate, each standard and sample had four replicates, wherein two wells contained AM-O Assay Mixture and two wells with AM Assay Mixture.

The microplate was analysed by a temperature-controlled plate reader (Thermomax, Molecular Devices Corp., USA). The linear rate of oxidation of NADH to NAD⁺ was measured at 340 nm for 10 minutes at 24°C. The Na⁺/K⁺-ATPase activity was calculated as the difference of ATP hydrolysis in the absence and presence of ouabain, which was

then related to the amount of protein (see below) in the sample and expressed as $\mu\text{mole ADP/mg protein/hour}$.

Protein was calculated using the BCA (Bicinchoninic acid) Protein Assay Reagent Kit (Pierce, Illinois, USA).

The standards were prepared as follows:

Standard ($\mu\text{g}/10\mu\text{l}$)	2mg/ml BCA standard (μl)	Distilled water (μl)
0	0	100
5	25	75
10	50	50
20	100	0

The Working Solution (WS) was prepared by mixing 20 ml of Reagent A (Na_2CO_3 , NaHCO_3 , Pierce BCA detection reagent, $\text{Na}_2\text{C}_4\text{H}_4\text{O}_6$ in 0.1 N NaOH) and 400 μl Reagent B (BCA Protein Assay Reagent). The reaction mixture was composed of 10 μl standard (for the standard curve), 10 μl sample and 200 μl WS. The standard and samples were incubated at 37°C for 60 minutes. The plate was placed in the temperature-controlled plate reader (Thermomax, Molecular Devices Corp., USA), and measured in three replicates at 540 nm in 12 seconds.

2.7 Statistical Analysis

All statistical analyses were computed using SPSS Statistics v.22. The results for plasma osmolality, plasma chloride, gill EROD activity, and bile PAH metabolites, i.e. naphthalene-type, pyrene-type and benzo(a)pyrene-type did not satisfy the assumptions of normal distribution required for running the parametric Analyses of Variance (ANOVA), thus a non-parametric test (Kruskal-Wallis) were used to analyse the data for possible treatment effects, followed by pairwise Bonferonni post-hoc tests in cases where overall significant effects were obtained. The gill Na^+/K^+ -ATPase activity data was analysed using ANOVA after satisfying the Shapiro-Wilk test for normality and Levene's test for homogeneity of variance. Tukey's HSD was used for pairwise, post hoc analysis. A probability level of $p < 0.05$ was considered significant for all the tests.

3 RESULTS

3.1 Gill EROD Activity

The mean (\pm S.E.) gill EROD activity of the control and CD were 4.56 ± 0.18 and 9.69 ± 2.62 pmol resorufin min^{-1} mg protein $^{-1}$, respectively. The mean (\pm S.E.) activity at the mechanically dispersed group range between 4.37 ± 0.69 and 4.40 ± 0.32 pmol resorufin min^{-1} mg protein $^{-1}$, while the chemically dispersed group has a range between 4.38 ± 0.49 and 5.61 ± 1.03 pmol resorufin min^{-1} mg protein $^{-1}$. There is no significant difference between the control and oil exposed fish, and no significant differences between mechanical and chemical dispersion treatments ($p > 0.05$) (Fig. 3).

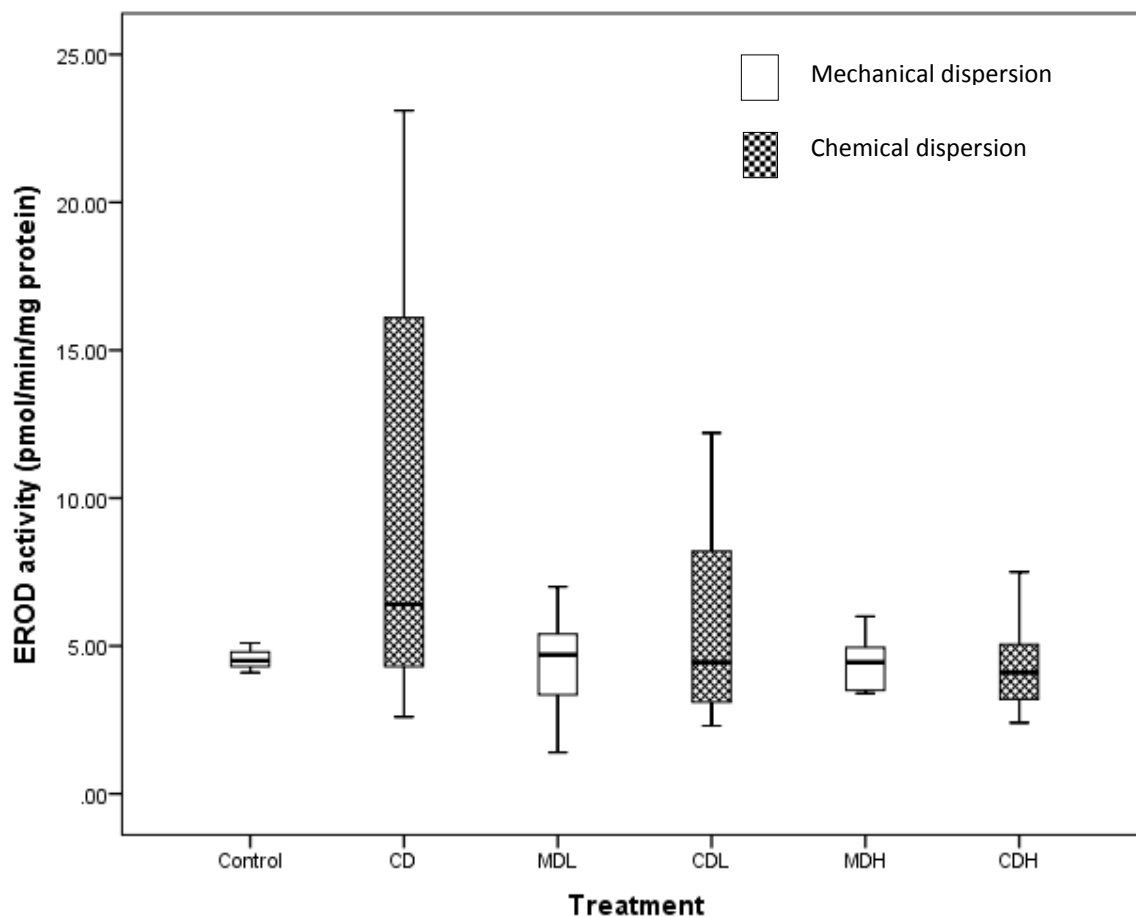


Figure 3. Box plot of gill EROD activity (pmol/min/mg protein) of juvenile lumpfish (*Cyclopterus lumpus*) after exposure for 48 hours to seawater added North Sea Troll crude oil (mechanical dispersion: MDL and MDH), North Sea Troll crude oil and Dasic NS dispersant (chemical dispersion: CDL and CDH), Dasic NS dispersant alone (CD), and control (seawater only). Values are shown as median, quartile range, upper and lower limits. Kruskal-Wallis was used to test for significance.

3.2 Bile PAH metabolites

Results of the Synchronous Fluorescence Scan showed three peaks, corresponding to 2-, 4-, and 5-ring PAHs (Fig. 4). The highest peak corresponds to the naphthalene-type metabolites, which lies at the excitation/emission of 290/335 nm, followed by the pyrene-type metabolites, which lies at the excitation/emission of 341/383 nm. The

lowest peak corresponds to the benzo(a)pyrene-type metabolites, that lies on excitation/emission of 380/430 nm.

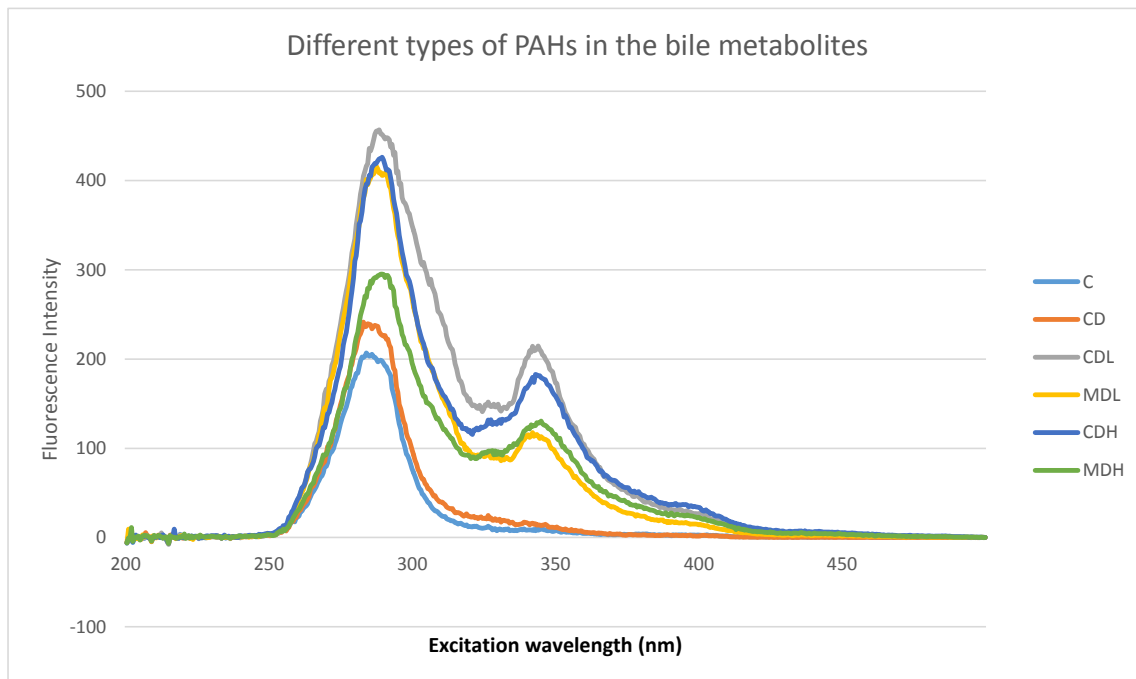


Figure 4. Different types of PAHs metabolites, i.e. naphthalene-type, pyrene-type, and benzo(a)pyrene type, with corresponding intensity found in the bile of juvenile lump sucker (*Cyclopterus lumpus*) after exposure for 48 hours to seawater added North Sea Troll crude oil (mechanical dispersion: MDL and MDH), North Sea Troll crude oil and Dasic NS dispersant (chemical dispersion: CDL and CDH), Dasic NS dispersant alone (CD), and control (seawater only).

3.2.1 Naphthalene-type metabolite

The amount of naphthalene-type metabolites (Fig. 5) differed significantly between the treatment groups ($\chi^2(5)=21.84$, $p=0.001$). Bonferonni post-hoc tests showed that the control group had significantly lower levels of naphthalene-type metabolites than the MDL ($p=0.002$), CDL ($p=0.000$) and CDH ($p=0.009$) groups.

Among the exposed groups, the levels in the CD group were significantly lower than the levels in the CDL ($p=0.001$), MDL ($p=0.007$) and CDH ($p=0.037$) groups, whereas the level in the CDL group was significantly higher than in the MDH group ($p=0.048$).

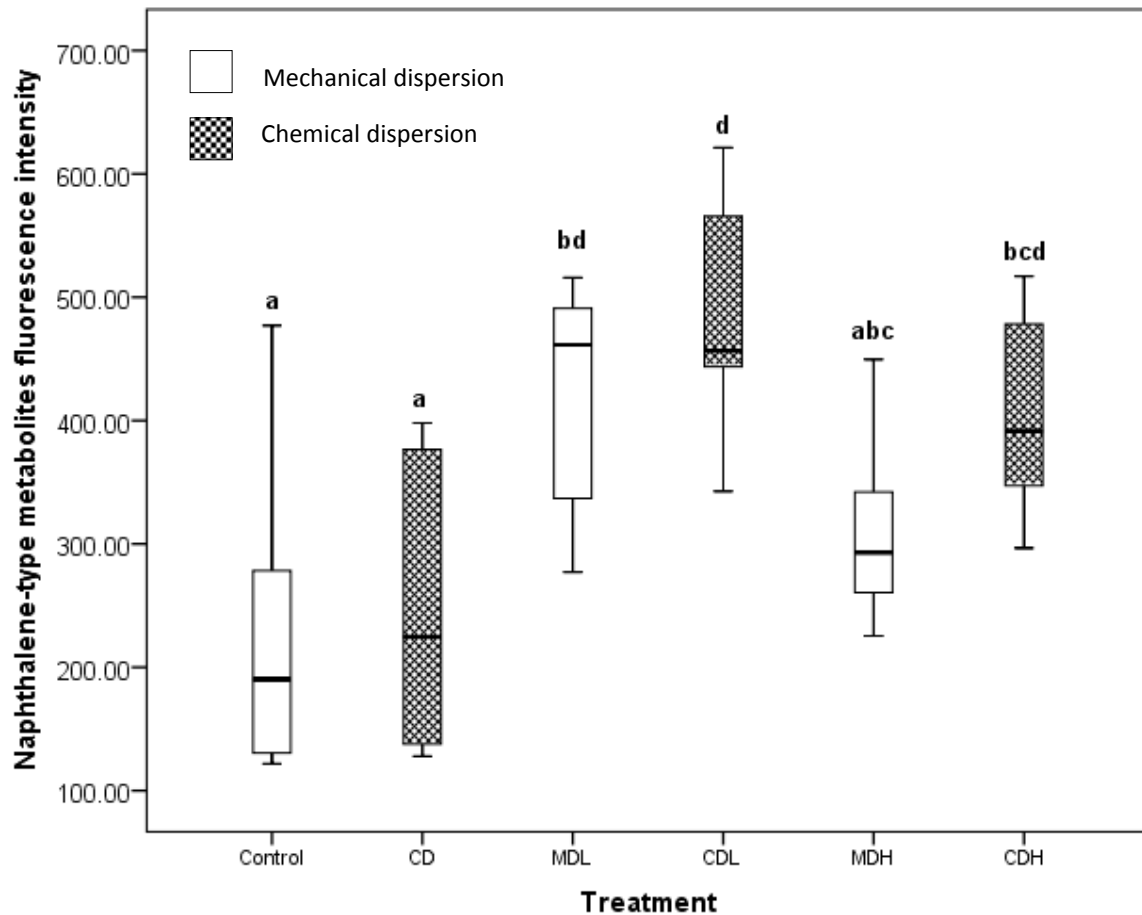


Figure 5. Box plot of the level of naphthalene-type metabolites in the bile of juvenile lumpfish (*Cyclopterus lumpus*) after exposure for 48 hours to seawater added North Sea Troll crude oil (mechanical dispersion: MDL and MDH), North Sea Troll crude oil and Dasic NS dispersant (chemical dispersion: CDL and CDH), Dasic NS dispersant alone (CD), and control (seawater only). Values are shown as median, quartile range, upper and lower limits. Kruskal-Wallis was used to test for significance, followed by Bonferonni post hoc test. Groups denoted with different letters are significantly different.

3.2.2 Pyrene-type metabolite

There was a significant difference ($X^2(5) = 12.08, p=0.034$) in the fluorescence intensity of pyrene-type metabolites among the groups (Fig. 6). Bonferonni post-hoc tests showed

that CDH had a significantly higher pyrene-type metabolite level than the Control ($p=0.000$) and CD ($p=0.000$) groups. The levels in the MDL, CDL and MDH were intermediate and did not differ significantly from the rest of the groups.

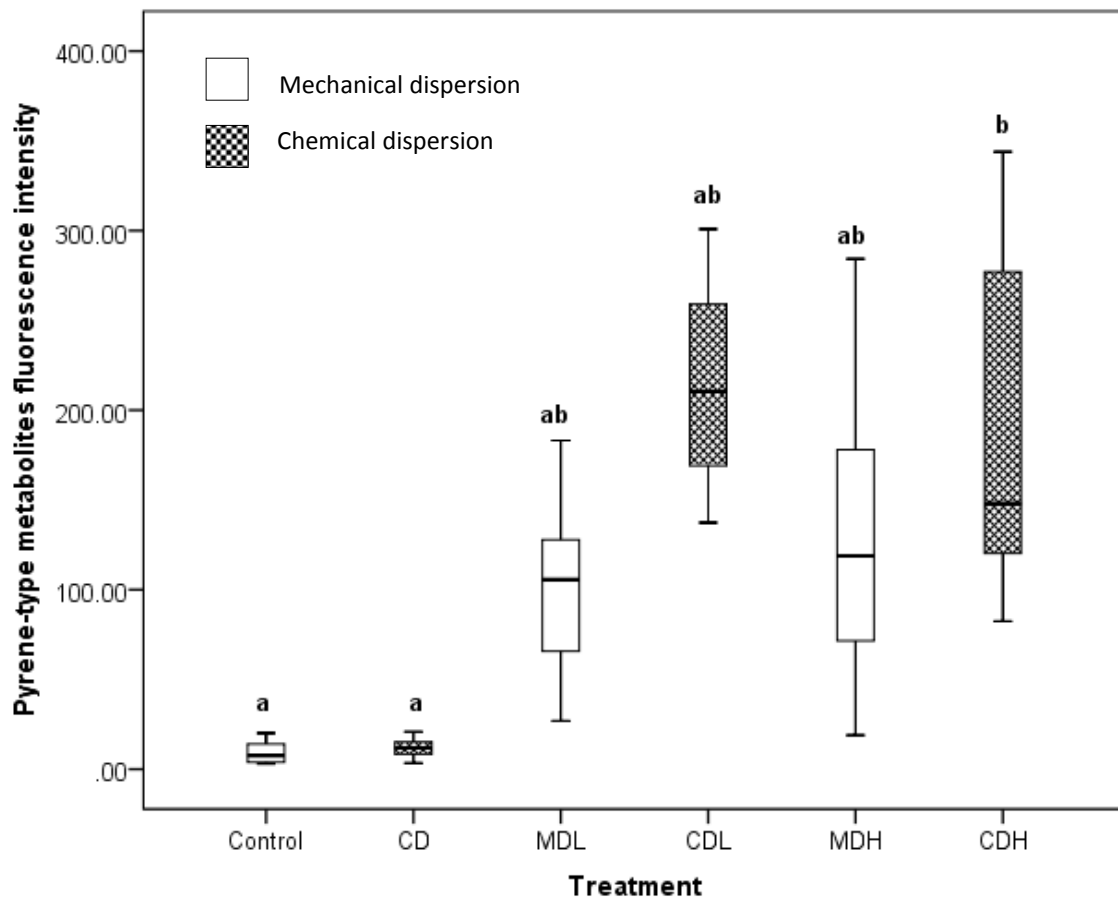


Figure 6. Box plot of the level of pyrene-type metabolites in the bile of juvenile lump sucker (*Cyclopterus lumpus*) after exposure for 48 hours to seawater added North Sea Troll crude oil (mechanical dispersion: MDL and MDH), North Sea Troll crude oil and Dasic NS dispersant (chemical dispersion: CDL and CDH), Dasic NS dispersant alone (CD), and control (seawater only). Values are shown as median, quartile range, upper and lower limits. Kruskal-Wallis was used to test for significance followed by Bonferonni post hoc test. Groups denoted with different letters are significantly different.

3.2.3 Benzo(a)pyrene-type metabolite

Significant differences ($\chi^2(5) = 13.43, p=0.020$) were found in the fluorescence intensity of benzo(a)pyrene-type metabolites among the groups (Fig. 7). Bonferonni post-hoc tests

showed that the CDH group had significantly higher level of benzo(a)pyrene-type metabolites than the Control ($p=0.000$), CD ($p=0.000$), MDL ($p=0.004$), and MDH ($p=0.021$) groups, whereas the level in the CDL group was intermediate and not significantly different from the levels in the other groups.

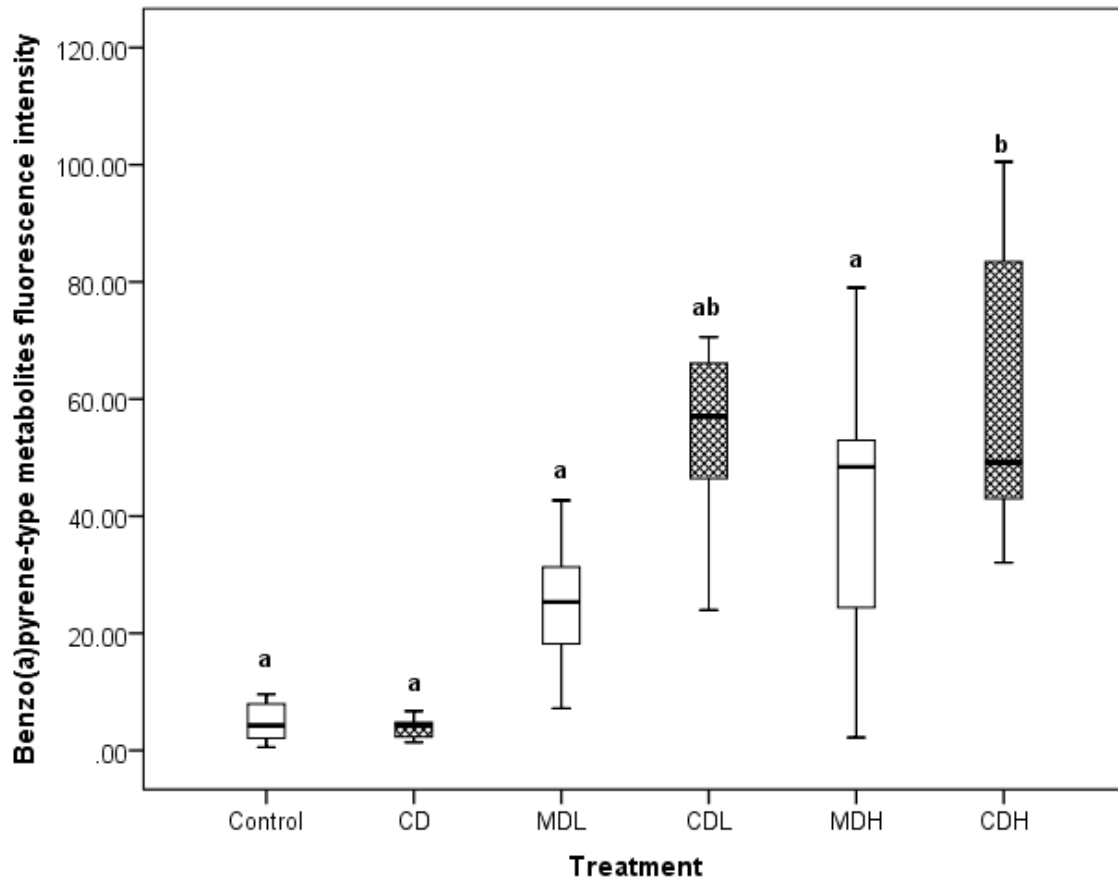


Figure 7. Box plot benzo(a)pyrene-type metabolites in the bile of juvenile lump sucker (*Cyclopterus lumpus*) after exposure for 48 hours to seawater added North Sea Troll crude oil (mechanical dispersion: MDL and MDH), North Sea Troll crude oil and Dasic NS dispersant (chemical dispersion: CDL and CDH), Dasic NS dispersant alone (CD), and control (seawater only). Values are shown as median, quartile range, upper and lower limits. Kruskal-Wallis was used to test for significance followed by Bonferonni post hoc test. Groups denoted with different letters are significantly different.

3.3 Plasma osmolality

Plasma osmolality in the different treatment groups are shown in Fig. 8. There was an overall, significant effect of oil treatment on plasma osmolality ($X^2(5) = 67.81, p=0.000$). Post-hoc Bonferonni test showed that the Control group had a significantly lower plasma osmolality than the MDL ($p=0.001$), CDL ($p=0.000$), MDH ($p=0.000$), and CDH group ($p=0.000$). There were no significant differences between the Control group and the CD group ($p > 0.05$) or between the mechanical- and chemical dispersed groups exposed to the same oil concentration ($p > 0.05$), but the high dose groups had a higher plasma osmolality than the low dose groups ($p = 0.000$).

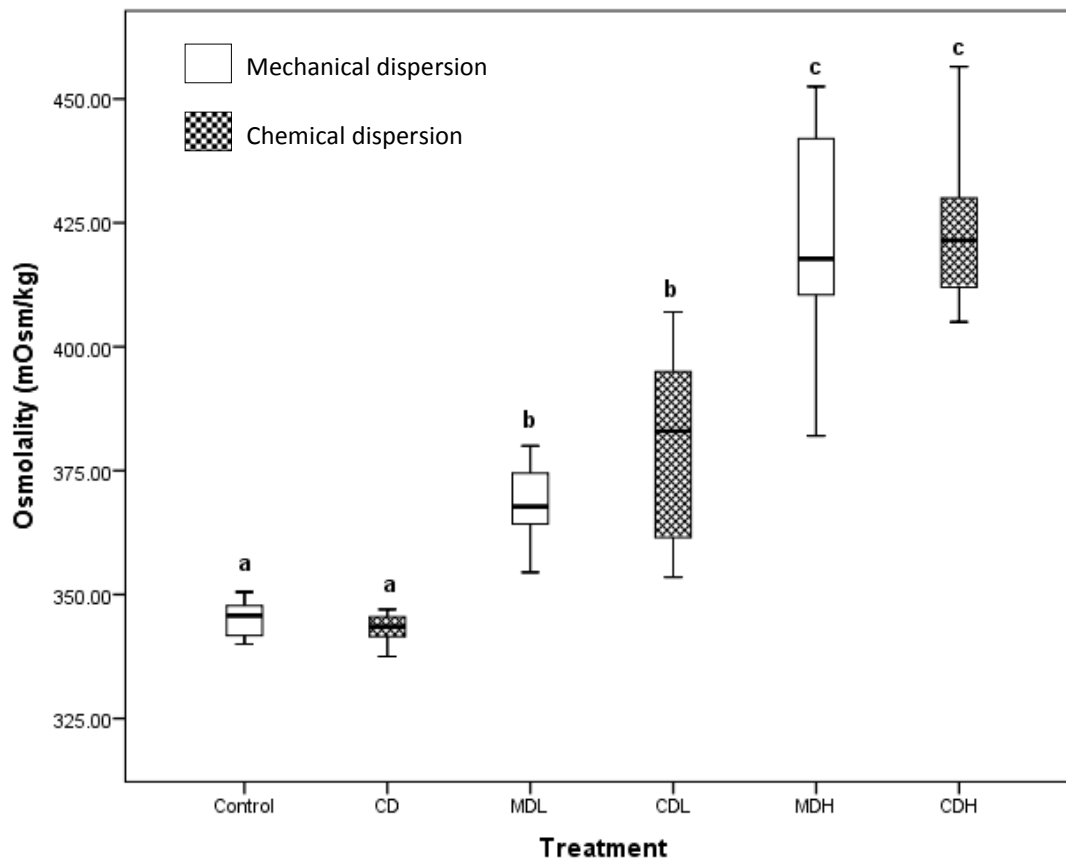


Figure 8. Box plot of plasma osmolality of juvenile lumpsuckers (*Cyclopterus lumpus*) after exposure for 48 hours to seawater added North Sea Troll crude oil (mechanical dispersion: MDL and MDH), North Sea Troll crude oil and Dasic NS dispersant (chemical dispersion: CDL and CDH), Dasic NS dispersant alone (CD), and control (seawater only). Values are shown as median, quartile range, upper and lower limits. Kruskal-Wallis was used to test for significance followed by Bonferonni post hoc test. Groups denoted with different letters are significantly different.

3.4 Plasma chloride concentration

Oil concentration had a significant effect on plasma chloride level, $X^2(5) = 64.31, p=0.000$, which increased with increasing oil concentration (Fig. 9). Post-hoc Bonferonni test showed that the plasma chloride level in the Control group was significantly lower than that in the MDL ($p=0.001$), CDL ($p=0.000$), MDH ($p=0.000$), and CDH ($p=0.000$) groups and that the high dose groups had significantly higher plasma chloride concentrations than the low dose groups ($p=0.000$). There were no significant differences ($p>0.05$) between mechanically- and chemically dispersed groups exposed to the same oil concentration.

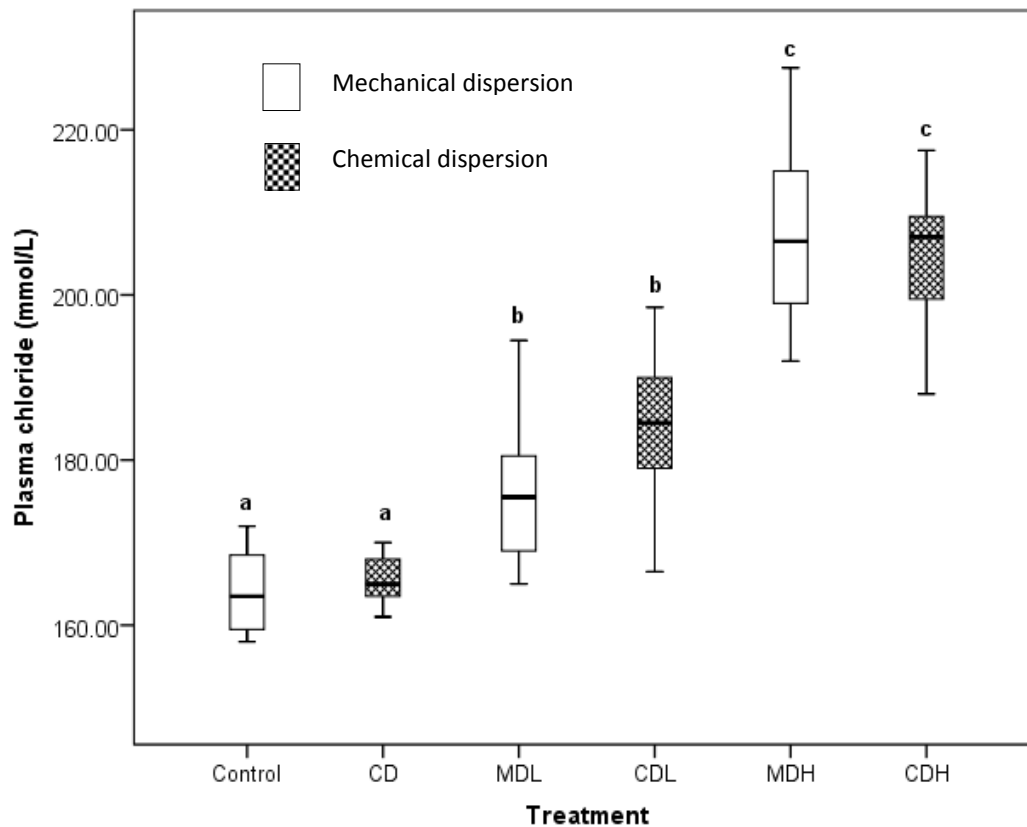


Figure 9. Box plot of plasma chloride of juvenile lumpsucker (*Cyclopterus lumpus*) after exposure for 48 hours to seawater added North Sea Troll crude oil (mechanical dispersion: MDL and MDH), North Sea Troll crude oil and Dasic NS dispersant (chemical dispersion: CDL and CDH), Dasic NS dispersant alone (CD), and control (seawater only). Values are shown as median, quartile range, upper and lower limits. Kruskal-Wallis was used to test for significance followed by Bonferonni post hoc test. Groups denoted with different letters are significantly different.

3.5 Gill Na^+/K^+ -ATPase activity

Oil exposure had an overall, significant effect on the gill Na^+/K^+ -ATPase activity ($F(5,69) = 2.77, p=0.024$). Tukey's HSD post-hoc test showed that the Control group has a significantly lower activity than the MDL group ($p=0.044$), and that the MDL group had a significantly higher activity than the MDH group ($p=0.046$). There was no significant difference between the Control and MDH groups. The gill Na^+/K^+ -ATPase activities in the

other groups were intermediary and not significantly different from the Control, MDL and MDH groups (Fig. 10).

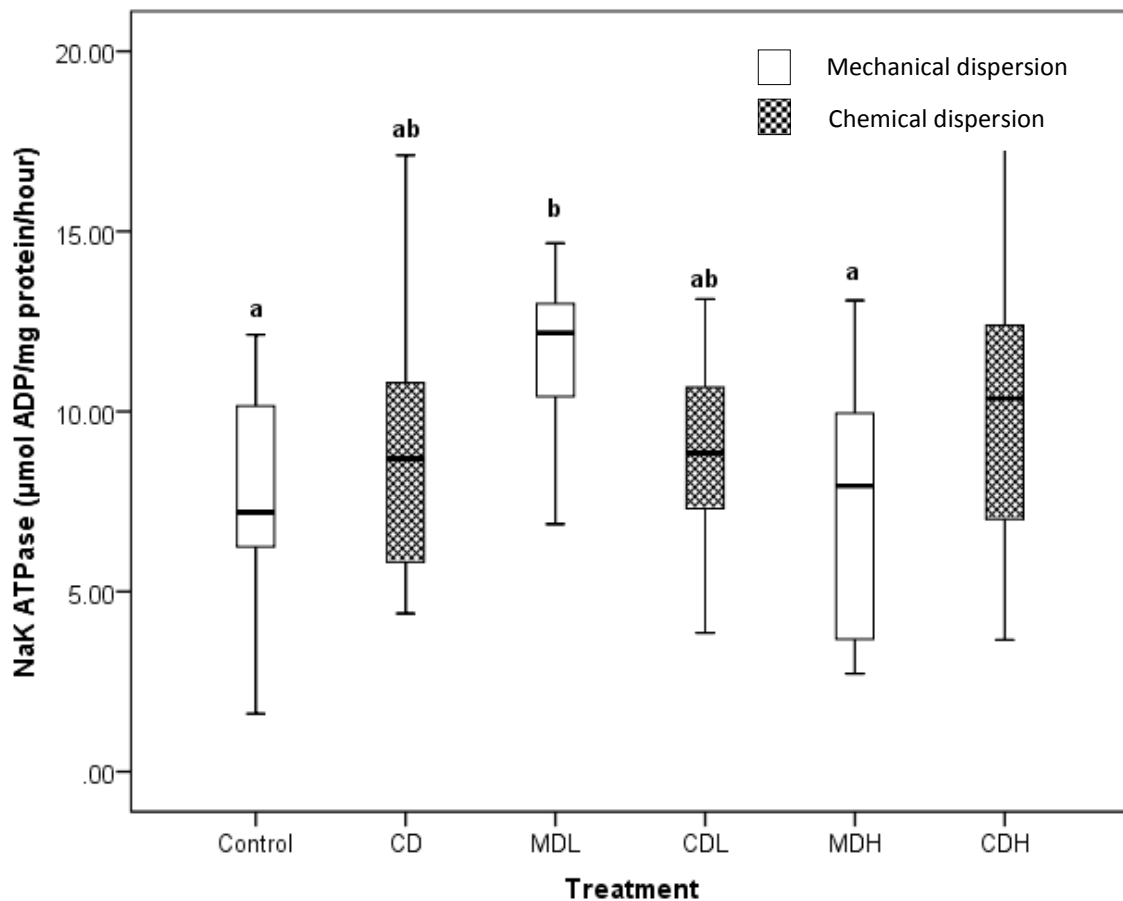


Figure 10. Box plot of gill Na^+/K^+ -ATPase activity ($\mu\text{mol ADP}/\text{mg protein}/\text{hour}$) of juvenile lump sucker (*Cyclopterus lumpus*) after exposure for 48 hours to seawater added North Sea Troll crude oil (mechanical dispersion: MDL and MDH), North Sea Troll crude oil and Dasic NS dispersant (chemical dispersion: CDL and CDH), Dasic NS dispersant alone (CD), and control (seawater only) Values are shown as median, quartile range, upper and lower limits. One-way ANOVA followed by Tukey's HSD post hoc test. Groups denoted with different letters are significantly different.

3.6 Other indicators of oil exposure and impaired hypo-osmoregulation

Out of 84 fish, one has died from the CDH group of unknown reason.

During dissection, fishes with swollen stomachs that are filled with water was notable, i.e. some fishes from MDL and CDL groups, 80% of the fish in MDH group, and 90% of the fish in CDH group.

Moreover, black spots on gills and notable oil smell were also observed in CDL (15% of the fish), MDH (25% of the fish) and CDH groups (more than 50% of the fish).

4 DISCUSSION

The present study has investigated possible acute toxic effects of dispersed crude oil exposure on the ion- and osmoregulation mechanisms, and potential biomarker (gill CYP1A and bile metabolites) responses in juvenile lumpsucker.

4.1 PAH metabolization

4.1.1 Gill CYP1A activity

To date, no published data was found on lumpsucker's gill EROD activity to compare the result of this study. However, when compared with polar cod, the gill EROD activity of the control fish in this study was 100 times higher than the gill EROD activity of the unexposed polar cod (*Boreogadus saida*), and at a similar level as in polar cod exposed to high oil concentration, in which CYP1A was induced (Nahrgang et al., 2010).

The substantially higher gill EROD activity of the control fish than the unexposed polar cod may indicate a constant high concentration of the enzyme in the gills of lumpsuckers. Perhaps an adaptation to compensate for being poor swimmers. In the presence of stressors, e.g. pollutants, it will be advantageous to have high concentration of this enzyme as they cannot hastily escape from the stressors. However, since the CYP1A enzyme is an inducible enzyme system in vertebrates, as supported by studies in both polar cod (Nahrgang et al., 2010) and other fish species (Jönsson et al., 2002, 2004) in which there has been shown a strong induction by Ahr agonists, e.g. PAHs, PCBs, etc., it is

more likely that the CYP1A activity level in the control lump suckers is not a baseline level, but rather an induced level. If this is the case, the possibility of contamination in the control tanks cannot be excluded. The hydrocarbons present in the air during the exposure experiment might have diffused into the water column, i.e. hydrocarbon molecules moved from higher concentration (in the air) to lower concentration (in the control tanks). In the first study, chemical analyses of the water sample showed presence of PAHs at the control tanks (although in a very minimal amount) (Frantzen et al., 2015). Since this study has used the same experimental set-up as the first study, there can be a possibility that the control tanks were also contaminated in this study.

In support of the abovementioned possibility that the gill CYP1A activity in the control group in the present study was induced by PAHs, no increase in gill CYP1A activity could be seen in fish exposed to neither mechanically nor chemically dispersed oil (Fig. 3). The lack of response was surprising, and contrary to other similar oil exposure studies (Abrahamson et al., 2007; Jönsson et al., 2009; Nahrgang et al., 2010). Another possible explanation of the lack of gill CYP1A induction can be the inhibition of CYP1A activity, as seen in zebra fish and rainbow trout (Jönsson et al., 2009), sea bream, *Sparus aurata* (Correia et al., 2007; Kopecka-Pilarczyk and Correia 2009a, 2009b; Atlantic cod (Lyon et al., 2011) and rainbow trout (Ramachandran et al., 2004) upon exposure to oil concentrations above certain levels.

When comparing the hepatic EROD activity of the first study (Frantzen et al., 2015) with the gill EROD activity of this study, the gill EROD activity of the control fish and fish that

were exposed only to dispersant(CD) were higher than the hepatic EROD activity in control and CD, the gill EROD control being 10 folds higher. If the assumption on contaminated control water tanks is correct (both in the first study and this study), then the 10 fold higher EROD activity in the gill suggests that gill CYP1A enzyme are more sensitive to the presence of PAHs than the liver, i.e. inducible at a lower PAH concentration. This result is similar to what has been reported in rainbow trout wherein gill CYP1A was induced at a low concentration but not the liver CYP1A (Jönsson et al., 2006; Abrahamson et al., 2007). However, it is also possible that since gill tissues are directly exposed to PAHs present in the water (compare to the liver in which the exposure is through the blood), the PAHs might have already been subjected to some biotransformation in the gills before they reached the liver (Andersson and Part, 1989; Jönsson et al., 2004; Jönsson et al. 2006; Griffitt et al., 2007).

When comparing the EROD activity at the low and high oil concentration of mechanical dispersion treatment, the gill EROD activity of this study was lower than the hepatic EROD activity of the first study. This might indicate an inhibition of CYP1A in the gill. As previous studies have shown, the CYP1A induction may be inhibited when the exposure is above certain levels (Ramachandran et al., 2004; Correia et al., 2007; Jönsson et al., 2009; Kopecka-Pilarczyk and Correia 2009a, 2009b; Lyon et al., 2011). There is a possibility that the oil concentrations used in the present study, even the lowest one, were above the threshold level to cause CYP1A enzyme inhibition at the gill.

The use of EROD activity as a biomarker of fish' exposure to pollutants has increased over the years. It is a very sensitive indicator of the presence of AhR agonists such as many PAHs, and is usually one of the first detectable, quantifiable responses to exposure (Stegeman, 1992). In the Arctic, it can be a valuable tool for monitoring and screening oil exposure during oil spills. The use of gill tissues in measuring EROD activity is particularly important since they are directly exposed to toxicants, and considered to be the principal site of xenobiotic uptake in fish (Jönsson et al., 2004; Griffitt et al., 2007). Moreover, this method can be performed without sacrificing the fish. The lack of gill CYP1A induction in the lumpsuckers exposed to oil in the present study is not in favor of using this species for monitoring purposes but further investigations should be carried out to reveal the cause of this lack in induction.

4.1.2 Bile PAH metabolites

The exposure, uptake, and metabolisation of PAH compounds were confirmed by the presence and higher concentration of bile metabolites in the oil exposed fish than in the controls. Three types of metabolites were detected, namely, the naphthalene-, pyrene-, and the benzo(a)pyrene-type. Among the three, naphthalene-type was found to have the highest concentration, followed by the pyrene-type. The bioavailability of these substances is related to their molecular weight, i.e. the lower the molecular weight, the higher is the bioavailability (AMAP, 2010). This finding is similar to what have seen in

muscle tissue in the first study, wherein the PAH type was dominated by naphthalene and substituted naphthalene homologues (Frantzen, et al., 2015).

Overall, there were no significant difference in the amount of bile metabolites between the high- and low-dose oil exposure groups. The only exception is the amount of benzo(a)pyrene-type in CDH, which was significantly higher than the MDH group. This is contrary to what have seen at the PAH in muscle tissue from the first study wherein there was an apparent exposure concentration-dependent increase in body burden (Frantzen et al., 2015). Since liver is considered the main organ for metabolizing PAHs, it is expected that a concentration-dependent increase will also be seen at a certain concentration before it will plateau or decrease. However, this is not what have been seen in this study, and the results are not able to provide explanation for this.

Bile metabolites have been used in other studies as a suitable endpoint for detecting oil contamination and exposure (Lee and Anderson, 2005). Similarly, as seen in this study, the lumpsuckers' PAHs exposure and assimilation were confirmed by the presence of PAHs metabolites in the bile.

4.2 Ion- and osmoregulatory effects

Under normal conditions, a seawater teleost fish is able to maintain, and dependent on maintaining, a relatively constant osmolality and ion concentration in their body fluids despite the large gradient in osmolality and ion concentration between their body fluids

and the external environment. This is achieved through the combined action of the gills, gut and kidneys in excretion of surplus ions and retainment of water (Eddy, 1981; McCormick, 1995; Fuentes and Eddy, 1997). However, stressors like handling, transport, and pollutants (Swift 1981; Stagg and Shuttleworth, 1982; Gluth and Hanke, 1984; Edwards et al 1987; Jones et al 1987; Goss and Wood, 1988; Allen, 1994) can disturb the mechanism of ion- and osmoregulation. Petroleum hydrocarbons, as a stressor, have been reported to have profound effects on plasma Na^+ , K^+ and Cl^- concentrations that increases in fish exposed to crude oil in seawater (Thomas et al., 1980; Al-Kindi et al., 2000; Kennedy and Farrell, 2005).

Based from the actual count of affected fish, the swelling of stomachs filled with water increases as the concentration of oil increases. This findings were similar to the study of Rørvik et al. (2000), wherein the frequency of trout with water-filled stomach increased significantly with increasing plasma osmolality. The swelling of stomachs is an indication of sub-lethal osmoregulatory stress.

The frequency of occurrence of black spots on gills and oil smell also increased as the oil concentration increases. These black spots may also contribute to the impairment of hypo-osmoregulation by acting as a barrier between the external environment and the cell membrane, thereby modifying the ion transport process involved in ion regulation.

This study also showed that exposure to crude oil has affected the ion- and osmoregulation capacity of the juvenile lumpsuckers in a dose-dependent manner, i.e. as the oil concentration increases, the plasma osmolality and plasma chloride concentration also increased. The dispersant, *per se*, did not seem to pose an additional load, as there was no significant difference found between fish exposed to mechanically and chemically dispersed oil.

One possible cause of the impaired hypo-osmoregulation can be the structural damage caused by hydrocarbons to the gill membrane (Fig. 11). Lipophilic hydrocarbons attach to, and accumulate in the membrane lipid bilayer. This accumulation of hydrocarbon molecules will eventually destroy the membrane's integrity and thus, increasing its permeability to ions and protons (McKeown and March, 1977, 1978; AMAP, 2010). The gradient difference between the cell and the external environment is used as a secondary transport process to drive selective uptake and excretion of solutes (Sikkema et al., 1995). However, when the membrane is destroyed, the permeability of the cell to ions also increases, and this increased permeability disturbs the gradient difference and the regulatory function of the gill.

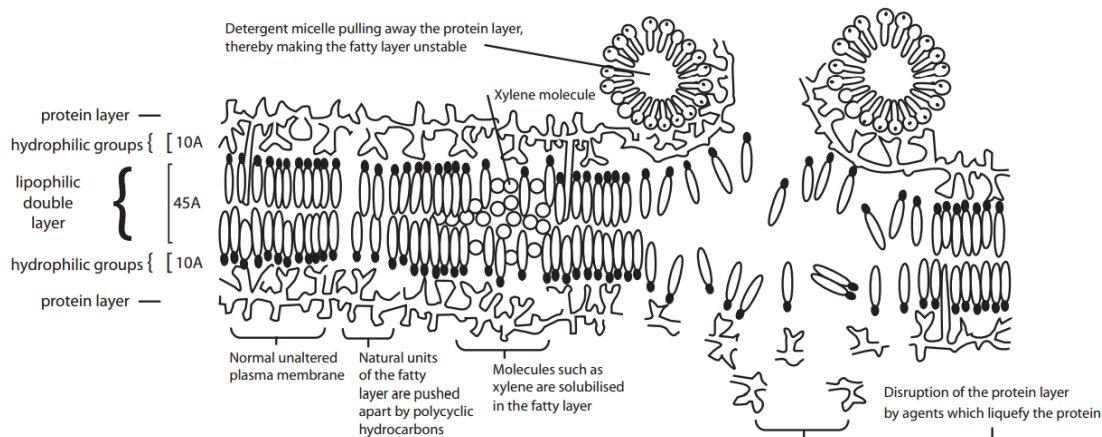


Figure 11. Schematic model of the mechanism how the hydrocarbons and other molecules alter the integrity of the lipid bilayer of the cell membrane (Source: van Overbeek and Blondeau, 1954).

Histological analysis of gills of coho salmon (*Oncorhynchus kisutch*) and starry flounder (*Platichthys stellatus*) showed loss of surface cells or the first two to three layers of cells after exposure for five days to 100 µg/L of WSF of Prudhoe Bay crude oil (Hawkes, 1977). Gill lesions, such as hyperplasia and hemorrhage were also observed on mummichogs when they were exposed to naphthalene (AMAP, 2010). Similar gill lesions, such as epithelial hyperplasia, epithelial lifting and clubbing were also observed in the first study. These lesions were not significant between the control and the treatment groups (Frantzen et al., 2015). It is probable that the reason the control fish also developed these lesions was because of the presence of PAHs detected in the control tanks (Table 1). However, again, this assumption needed to be further investigated.

Besides plasma osmolality and chloride concentration, the Na^+/K^+ -ATPase activity can also be used to reveal the hypo-osmoregulatory capacity of a fish in seawater. In fish, the Na^+/K^+ -ATPase pump is mandatory in ionic balance- and osmoregulation (Richards et

al., 2003), and the ability to hypo-osmoregulate is strongly linked with high gill Na^+/K^+ -ATPase activity (Glover et al., 2007).

This study did not see any effect of oil exposure to the gill Na^+/K^+ -ATPase activity, except at the MDL group (Fig. 10). This is surprising, though, and contrary to what have seen in other studies. The increase in gill Na^+/K^+ -ATPase activity in the MDL group may be seen as an attempt to counter-act the increased ion inflow and water loss. However, when exposed to higher concentrations, the gill Na^+/K^+ -ATPase decreased. This may indicate that the concentration of oil caused an inhibition on the ability of the gill to mount a higher gill Na^+/K^+ -ATPase activity in response to the increased ion inflow and water loss. Some studies have shown a reduction or inhibition of Na^+/K^+ -ATPase activity upon exposure to PAHs, which was caused by the alteration of membrane fluidity and structure (Bystriansky and Ballantyne, 2007). Besides altering the structural integrity of the cell membrane, PAHs have also been observed to inhibit the enzyme systems associated with membranes, notably the ATPases (Darville et al., 1983; Li et al., 2011). Gilthead sea bream (*Sparus auratus*) treated with 200 mg/g body wet of 4-nonylphenol displayed a clear reduction in kidney Na^+/K^+ -ATPase activity (Carrera et al., 2007). Gill Na^+/K^+ -ATPase activity was also reduced in Pacific staghorn sculpin (*Leptocottus armatus*) when exposed to refinery wastewater (Boese et al. 1982).

This study showed that the gill ATPase activity of juvenile lumpsuckers lies between (mean \pm SD) 7.33 ± 3.11 and 11.36 ± 2.5 $\mu\text{mol ADP mg}^{-1}$ protein hr^{-1} (Fig. 10). Compared with other species' baseline gill ATPase activity, i.e. rainbow trout ($1.5 \mu\text{mol ADP mg}^{-1}$ protein hr^{-1} , Nawata et al. 2007) and Arctic char in seawater ($2 \mu\text{mol ADP mg}^{-1}$ protein hr^{-1} , Bystriansky and Ballantyne, 2007), the juvenile lumpsuckers' were relatively higher. This suggests that either the juvenile lumpsuckers have a default high gill Na^+/K^+ -ATPase, or, the Na^+/K^+ pump is actually working at a maximum rate. The high activity in the control (similar to what was seen in the high groups) may be due to the presence of oil at the control tanks, as was assumed previously (Table 1).

The juvenile lumpsuckers in the first study have recovered after the acute exposure, and there was no sub-lethal long-term impacts seen from both the mechanical and chemical treatments (Frantzen et al., 2015). Thus, their study have shown that the exposure to oil in a short time will not be lethal to the juvenile lumpsuckers. In terms of long term exposure, though, other studies have shown that impaired ion and osmoregulation were fatal to the animals. In this study, the increasing level of plasma osmolality and plasma chloride, and the swelling of stomachs were all indications of impaired ion and osmoregulation. In the first study, all those fishes that died have swollen stomachs that are filled with water. Thus, it can also be probable that prolonged exposure will be fatal to juvenile lumpsuckers. However, this still needs to be confirmed.

5 CONCLUSION

This study has shown that juvenile lumpstickers exposed to environmentally realistic oil concentrations for an acute period resulted to impairment of ion and osmoregulation mechanism. Although the mechanisms involved were not clearly elucidated in this study, it is possible that the dysfunction and impairment are related to the integrity of the membrane, since the gill *ATPase* activity was not reduced nor inhibited (contrary to published oil exposure studies), and that the activity of the Na^+/K^+ pump was actually relatively higher to the baseline activity of other species, to which suggests a pump that might be functioning at a maximum rate.

Moreover, these findings also reveal that an oil spill have dramatic effects on fish in the spill area that are not able to escape, such as juvenile lumpstickers. Although acute exposures are not fatal (a finding similar to what was seen in the first study), the long exposure might be lethal, especially to species that are poor swimmers.

This study also showed that mechanical dispersion and chemical dispersion does not differ significantly. They had the same effect on the gill EROD activity, bile PAH metabolism, plasma osmolality, plasma chloride concentration, and gill Na^+/K^+ -*ATPase* activity. Based on these findings, it can be concluded that the use of dispersants to speed up the dissolution of oil in the water does not cause more serious detrimental effects on lumpstickers than the oil that is dispersed by natural means, e.g. waves.

The seemingly lack of induction at the gill EROD activity, if correct, does not make juvenile lumpstickers a good candidate for the detection and monitoring of oil spill.

However, as mentioned, since the results obtained were inconclusive due to the possibility of contamination of the control tanks, further investigation on gill CYP1A enzyme of lumpsuckers is recommended. Bile PAH metabolites of juvenile lumpsuckers, on the other hand, this study has shown the presence of PAH metabolites in the bile, a biomarker that can be used to detect oil exposure. Aside from the possibility of detecting the exposure and semi-quantification of metabolites, this assay is also cheap and not complicated to perform.

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7 APPENDIX I

Preparation of solutions for the analysis of Na⁺/K⁺-ATPase activity

SEI Buffer

Prod. No.	Chemical	Concentration (mM)	Amount (g)
102745C, <i>Analar BDH</i>	Sucrose	150	26.67
CAS # 6381-92-6, <i>Sigma-Aldrich</i>	Na ₂ EDTA	10	1.86
CAS # 288-32-4, <i>Sigma-Aldrich</i>	Imidazol	50	1.70

Mix sucrose, Na₂EDTA and Imidazol in 475 ml in distilled water and adjust the pH to 7.3 with HCl (1M). Afterwhich, add more distilled water to make up a total volume of 500 ml. It can be stored in the fridge (4°C) for up to 3 months.

0.5% SEID

Mix 0.1 g of sodium deoxycholate (Sigma, D-6750) with 20 ml SEI buffer.

Imidazole Buffer (IB)

Mix 1.702 g Imidazole (50mM) (Sigma-Aldrich, CAS # 288-32-4) with 475 ml distilled water and adjust the pH to 7.5 with HCL (1M). Afterwhich, add more distilled water to make up a total volume of 500 ml.

Salt

Prod. No.	Chemical	Concentration (mM)	Amount (g)
Lot # K36586304638, <i>Merck</i>	NaCl	189	5.52
Lot # MKBK C7 S0V	MgCl ₂ * 6H ₂ O	10.5	1.07
CAS # 7447-40-7, <i>Sigma-Aldrich</i>	KCl	42.0	1.57

Mix NaCl, MgCl₂ and KCl in 500 ml Imidazole buffer and store in the fridge (4°C). It can be stored for up to 3 months.

Phosphoenolpyruvate (PEP)

Mix 0.491 g phosphoenolpyruvate (Sigma-Aldrich, P7002-250mg) in 100 ml Imidazole buffer. Transfer 50 5 ml and 25 10 ml to eppendorf tubes and store in -80°C.

Oubain

Mix 0.382 g Oubain (Sigma, O-3125) in 50 ml IB. Put in the water bath with constant stirring. After the solution has cooled down, it can be stored inside the fridge (4°C).

Sodium Acetate Buffer

Mix 0.4627 g sodium acetate (57 mM) (Sigma S-8625) in 100 ml distilled water and adjust the pH to 6.8.

ADP standard

Mix 0.0489 g ADP (Sigma A-2754) in 25 ml sodium acetate buffer. Transfer the solutions to 300 µl eppendorf tubes and store in 80°C.

AM-Medium

Mix 105 µl pyruvate kinase (Sigma, P-1506) and 16.5 µl lactic dehydrogenase in a 0.5 ml eppendorf tube (placed on ice). Centrifuge the mixture at 4630 rot/min, 10 minutes at 4°C. Remove the supernatant and pipette the precipitate to the 50 ml graduated cylinder containing 20 ml Imidazole buffer (IB) (placed on ice). Add also 5 mg NADH (Sigma, 340-105), 5 ml PEP (Sigma, P-7002) and 0.0145 g ATP (Sigma, A7699).

AM-O Assay Mixture (AM-O)

Mix 16 ml AM-Medium with 1.25 ml Oubain in 50 ml Eppendorf tube (placed on ice).

AM Assay Mixture (AM)

Mix 16 ml AM-Medium with 1.25 ml IB in 50 ml Eppendorf tube (placed on ice).

Final Assay Mixture

Below is the final mixture that will be pipetted into the plate.

	Assay Mixture (ml)	Salt solution (ml)
AM Reservoir	8.1 AM	2.7
AM-O Reservoir	8.1 AM-O	2.7