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Biological Effects of Produced Water on Various Life Stages of Marine Fish

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BIOLOGICAL EFFECTS OF PRODUCED WATER FROM OFFSHORE CANADIAN ATLANTIC OIL AND GAS PLATFORMS ON VARIOUS LIFE STAGES OF MARINE FISH

by:

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Abstract

Canada remains dependent on oil and gas for much of its energy requirements. To meet anticipated future demands, there has been a rapid increase in offshore oil and gas exploration, production and transport during the last decade, particularly on the East Coast. Scientific information is required for environmental risk assessments that support the authorization of new developments across the country. In terms of operational risks from on-going and planned offshore production operations, a priority concern of regulators and the environmental community is that production water discharges may cause contamination and ill effects on fish and fish habitat. The development of sensitive ecologically relevant biotests for produced waters effects is needed for assessment of risk to the aquatic ecosystem and future field monitoring programs. For this project, experiments were conducted to determine the effects of produced water on various life stages of Atlantic cod (*Gadus morhua*) and Atlantic herring (*Clupea harengus*). Sensitive life stages were investigated to establish threshold concentrations and compare those thresholds to environmentally relevant concentrations of produced water.

In the first part of this study, Atlantic cod eggs, larvae and juveniles were exposed for 24 hours to a range of dilutions of five produced waters and lethality was assessed for larval and juvenile cod. Fertilization and hatching success were affected by produced water (PW) from the 2 gas platforms (Venture and Thebaud). The short-term exposure (24 h and 48 h) results show that the early life stages of cod are not affected by environmentally relevant concentrations of the PW tested. However, results suggest that chronic exposure to environmentally relevant concentrations of PW from some oil and gas platforms could pose a risk.

The objective of the next part of the study was to utilise the Atlantic herring sticky-egg bioassay to evaluate the toxicity of PW in chronic exposures during the entire embryonic period and measure impacts on embryonic survival, heart rates, size-at-hatch, time-to-hatch and morphological abnormalities. We also evaluated if prolonging the exposure period to include the first week following hatch could increase PW toxicity. Finally, we evaluated the influence of water temperature on PW toxicity. Produced water from the gas platforms proved more toxic than PWs from the oil platforms, the larval stage proved more sensitive than the embryo stage, and toxicity was increased by warmer temperatures, at least for the cold-adapted spring-spawning fish tested. Because of high dispersion rates of PW in the marine environment, the toxicity observed in the present study following chronic exposure to PW would not likely occur in the environment or, would occur only at very close range to the PW discharge.

The results of these laboratory tests indicate a potential for effects of PW on fish. Whether such effects actually occur around oil and gas platforms off Canada east coast can only be answered by field studies of fish health and PW concentrations. We recommend such studies as a next step in this research. An overview of the produced waters used and the endpoints tested is provided in the matrix below:

		Hibernia	Terra Nova	Venture	Sea Rose	Thebaud
Atlantic Cod (*Gadus morhua)* Endpoints	Hepatic EROD induction	↑		no significant difference		
	24h larval lethality	no effect	↑	**↑**	no effect	**↑**
	24h juvenile lethality	↑	↑	**↑**	↑	**↑**
	Juvenile growth	no effect	no effect	no effect	**↓**	**↓**
	Juvenile food consumption*		no effect	↑	↑	↑
	fertilization rate	constant	constant	↓		**↓**
	egg survival	constant	constant	↓		**↑**
	hatching success	no effect	no effect	↓		↓
	Vitellogenin	no effect				
Atlantic herring *(Clupea harengus)* Endpoints	Time-to-hatch	**↓**	**↓**	**↓**		
	Size-at-hatch	**↓**	**↓**	**↓**		
	Morphological abnormalities	↓	↓	↓		
	Survival	no effect	no effect	↓		

↑ indicates increase
↓ indicates decrease
(**Bold** is significant)
* Preliminary result

The two data chapters constituting this report are supported by, and refer to, the following materials which are presented as appendices:

1. Lyons, M.C., D.K.H. Wong, K. MacKeigan, L.E. Burridge, K. Lee and B. Robinson. The effect of chronic exposure to produced water on growth and food consumption of juvenile Atlantic cod (*Gadus morhua*). Manuscript in preparation.

2. Lyons, M., D. Wong, K. MacKeigan, L. Burridge, K. Lee and B. Robinson. 2012. The effect of chronic exposure to produced water on growth and food consumption of juvenile Atlantic cod (*Gadus morhua*). Poster presented at the 39th Annual Aquatic Toxicity Workshop: September 30 to October 3, 2012, Sun Peaks, British Columbia.

3. Wong, D., M. Lyons, L. Burridge and K. Lee. 2010. Fertilisation and hatching success of Atlantic cod (*Gadus morhua*) eggs when exposed to various concentrations of produced water. Poster presented at the 37th Annual Aquatic Toxicity Workshop: October 3-6, 2010, Toronto Ontario.

4. Lee, K., S.E. Cobanli, B.J. Robinson and G. Wohlgeschaffen. 2011. Application of microbiological methods to assess the potential impact of produced water discharges. P. 353-373 In: Lee, K. and J. Neff (eds.) Produced water: environmental risks and advances in mitigation technologies. Springer, New York.

5. Burridge, L., M. Boudreau, M. Lyons, S.Courtenay and K. Lee. 2011. Effects of production water from the Hibernia platform on the survival, growth and biochemistry of juvenile Atlantic cod (*Gadus morhua*) and mummichog (*Fundulus heteroclitus macrolepidotus*). P. 329-344 In: Lee, K. and J. Neff (eds.) Produced water: environmental risks and advances in mitigation technologies. Springer, New York.

Chapter 1: Effects of produced water on a commercially important indigenous species, Atlantic cod (*Gadus morhua*)

Introduction

Produced water (PW) is formation and injected water containing production chemicals and represents the largest volume waste stream in oil and gas production operations on most offshore platforms. There is considerable concern about the ocean disposal of produced water because of the potential danger of chronic ecological harm (Neff *et al*, 2011). With anticipated increases in the number of new offshore platforms globally, PW discharge has been identified as an issue of concern by both regulators and environmental groups (Zhao *et al*. 2008). The chemical characteristics of PW are different for each production platform or formation from which the oil is extracted. It is typically highly saline and contains elevated levels of heavy metals, hydrocarbons such as polycyclic aromatic hydrocarbons (PAHs), alkyl phenols, ammonia and radionuclides relative to the receiving environment (Lee *et al*. 2005; Burridge *et al*. 2011; Appendix 5). The physical and chemical properties of PW vary widely depending on the geologic age, depth, and geochemistry of the hydrocarbon-bearing formation, as well as the chemical composition of the oil and gas phases in the reservoir and process chemicals added during production. Because no two PW are alike, region specific studies are needed to address the environmental risks from its discharge (Neff *et al*, 2011). The plume of PW found in the nearfield may be quickly dispersed and be found in patches or pockets further away from the platform. Actual horizontal and vertical concentrations of PW constituents may be driven by winds and currents, densities and solubilities. Although the acute toxicity of PW discharges into the environment for marine organisms is probably a threat only within the direct zone of the discharge, the effects of chronic exposure to PW on the biology and/or physiology of organisms are mostly unknown (Pérez-Casanova *et al*., 2010). Continual chronic exposure may cause sub-lethal changes in populations and communities, including decreased community and genetic diversity, lower reproductive success, decreased growth and fecundity, respiratory problems, behavioral and physiological disorders, decreased developmental success and endocrine disruption (Neff *et al*, 2011).

Fish respond to stressors by eliciting a generalized physiological stress response, which is characterized by an increase in stress hormones and consequent changes that help maintain the animal's normal or homeostatic state. These physiological alterations are grouped as primary responses, which include hormonal changes and secondary responses, which include changes in metabolites, blood ions and hematology (Barton, 2002; Iwama *et al*., 2004). The tertiary response represents whole-animal and population level changes associated with stress. Exposure to stressors, depending on the intensity and duration can lead to decreases in growth, disease resistance, reproductive success, swimming performance and other characteristics of the whole animal or population (Iwama *et al*., 2004).

Food intake and nutritional status of fish can be assessed by using body morphometrics and daily food consumption measurement. Change in weight (mass) is the most commonly used assessment for growth performance. When growth rate is exponential, as it usually is over intervals of a year or less, growth

can be expressed as an instantaneous growth rate or as a percentage of instantaneous growth called specific growth rate (Busacker *et al*. 1990).

Atlantic cod is a species of considerable ecological and economic importance for Canada. Examining the chronic effects of PW on cod growth and feeding will shed light on the potential long-term impact of oil and gas offshore developments on stocks (Hamoutene *et al*. 2011). Metabolic capacities, feeding and digestive physiology of fish are influenced by environmental parameters. Chronic exposure to PW might potentially affect feeding, which in turn might have consequences on growth/health status of fish populations (Hamoutene *et al*. 2011).

Atlantic cod spawn in the Northwest Atlantic Fisheries Organization (NAFO) areas 4W (Scotian Shelf) and 3L (Grand Bank) (Brander, 1993; ICES, 2005). Atlantic cod eggs are pelagic and buoyant. When larvae hatch, they occur from near-surface to depths of 75 m, and they move deeper with growth. Transformation to the juvenile stage occurs at sizes greater than 20 mm and descent from the water column to bottom habitats occurs at sizes of 2.5 - 6 cm. By day, the young juvenile cod remain on the bottom, but at night they rise several meters into the water column and drift in the tidal current while feeding. Early juvenile cod consume more pelagic than benthic invertebrates and medium cod consume more benthic invertebrates and fish. A small number of these passive swimmers could get caught in the outflow of PW or juvenile cod could swim in and out of the plumes or patches and therefore get multiple exposures. Opportunities for chronic exposure are low but looking at actual PW concentrations in the field, and actual health of fish in those areas should be studied.

The areas of the northwest Atlantic that have the highest abundance of Atlantic cod are in Canadian waters and include the eastern coast of Labrador south of Cape Harrison, off eastern Newfoundland, the Flemish Cap, the Grand Banks, the Gulf of St. Lawrence, and the Scotian Shelf (Lough, 2004). All five of the PWs used in this study are discharged into Canadian waters where Atlantic cod may be chronically exposed.

Of the five PW used in this study two were from gas platforms, Venture and Thebaud. These platforms are off the coast of Nova Scotia, Canada, in the Scotian Shelf region of the Atlantic Ocean. Water depths at Thebaud and Venture platforms are 29 m and 22 m respectively. Venture platform does preliminary dehydration of the natural gas from its wells and then transports it to Thebaud for further processing. Thebaud platform is the hub of the Sable Offshore Energy Project's activity. Preliminary processing of the gas from the Thebaud wells, as well as gas from the existing satellite platforms – North Triumph and Venture – is done at Thebaud and then sent through a pipeline to the Goldboro Plant in Guysborough County, Nova Scotia (Sable Offshore Energy Project, 2012). The other three PWs studied were from Hibernia, Sea Rose and Terra Nova oil fields. Hibernia platform sits in 80 m of water off the coast of Newfoundland, Canada on the eastern edge of the Grand Banks in the North Atlantic Ocean. Sea Rose is a floating production storage and offloading vessel (FPSO) located in the White Rose oil and gas field, off the coast of Newfoundland, Canada on the eastern edge of the Grand Banks in the North Atlantic Ocean. The water depth at the southern part of the White Rose area ranges from 115 to 130 m (Canada-Newfoundland and Labrador Offshore Petroleum Board, 2000). Terra Nova is a FPSO located south of White Rose. Water depths at Terra Nova are between 90 to 100 m (Offshore Technology, 2012).

Purpose/objectives

We investigated sensitive life stages of Atlantic cod, establishing threshold concentrations and compared these thresholds to environmentally relevant concentrations. Exposure to PW may be short-term (hours), pulsed i.e. repeated short-term or chronic (days or weeks). While it is unlikely that we can investigate lethal and sub-lethal responses of fish to all the possible exposure regimes we hope to at least conduct range-finding tests, the results of which can be used to plan future experiments. By establishing dose-response relationships with PW and Atlantic cod investigators can begin to develop a suite of "health" indicators for Atlantic cod. Some of these indicators will be specific to hydrocarbon exposure while others may be of a more general nature.

Acute lethality tests were performed on larval and juvenile cod to determine the 24 h LC_{50}'s of the five PWs. LC_{50} is the calculated concentration where 50% of the animals die over a specified time period after exposure to a range of concentrations. Atlantic cod eggs were exposed to a range of concentrations of PW to identify effects on fertilisation, egg survival and hatching success.

Juvenile Atlantic cod were chronically exposed to two environmentally relevant concentrations of PW and the potential effects on growth parameters and food consumption were assessed. Intermittent exposure to PW concentrations of 100 ppm (10 000X dilution) and 200 ppm (5000X dilution) was used in an attempt to mimic the rapid dilution taking place after release in the environment. Rapid dilution of PW by at least 240X occurs within 50-100 meters, 1000X by 4-5 km and up to 9000X at 20 km from the discharge site (Somerville *et al*., 1987; Murray-Smith *et al*., 1996; Pérez-Casanova *et al*., 2010). Neff (2002) notes that while field measurements and dispersion modelling studies of the fate of PW in various marine environments show differences in the details, all predict a rapid dilution of PW.

PAHs and alkyl phenols are oestrogenic compounds and so PW exposure may adversely affect the hormonal system of fish. It is accepted that exposure to oestrogenic-like compounds in the aquatic environment may result in production of the yolk protein, vitellogenin (VTG) in male fish (Allen *et al.,* 1999). An enzyme linked immunosorbent assay (ELISA) for VTG in cod has been developed and is sensitive to environmentally relevant concentrations of contaminants (Burridge *et al*., 2011; Appendix 5). Vitellogenin was measured in the plasma of juvenile cod chronically exposed to Hibernia PW.

PAH exposure can be estimated by a standardized laboratory assay of cytochrome P-450 (CYP1A) induction (Hodson *et al*., 1996). Mixed-function oxygenase (MFO) enzymes are a family of membrane-bound enzymes which increase the water solubility of aromatic and lipophilic compounds. The terminal oxidase enzyme of the MFO system is the iron-containing hemoprotein CYP1A. The CYP1A enzyme catalyzes the hydroxylation of PAH to a more soluble and excretable form in the bile. Assays of liver CYP1A activity provide a good biomarker of PAH exposure in fish (McCarty *et al*., 2002). Ethoxyresorufin *O*-deethylase (EROD) is part of the family of cytochrome enzymes and is induced in the presence of xenobiotic compounds including PAHs (Hodson *et al*., 1991). EROD was measured in the liver of juvenile cod chronically exposed to Hibernia, Sea Rose, Thebaud and Venture PW.

Materials and Methods

Produced water

Acid-washed (1 M HCl) nalgene high-density polyethylene jerricans were provided to platform staff for the collection of PW. Instructions for collection were to fill the jerricans to overflowing to eliminate any headspace. Collection of PW was coordinated so that the samples were returned to scientific staff as soon as logistically possible. The containers were sealed with electrical tape, refrigerated and transported to the Bedford Institute of Oceanography, Dartmouth, Nova Scotia and then on to St. Andrews Biological Station (SABS), St. Andrews, New Brunswick where they were refrigerated at 4°C for the duration of the study.

Cod eggs and milt

Cod eggs and milt were obtained from broodstock held in +6°C sand filtered sea water at SABS, St. Andrews, New Brunswick, Canada. Fish were stripped by hand and single pair fertilisation performed for each experiment.

Fish stocks

Juvenile Atlantic cod (*Gadus morhua*) were obtained in 2006 and 2010 from the hatchery at SABS, St. Andrews, New Brunswick, Canada. Juvenile cod were obtained in 2011 and 2012 from Great Bay Aquaculture, Portsmouth, New Hampshire, USA. The fish were held in flow through filtered sea water at ambient temperature and under simulated natural photoperiod. Dissolved oxygen and water temperature were recorded daily. The fish were hand fed once daily with dry pellet of EWOS marine sinking cod diet from EWOS Canada Ltd. (Surrey, British Columbia, Canada) in 2006 or Gemma starter feed and then Europa, 2.0 mm feed from Skretting North America (Bayside, New Brunswick, Canada) in 2010, 2011 and 2012.

Acute lethality of PW to larval Atlantic cod

Cod eggs were fertilised using standard procedures then held in larval pots at +6°C until they hatched. Larvae (<3 days old), before depletion of their yolk sac occurred, were then used for acute lethality experiments to determine the LC_{50} of the PWs. Larvae were exposed by adding newly hatched larvae (>50) into beakers containing the appropriate PW dilution (Table 1). Larvae (50 per concentration) were then transferred along with up to 200 µL of the PW to 96 well plates (1 larva per well) and held at +6°C for 24 h. Larvae were monitored at 0, 1, 3, 6, 12 and 24 h post start of exposure and the number of mortalities recorded. All tests were performed in a cold room where temperature was maintained. The LC_{50} was calculated according to Stephan (1977) using ToxStats software.

Acute lethality of PW to juvenile Atlantic cod

Juvenile cod were exposed for 24 hours to a range of concentrations of PW and a sea water control. Five fish per concentration were exposed at temperatures of 12°C to 14°C in aerated glass test chambers in a water bath to maintain temperature (Table 1). The exposures were repeated 3 times. Fish behaviour and mortality were observed at 1, 3, 6, 12 and 24 h and length and weight were recorded at the end of the test. Dissolved oxygen and temperature were recorded throughout the test. The LC_{50} was calculated according to Stephan (1977) using ToxStats software.

Table 1. Exposure concentrations for 24 h lethality tests.

Produced water	**Larval cod Exposure Conc. (% v/v)**	**Juvenile cod Exposure Conc. (% v/v)**
Hibernia	0 2.22 6.67 20 60 75 100	0 13 22 36 60 100
Sea Rose	0 1.2 3.7 11.1 33.3 100	0 7.8 13 22 36 60
Terra Nova	0 0.74 2.22 6.67 20 60 100	0 11 18 36 60
Thebaud	0 0.15 0.44 1.33 4 12	0 1.8 3.6 6 10

Venture	0 0.15 0.44 1.33 4 12	0 1.8 3.6 6 10

Fertilisation and hatching success of Atlantic cod eggs

Preparation of produced water dilutions

Neat solutions of PW were diluted with UV disinfected sea water to give appropriate exposure concentrations for the fertilisation and lethality experiments (Table 2).

Table 2. Concentrations for egg exposures to PW.

Experiment	Exposure concentrations (% v/v)			
	Hibernia	Terra Nova	Thebaud	Venture
Fertilisation & Hatching	0 0.32 1.08 3.6 12	0 0.32 1.08 3.6 12	0 0.32 1.08 3.6 12	0 0.32 1.08 3.6 12

Preparation of high salinity sea water sample

The salinity of Venture and Thebaud PWs was very high compared to Terra Nova and Hibernia; exposures were conducted using dilutions of a high salinity sea water stock to eliminate the possibility that this was a factor in their fertilisation success. To mimic the salinity of Thebaud and Venture PWs, sand filtered sea water was boiled down until its salinity was similar to that of the PWs (*ca* 200 ‰) The high salinity preparation was used as a control for the gas field PWs in addition to the regular sea water control (Figure 1).

Fertilisation and hatching success exposures

Cod eggs were fertilised with milt in 0, 0.32, 1.08, 3.6 and 12% v/v PW concentrations. Eggs were rinsed with the respective PW solutions then held for a further 24 h in the same solutions after which time they were rinsed with clean UV disinfected sea water and the fertilisation success assessed. Fertilisation success was normalised against seawater controls using the following equation:

$$\text{fertilisation success}(\%) = \frac{\text{exposed fertilisation rate}(\%)}{\text{control fertilisation rate}(\%)} \times 100$$

Viable fertilised eggs (≤ 96) from each exposure concentration were transferred to 96 well plates (1 egg per well) containing UV disinfected sea water and incubated at +6°C in the dark. No water changes were performed and the plates were monitored throughout the incubation period until the eggs hatched (~17 days). Experiments were conducted in triplicate for each PW. Very poor egg quality in 2012 meant no usable results for Sea Rose PW.

Sublethal exposure of juvenile cod to PW

Juvenile cod were exposed for 48 h to one of five concentrations (0.06, 0.19, 0.56, 1.67 and 5%) of Hibernia PW. Hepatic EROD activity was measured. Experimental design is described in Burridge *et al*., 2011 (Appendix 5).

Chronic exposure of juvenile Atlantic cod

Juvenile cod (8.8±0.7 g) in 2007, (9.1 ± 3.9 g) in 2010, (12.7 ± 2.4 g) in 2011 and (13.9 ± 2.2 g) in 2012 were anaesthetized with tricaine methanesulfonate (MS222; Syndel Laboratories Ltd, Vancouver, BC, Canada), tagged with Passive Integrated Transponders (PIT) and placed in 400 L fibreglass tanks with flow through filtered seawater. Fish length and weight were recorded. The fish were allowed to recover in ambient seawater for a few weeks before chronic exposures were started.

Hibernia PW

In January 2007 seventy juvenile cod were exposed continuously for 45 days to 0.05% v/v PW (2000X dilution). Growth of fish, plasma vitellogenin and hepatic EROD activity were measured. Experimental design is described in Burridge *et al*., 2011 (Appendix 5).

Sea Rose, Terra Nova, Thebaud and Venture PW

Three times a week the fish in each tank were slowly hand fed a preweighed amount of food (Specific feeding rate, SFR = 3% to 4%). On the same days, for fourteen weeks, the 100 ppm (10 000X dilution) and the 200 ppm (5000X dilution) groups were exposed to one pulse of PW from either Sea Rose, Terra Nova, Thebaud or Venture platforms. After four hours the standpipes were briefly lifted in each tank and the uneaten food was collected and allowed to air dry in a fume hood for five days before weighing. The fish were held in 250 L of seawater and the water flow was maintained at 2.5 L min^{-1} so that the initial

pulse dose was gradually diluted over a period of 8 h. Ambient water temperatures during the exposures were 7 to 15°C.

At five, ten and fourteen weeks, five fish per tank were sacrificed. Fish were stunned with a sharp blow to the head and blood was collected from the caudal vein with a heparinized disposable syringe and centrifuged at 3500 x g for 10 minutes at 4°C. Plasma was aliquoted and stored at -80°C. Liver and brain tissue were taken and stored for biochemical analyses. Tissues were flash frozen in liquid nitrogen and stored at -80°C. Length, weight, liver weight, blood volume and sex were recorded for each fish.

Remaining fish were anaesthetized and length and weight were recorded. The specific growth rates of each fish based on weight (SGR) were calculated for the sampling time periods: PIT tag date to five week sampling date, five to ten week sampling date and ten to fourteen week sampling date. The SGRs were calculated using the following formula:

SGR= $((\log_e Y_2 - \log_e Y_1)/ (t_2 - t_1)) * 100$ where Y_2 is the weight at sampling, Y_1 is the weight at the previous sampling , t_2 is the Julian day at sampling and t_1 is the Julian day at the previous sampling. Food consumption (FC) was calculated as: FC (g $day^{-1)}$ = g food into tank – g uneaten food.

Water analysis

PW collected from Hibernia, Sea Rose, Terra Nova, Thebaud and Venture platforms was analysed at the Bedford Institute of Oceanography, Dartmouth, Nova Scotia. All PW samples used in this study were analysed for a suite of organic and inorganic constituents, and the analytical methods used for analysis are summarized elsewhere (Lee *et al*., 2011; Appendix 4).

Statistical analysis

The LC_{50} estimates were determined according to Stephan (1977) using the Toxstats program. All LC_{50}'s were calculated using a Spearman-Karber analysis. Specific growth rates based on weight (SGR) were calculated for the chronic exposure studies prior to statistical analysis. All data were analyzed for homogeneity of variance using Levene's test. Dunnett's post hoc comparison test was used to compare the SGRs of the control fish with those of treated fish if equal variances were assumed and Dunnett T3 test was used if equal variances were not assumed. Analyses of variance (ANOVA) were performed for EROD activity values that had been log transformed to achieve normal distribution (Hodson *et al*., 1996). The *post hoc* Tukey's HSD was used to make all pair-wise comparisons and to identify significant differences in EROD activity between treated fish and seawater controls. All of the growth, food consumption and EROD statistical analyses were conducted using IBM SPSS Statistics Version 19.0 (SAS Institute Inc., Cary, NC, USA). Statistical analysis of hatching success was performed using SPSS version 19.0 software (IBM, USA). Generated data were analysed using a one way ANOVA test followed by the *post hoc* Tukey pairwise multiple comparisons test.

Results

Acute lethality of PW to larval and juvenile Atlantic cod

Lethality of the PWs to newly hatched cod larvae followed the same trend as for fertilisation and hatching success. Hibernia and Sea Rose PW were the least toxic to larvae because even when exposed to the neat solution (100% v/v), less than 50% of test animals were affected, thus no LC_{50} could be determined. Terra Nova gave an LC_{50} of 42.51% v/v indicating that it was more toxic than Hibernia and Sea Rose PW. Thebaud and Venture were the most toxic. A summary of the calculated 24 h LC_{50}'s for both larval and juvenile cod are presented in Table 3.

Table 3. Calculated 24 h LC50 results for larval and juvenile cod.

Produced water	**Larval cod 24 h LC50 (% v/v)**	**Juvenile cod 24 h LC50 (% v/v)**
Hibernia	n/c#	43.53
Sea Rose	n/c#	22.53
Terra Nova	42	36.53
Thebaud	1.25	3.68
Venture	0.88	4.82

= not calculable since >50% of test animals survived in 100% solution of PW

Fertilization and hatching success of Atlantic cod eggs

<u>Fertilisation and hatching success</u>

The fertilisation rate for eggs exposed to Thebaud declined steadily from 1.08% v/v until no viable fertilised eggs were present after 24 h at 12% v/v. The same pattern was seen for Venture PW except the rate of decline was slower than for Thebaud. Terra Nova and Hibernia showed similar fertilisation

rates over the full concentration range. The high salinity sample also showed similar, although slightly lower, fertilisation rates over the full exposure to Terra Nova and Hibernia (Figure 1).

Hatching success from transferred viable fertilised eggs showed that for Hibernia, Terra Nova and the high salinity exposures, there were no significant differences ($p < 0.05$) when compared to controls. There was however a slight decrease in hatching rate at the 12% v/v exposure concentration for Terra Nova and the high salinity samples although these were not statistically significant from the controls. For Thebaud, there was a steady decline in hatching from 0.32 to 3.6% v/v followed by a dramatic reduction at 12% v/v ($p < 0.005$). For Venture, no reduction in hatching success was noted until 3.6% v/v ($p < 0.005$) with no hatching seen at 12% v/v (Figure 2). The high salinity exposure showed a lowered hatching success at 12% v/v but this was not significantly different from the control.

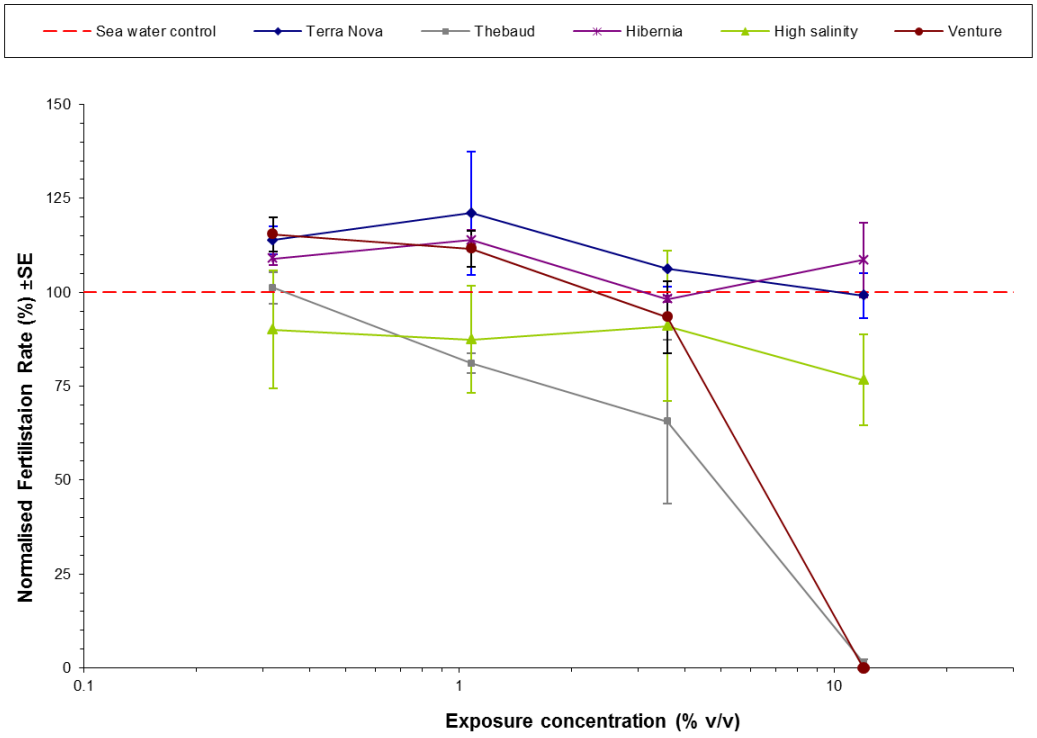

Figure 1. Fertilisation rate of cod eggs exposed to various concentrations of Hibernia, Terra Nova, Thebaud and Venture produced waters and high salinity sea water normalised against sea water controls (n=3).

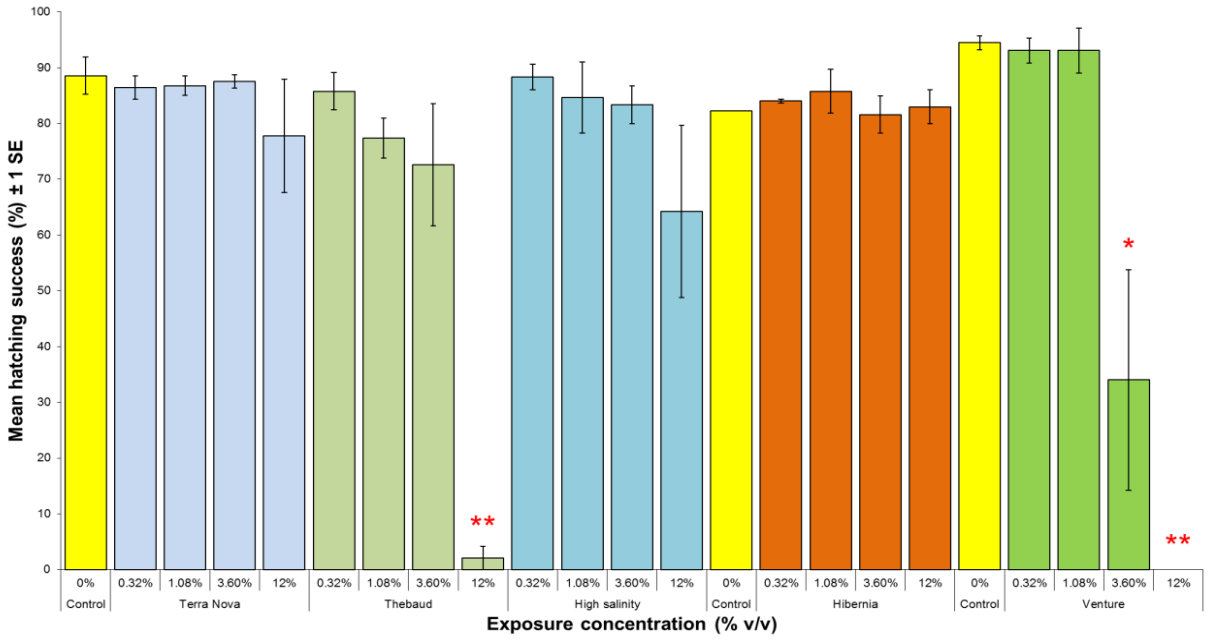

Figure 2. Hatching success of viable fertilised cod eggs following exposure to Hibernia, Terra Nova, Thebaud and Venture produced waters and high salinity sea water (n=3; * = p<0.05; ** = p<0.005).

Sublethal exposure of juvenile cod to PW

The level of EROD activity in livers collected from cod exposed to a range of Hibernia PW concentrations ranged from 0.2 to 12.6 pmol·min^{-1}·mg^{-1}. Only in Hibernia PW concentrations of 1.67 and 5 % was EROD activity significantly (P<0.05) elevated compared to controls. Detailed experimental results are described in Burridge *et al*., 2011 (Appendix 5).

Chronic exposure of juvenile Atlantic cod

<u>Hibernia</u>

During the 45-day exposure of juvenile cod to 0.05 % PW from Hibernia there was no significant difference (P<0.05) in hepatic EROD activity between treated and untreated fish. EROD activity was low throughout the study. The maximum measured value was approximately 5 pmol·min^{-1}·mg^{-1}. Vitellogenin was measured in the plasma of juvenile cod chronically exposed to Hibernia PW. There was no significant change in EROD activity, growth or plasma vitellogenin for juvenile cod exposed chronically to 0.05% v/v Hibernia PW compared to unexposed fish. Detailed water analysis and experimental results are described in Burridge et al., 2011 (Appendix 5).

Sea Rose, Terra Nova, Thebaud and Venture PW

Growth and food consumption experimental results are described in detail in Lyons *et al.*, unpublished (Appendix 1). Comparisons of mean food consumption of control and treated juvenile cod showed that food consumption was affected for 100 and 200 ppm Sea Rose PW, 100 and 200 ppm Thebaud PW and 100 ppm Venture PW treated fish in the final third of the exposure period (Dunnett's $P<0.005$). Although the daily food consumption of cod exposed to 200 ppm Venture PW (14.01 g ± 0.27, n = 11) was less than food consumption of control fish (14.77 g ± 0.31, n = 11), the difference was not significant (Dunnett's). Terra Nova PW had no effect on food consumption of juvenile Atlantic cod when compared to control fish. There were no significant differences in food consumption between control and treated fish in the first 10 weeks in any of the studies.

Comparison of the mean SGRs of control and treated juvenile cod showed that mean SGRs of treated fish were significantly different from controls in the final third of the exposure period (weeks 10 to 14) for two treatments -100 ppm Thebaud PW (Dunnett's $P<0.005$) and 200 ppm Sea Rose PW (Dunnett's $P<0.05$). There were no significant differences between mean SGR of treated fish and control fish in the first ten weeks of the studies.

During the 14 week chronic exposures of juvenile cod to PW (Sea Rose, Thebaud and Venture) there were no significant differences ($P<0.05$) in hepatic EROD activity between treated and untreated fish. The level of EROD activity in livers collected from control cod ranged from 1.5 to 9.2 $pmol \cdot min^{-1} \cdot mg^{-1}$. The level of EROD activity in livers collected from cod exposed to a range of Sea Rose PW concentrations ranged from 0.3 to 10.6 $pmol \cdot min^{-1} \cdot mg^{-1}$. The level of EROD activity in livers collected from cod exposed to a range of Thebaud PW concentrations ranged from 0.3 to 9.2 $pmol \cdot min^{-1} \cdot mg^{-1}$. The level of EROD activity in livers collected from cod exposed to a range of Venture PW concentrations ranged from 0.3 to 16.8 $pmol \cdot min^{-1} \cdot mg^{-1}$. A summary of the PW organic components is presented in Table 4. A detailed account of PW analysis results is presented in Lee *et al. (2011)* (Appendix 4).

Table 4. Organic components of four produced waters, n = 1 for each raw PW sample. Values are a sum of a number of individual compounds with detection limits as follows: Alkanes/PAH/Alkylated PAH = 0.0001 mg/L (or 100 ng/L) for each individual compound, Phenols = 0.000001 mg/L (or 1 ng/L) for each individual compoundBTEX = 0.001 mg/L (or 1000 ng/L) for each individual compound.

	Sea Rose	**Terra Nova**	**Thebaud**	**Venture**
PW component	**Concentration (mg/L)**			
Alkanes	1.69	0.83	0.55	0.32
Alkylated PAHs	0.52	0.26	0.47	0.30
PAHs	0.30	0.13	0.41	0.19
Phenols	3.52	4.04	16.54	10.62
BTEX	15.42	8.80	11.43	12.95

Discussion

The discharge of produced water (PW) from offshore oil and gas facilities remains an environmental concern due to uncertainty regarding its fate, transport and potential biological effects. The plume of PW found in the nearfield may be quickly dispersed and be found in patches or pockets further away from the platform. Actual horizontal and vertical concentrations of PW constituents may be driven by winds and currents, densities and solubilities so require more study. Furthermore, PWs differ in their compositions, making it difficult to apply knowledge of one PW to a broad range of PWs (see Appendix 1, Table 3). Additionally, by boiling seawater to create a high salinity control for fertilization and hatching tests, we acknowledge that it is not possible to mimic the exact ratio of inorganic ions of PW.

Sensitive life stages of Atlantic cod, *Gadus morhua* may be exposed to PW for a short-term (hours), in pulsed doses (repeated short-term) or chronically (days or weeks). Atlantic cod eggs, larvae and juveniles were exposed for 24 hours to a range of dilutions of five PWs and lethality was assessed for larval and juvenile cod. The order of lethality of PW to juvenile cod was: Hibernia < Terra Nova < Sea Rose < Venture < Thebaud. The order of lethality of PW to larval cod was: Sea Rose = Hibernia < Terra Nova < Thebaud < Venture. The PW from the 2 gas platforms Venture and Thebaud were the most lethal to larvae and juvenile of the 5 PWs tested. The high salinity of the Thebaud and Venture PWs was determined not to be the reason for the higher toxicity. The LC_{50} values reported for larval and juvenile cod are not environmentally relevant concentrations. Rapid dilution of PW by at least 240X occurs within 50-100 m, 1000X by 4-5 km and up to 9000X at 20 km from the discharge site (Somerville *et al*., 1987; Murray-Smith *et al*., 1996; Pérez-Casanova *et al*., 2010). The most lethal of the PWs to larval cod were those from the gas platforms with 24 h LC_{50}'s of 0.88% v/v and 1.25% v/v which are equivalent to 114X and 80X dilutions. These dilutions would only be experienced by fish very close to a production platform.

Polycyclic aromatic hydrocarbon (PAH) exposure was measured by induction of cytochrome P-450 (CYP1A) as indicated by the enzyme ethoxyresorufin-*O*-deethylase (EROD) in the liver of juvenile cod exposed to Hibernia PW. EROD activity was significantly elevated in the liver of juvenile cod exposed for 48 h but only at concentrations greater than 1.67% by volume which is not an environmentally relevant concentration. Previous oil and dispersant studies with juvenile cod have shown that EROD is a sensitive indicator of PAH in liver. A dose response was seen for EROD and using five fish for each concentration was sufficient to see the response (Lyons et al., 2011). Stocking densities of fish in exposure tanks prevented using larger numbers.

Fertilization and hatching success of eggs were assessed. The order of lethality of PW to cod eggs was: Terra Nova = Hibernia < Venture < Thebaud. Fertilisation was affected equally at the two oil platforms and high concentrations were required to produce an effect. The gas sites affected fertilisation to a greater extent with Venture PW having a greater affect than Thebaud PW. Venture PW affected hatching at 3.6 and 12% v/v, Thebaud PW affected hatching at 12% v/v but no effects were seen for Terra Nova and Hibernia PWs. These concentrations would only be experienced very close to a production platform.

Marine fish may be chronically exposed to diluted PW at distances far from oil and gas production sites. Juvenile Atlantic cod were chronically exposed to 0 or 0.05% v/v Hibernia PW (2000X dilution) for 45 days. Juvenile Atlantic cod were exposed intermittently to 0, 100 or 200 ppm of PW (10 000X dilution and 5000X dilution) from eitherSea Rose, Terra Nova, Thebaud or Venture platforms for 14 weeks. Growth and food consumption were monitored. EROD was measured in the liver of juvenile cod for all chronic exposures of PW except Terra Nova. Vitellogenin was measured in the plasma of juvenile cod chronically exposed to Hibernia PW. There was no significant change in EROD activity, growth or plasma vitellogenin for juvenile cod exposed chronically to 0.05% Hibernia PW compared to unexposed fish. At the end of the 100 day chronic exposures significant effects on specific growth rate (SGR) were seen in fish exposed to 200 ppm Sea Rose PW ($P<0.05$) and 100 ppm Thebaud PW ($P< 0.005$). Effects on daily food consumption (FC) were significant ($P< 0.005$) for 100 and 200 ppm Sea Rose PW, 100 and 200 ppm Thebaud PW and 100 ppm Venture PW treated fish at the end of the exposure. Concentrations of 100 ppm and 200 ppm PW (10 000X dilution and 5000X dilution) could be experienced by juvenile cod at great distances from the platforms.

Conclusions

The short-term exposures (24 h) data presented here show that the early life stages of cod are not affected by environmentally relevant concentrations of PW. The end-points investigated: egg fertilization, hatching success, mortality were only observed at PW concentrations of 1% or greater. The short-term sublethal exposure (48 h) data presented show that juvenile cod are not affected at environmentally relevant concentrations of Hibernia PW. EROD was only elevated at 1.67% and 5% v/v PW which are equivalent to 60X and 20X dilutions. The chronic exposure of juvenile cod to 0.05% v/v Hibernia PW (2000X dilution) for 45 days produced no effect on growth, EROD induction or vitellogenin. These data show that indicators of exposure and of effects are only observed at high concentrations relative to expected environmental exposure. The concentrations of PW that produced measureable effects in the endpoints investigated would only be observed very close to the PW discharge meaning that risks associated with these endpoints are very small. When PW is discharged into the sea from offshore installations, it quickly becomes diluted. At a distance of 100 m, 5 km and 20 km from the installation it is diluted 240x (equivalent to 0.42%), 1000x (0.1%) and 9000x (0.011%) respectively (Somerville *et al*, 1987; Murray-Smith *et al,* 1996). As a result, the possibility of cod eggs and larvae being exposed to the concentrations used in this study for a sustained period of time is quite low. Therefore the risk to eggs and larvae being exposed to concentrations of PW similar to those in this study for the same amount of time in the real world environment is considered very low.

The 14 week chronic exposures to pulses 3 times per week of diluted PW (5000X and 10 000X dilution) used in this study to mimic the rapid dilution that follows discharge into the environment did affect juvenile cod growth and food consumption. Effects were seen for Thebaud, Venture and Sea Rose PW but not for Terra Nova PW. The concentrations of Sea Rose, Thebaud and Venture PW that produced measureable effects in the growth and feeding endpoints investigated could be observed at 10 000X dilution which can occur at distances of greater than 20 km from the PW discharge indicating that risk associated with these endpoints are possible. The hepatic EROD endpoint may be of little predictive

value for chronic exposures of PW. Dilutions of PW (2000X, 5000X and 10 000X) over several days did not induce EROD in cod liver.

Discharge of PW to the waters of the Scotian Shelf and edge of the Grand Banks, two highly productive areas that have some of the highest abundance of cod in Canadian waters could pose a risk to juvenile cod. The effects of chronic, low level exposures of PW on important marine species such as cod may become evident only after monitoring several life stages. Earlier life stages (egg, larval and juvenile) of cod are vulnerable as they have little control over their movement in the ocean currents and may be unable to avoid being caught in a plume of PW in the near-field or a patch or pocket in the far-field.

The chronic exposure data presented here indicate that effects can occur and need to be considered in a risk assessment context but generalizations about the effects of PW cannot be made. The complex nature of PW makes it challenging to try and relate biological effects to one or more specific compounds, and instead the toxicity of whole mixtures of chemicals should be considered. Chemical analysis of PW may be of little predictive value. The consequences of long-term effects on cod populations from offshore oil and gas facilities that have a 15-20 year life-cycle need to be better understood.

Chapter 2: Effects of produced water on a commercially important indigenous species, Atlantic herring (*Clupea harengus*)

Introduction

One conclusion of the 2007 International Produced Water Conference, sponsored by ESRF, (St. John's Newfoundland, Canada) was that tools and approaches are needed to assess potential and actual biological impacts of produced water (PW) associated with the growing offshore oil and gas industry in Atlantic Canada (Lee and Neff, 2011). While a number of options exist for assessing acute toxicity [i.e., median lethal concentration (LC50 tests)], effects of chronic exposure to low, environmentally relevant concentrations of PW have not received as much attention both because of inherent logistic difficulties and because appropriate assays have not been developed. Furthermore, the results of recent studies have clearly shown that acute toxicity is a non-issue within Atlantic Canada at the current discharge rates due to effective dispersion of the PW effluent stream (Burridge *et al*., 2011; Appendix 5). While some studies have found no significant ecological risk or health effects on fish from produced water *in situ* exposure (Neff *et al*., 2006; Mathieu *et al*., 2011), others have shown significant sub-lethal effects of chronic exposure to PW at considerable distances from discharge. Rabalais *et al*. (1992) observed significant changes in the benthic macroinfaunal community in coastal areas in Louisiana State (USA) with the reduction of the number of species and individuals as far as 800 m for the PW discharge. Discharge volumes in the Mississippi River Delta study area were between 3000-106,000bbl/day. Krause (1995) observed a reduction in fertilisation success of the purple sea urchin (*Strongylocentrotus purpuratus*) in Carpintenia, California following exposure of gametes to water samples collected by divers at 1000 m from the PW discharge. The Carpintenia facility discharged approximately 16,000bbl/day from onshore, releasing the produced water 200-300m off the coast at 12 m depth. Exploitation of offshore oil and gas is a relatively small and young industry in Atlantic Canada so volumes of PW have been small. With a growing industry and aging wells, volumes of PW will increase. For instance, the Tampen Region in the Norwegian section of the North Sea has 13 active platforms which discharge approximately 1,151,000bbl/day at depths between 1 and 40 m (discharge volumes for 1997 and 1999; Durell *et al*., 2006) compared to the Jeanne d'Arc Basin of Atlantic Canada which has 18 officially confirmed fields but only four active platforms (http://www.ogj.com/articles/print/volume-98/issue-32/special-report/grand-banks-jeanne-darc-basin-no-1-hot-spot-for-canadas-remote-offshore-ed.html), one of which is the Hibernia oil platform which discharges approximately 106,900 – 127,700bbl/day (July to September 2007; Reuters, 2007).

PW released from oil and gas platforms is a complex mixture composed of dissolved hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), phenols, organic acids, metals and high salinity which can vary considerably among oil and gas fields (Neff et al., 2011). Among its constituents, PAHs are considered the most toxic to early-life stages of fish and are the main cause for the observed toxicity following exposure to oils and dispersed oils (Billiard et al., 1999; Carls et al., 1999; Heintz et al., 1999). PAHs are also the primary compounds of concern in risk assessments of PW discharges (Neff et al., 2006). Polycyclic aromatic hydrocarbon exposure during the early life stages of fish can cause blue sac disease (BSD), an assemblage of developmental abnormalities characterized by vertebral abnormalities, edemas, hemorrhaging, reduced growth and survival (Billiard et al. 1999; Brinkworth et al. 2003; Carls et al. 2008;

Boudreau et al. 2009; McIntosh et al. 2010). The concentrations of PAHs estimated in the Tampen and Ekofisk regions of the North Sea were approximately 20-100 ng/L within 1 km of the PW discharges with concentrations dropped to back-ground levels at distances of 5-10km from the discharge. In these discharges, two-ring PAHs, naphthalenes, were the most abundant class (60%) followed by other two-ring and three-ring PAHs (40%; Durell et al., 2006). Three-ring PAHs, such as phenanthrene and dibenzothiophene, have been linked to cardiac dysfunction, the precursor to BSD (Incardona et al., 2004). Blue sac disease abnormalities were observed in mummichog embryos (*Fundulus heteroclitus*) following exposure to Hibernia PW (Burridge et al., 2011; Appendix 5), indicating that PAHs, such as the three-ring PAHs, may be in sufficient quantities in Hibernia PW to induce these morphological abnormalities in embryonic fish.

Bioassays with early-life stages of Pacific herring (*Clupea pallasi*) increased in frequency following the 1989 Exxon Valdez spill (Carls *et al*., 2002). Developed to enable *in situ* field studies, a "sticky-egg" bioassay for this species was employed to assess the chronic toxicity of oil in which herring eggs adhered to glass slides to facilitate controlled exposures (Kocan *et al*., 1996). This sticky-egg bioassay proved successful with Atlantic herring (*Clupea harengus*) and was employed in our laboratory to examine the toxicity of orimulsion (Boudreau *et al*., 2009). This bioassay was also utilised in our laboratory to assess the chronic toxicity of PW, in a preliminary study with Hibernia PW in 2005.

The main objective of the present study was to build on previous work done with the Atlantic herring sticky-egg bioassay to evaluate the toxicity of PW in chronic exposures during the entire embryonic period. Also, to better assess toxicity of PW, different conditions or exposure regimes were tested during this study. More specifically, our objectives were to 1) evaluate the chronic toxicity of three PW on Atlantic herring embryonic survival, heart rates, size-at-hatch, time-to-hatch and morphological abnormalities; 2) to determine if prolonging the exposure to PW during the first week following hatch could increase the toxicity of PW; 3) to compare the sensitivity of the embryonic vs the larval stage and how the sensitivity of these individual stages compares to the sensitivity observed when exposing both stages; 4) determine if environmental factors such as temperature can affect the toxicity of PW.

Materials and Methods

Produced Water

The PW tested in the present study were obtained from two oil platforms located on the Grand Banks, the Hibernia platform from the Hibernia oil field located approximately 315 km east-southeast of St. John's NF and Terra Nova platform from the Terra Nova oil field located approximately 350 km off the coast of NF and one gas platform, Venture, from the Sable Offshore Energy Project, 10 to 40 km north of the edge of the Scotian Shelf, offshore Nova Scotia. The Sable Offshore Energy Project is located at depths of 20-80m, while the Hibernia and Terra Nova oil fields are located at depths of 80m and 90-100m respectively. Samples of raw PW were obtained from Venture on 9 July 2009 and 20 March 2012, from Terra Nova on 2 February 2011 and Hibernia on 5 March 2011. The PW was collected in acid-washed (1 M HCl) nalgene high-density polyethylene jerricans (10L) or amber glass bottles of (4L) filled with no headspace and shipped in coolers to the Centre for Offshore Oil, Gas and Energy Research (COOGER) at the Bedford Institute of Oceanography, Dartmouth, NS. Produced water from all platforms

was kept at COOGER for chemical analyses and the remaining PW was sent to the Gulf Fisheries Centre in Moncton, NB where the bioassays were performed.

Raw Produced Water Chemistry and Spectrofluorometry Analysis of Test Solution

All PW tested in this study were analysed for a suite of organic and inorganic constituents. Benzene, Toluene, Ethyl benzene, Xylenes (BTEX), alkanes, PAHs and phenols were quantified by Gas Chromatography-Mass Spectrometry (GC-MS) while all metals were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS), except mercury which was quantified by Cold Vapour Atomic Fluorescence Spectroscopy (CV-AFS). These analytical methods are summarized elsewhere (Lee *et al*., 2011).

Total PAH equivalents (TPE) in test solutions were quantified by fluorescence spectrophotometry. Water samples (50mL) taken during the bioassays were placed in hexane-acetone rinsed glass test tubes, preserved with 50 μL 6N HCl, capped, sealed with Teflon tape and refrigerated until analyzed. However, because these samples were only analysed months after collection, levels of PAHs in these samples were below detection levels. Consequently, fresh samples were mixed the day of the analysis to better reflect actual exposure concentrations. Water samples were analyzed by synchronous scan fluorescence and followed the method described in Lyons *et al*. (2011) with the following modifications. Calibration lines were calculated by producing a series of six standards with concentrations ranging from 0.5 to 5 ug/L (Hibernia and Terra Nova) or 1 to 10 ug/L (Venture 2012) based on total PAH levels in each PW measured by GC-MS at COOGER. Standards and water samples were extracted with 4 ml of hexane for Hibernia and Terra Nova and with 4 ml of 1:1 hexane: dichloromethane (1:1, v/v) for Venture. Because of high levels of particulates in the Venture PW, 1 ml of 50% HCL was also added to the samples prior to extraction. The hexane or hexane/dichloromethane extract were transferred to disposable methacrylate cuvettes (Fisher Scientific, clear sided, 4.5mL, 10mm pathlength) or Quartz Suprasil cuvettes (101-QS, Hellma Analytics, clear sided, 4.5mL, 10 mm pathlength) and analysed at an excitation from 230 to 523 nm, $\Delta\lambda$57 nm or 230 to 525 nm, $\Delta\lambda$45 nm respectively using a Varian Cary Eclipse Fluorescence Spectrofluorometer with Varian BIO Package version 1.1 software (Varian Inc., Paolo Alto, USA). The TPE limit of detection was 4 μg/L.

Toxicity comparisons of PWs, in terms of PAH levels, are primarily based on results of GC-MS. Spectrofluorometry was employed primarily to confirm expected levels of TPE in test solutions.

Fertilisation and Test Conditions

Atlantic herring were collected at various fishing wharves throughout the Maritime Provinces and brought back to the laboratory at the Gulf Fisheries Centre. Fish were packed on ice with a layer of insulation, such as paper, to avoid direct contact with ice. At the laboratory, eggs from females were

extracted by ventral pressure and placed in a Petri dish. Milt from four to six males was also extracted by ventral pressure and placed in a 38 x 27 x 5 cm Pyrex dish with 30 ppt water (Kent Sea Salts in RO-treated municipal water). Eggs from four to six females were mixed, placed on a microscope slide and spread on the slide with a dissecting needle. There were approximately 80 to 100 eggs on each slide. The egg-covered slides were added to the milt solution and left for 10 – 15 min for fertilisation to occur. The slides were then rinsed with 30 ppt water and transferred to a second Pyrex dish with 30 ppt water. At approximately 1 hour post-fertilisation, the eggs were observed by microscope to determine fertilisation success with the presence of the raised fertilisation membrane. The slides were then transferred to the treatment solutions with various concentrations (% v/v) of PW and a negative control group. The 200 ml of test solution in each 250 ml Mason jar was renewed three times per week with freshly mixed solution and jars were kept at 10°C, 30 ppt and a photoperiod of 16:8 h light:dark with three replicates per concentration. Produced water from the Venture gas platform had a high salinity of approximately 200 ppt. This high salinity was corrected in the bioassay with Venture 2009 by diluting the PW with 0 ppt water, in addition to the 30 ppt water, to obtain the different test concentration, for a final salinity of 30 ppt. For bioassays with Venture 2012, the salinity was not corrected in order to assess the toxicity of all aspects of the PW. In these bioassays, salinities ranged from ~ 30 ppt in the lower concentrations (≤ 1%) to 90 ppt in the 32% concentration. Concentrations of PW tested ranged from 0.001 to 10% for Venture 2009, from 0.01 to 10% for Terra Nova and Hibernia and 0.01 to 32% for Venture 2012. The range of concentrations was modified among the different bioassays in order to include concentrations which would provide both LC50 and median effective concentration (EC50) or effective concentration to 75% of test animals (EC75). For the first objective, to evaluate the chronic toxicity of PW to herring embryos, embryos were exposed from fertilisation until hatch in three separate bioassays with Venture 2009, Terra Nova or Hibernia. For the second objective, to determine if a longer exposure to PW during the first week following hatch could reduce the LOEC, embryos were also exposed to Venture 2009 PW throughout the embryonic period as was done in the first series of bioassay. In addition, at hatch the larvae were transferred to a different Mason jar and were exposed for one week post-hatch. At the end of the one week larval exposure survival was noted. At hatch, in the higher treatment groups (0.1 to 3.2%), the larvae were very weak and lay on the bottom of the dish. Consequently, a very low survival to one week post-hatch was expected in these groups and may not be related to a prolonged exposure to PW as larvae, but to exposure to PW as embryos. To test this hypothesis, our third objective included three groups. Group 1 in which both embryos and larvae were exposed to PW; Group 2 in which embryos were exposed and the larvae were reared in clean water; Group 3 in which embryos were reared in clean water and the larvae were exposed to PW. The PW tested in the third objective was Venture 2012. Finally, the fourth objective of this study was to test if temperature influenced the toxicity of PW by rearing the embryos exposed to Venture 2012 PW throughout the embryonic period at temperatures of 7, 10 and 15°C.

Toxicological Response

Within 72 hours of fertilisation, unfertilised eggs were removed and live embryos were reduced haphazardly to 20 per slide. All slides were checked every other day to determine mortality rates and dead embryos were removed. Heart rates were measured on day 8 PF in bioassay 1 (Venture 2009). To measure heart rates, embryo-covered slides were placed in a temperature-controlled petri dish and heart rate was measured by counting the number of beats for 10 seconds under a dissecting microscope (see below). At hatch, time-to-hatch, length-at-hatch and the presence and severity of morphological abnormalities were noted. Presence and severity was recorded for spinal curvature, pericardial edema (accumulation of fluid in the pericardial sac), and yolk-sac edema (accumulation of fluid in the vitelline vasculature). Severities were determined using the graduated severity index method (Carls *et al.,* 1999) where 1 represented slight defect, 2 represented moderate defect, and 3 represented severe defect (Boudreau *et al.,* 2009; McIntosh *et al.,* 2010). Presence or absence was recorded for jaw malformation (inability to close lower jaw) and skin lesion (rough, darkened appearance of the epithelial tissue). Observations and measurements were made with a computer-based image analysis system (uScope PixeLINK, version 3.6, PixeLINK, Ottawa, ON, Canada) linked to a microscope (Leitz, Wild Photomakroskop M400, Leica Microsystems, Willowdale, ON, Canada) (16x-90x) through a video camera (PixeLINK USB 2.0 camera, PixeLINK, Ottawa, ON, Canada).

Statistical Analyses

Replication was the jar, with three jars per treatment group. Survival-to-hatch was analyzed by LC50. To calculate LC50s, top constraints were set at the average control values for each PW and lower constraints were set at zero. Abnormal development was analysed by EC75 for the toxicity comparison and EC50 was calculated for the temperature comparison with lower constraints set at control values and top constraints for both comparisons set at 100. A four parameter nonlinear regression was employed to calculate LC50s and EC50s and an ECanything nonlinear regression was employed to calculate the EC75. F-test and non-overlapping confidence limits established significant differences among the groups (Environment Canada 2005). An LC50 for the temperature comparison could not be calculated by non-linear regression due to ambiguous fit of the data and very wide confidence intervals. Therefore, the Spearman-Kärber method was employed to calculate the LC50. All values were calculated based on nominal concentrations.

Heart rates, time-to-hatch and length-at-hatch were analyzed by a 1-factor nested ANOVA for the toxicity comparison and a 2-factor nested ANOVA for the temperature comparison (temperature and concentration as factors). A nested design was employed to remove the variability associated with replicates (nested within concentration) to test the influence of treatment only. Interactions in the 2-factor ANOVA were explored by ANOVAs analyzing each factor separately. ANOVAs were followed by Tukey multiple comparison tests. Prior to analyses, data were tested for normality (probability plot) and homoscedasticity (Fmax test).

Analyses were performed with Systat version 11.0 (SPSS, Chicago, IL, USA), GraphPad Prism version 5.04 (San Diego, CA, USA) and Trimmed Spearman-Kärber Method program version 1.5. The level of

significance was $p<0.05$. Means, LC50s, EC50s and EC75s are accompanied by their 95% confidence interval, where possible.

Results

Chemistry

Concentrations of total metals (dissolved and particulate phase) present in the PW are listed in Table 5. Amounts of BTEX, alkanes, total PAHs and phenols in the raw PW are presented in Table 6. Results of synchronous scan fluorescence showed that concentrations of TPE were proportional to serial dilutions for Hibernia ($y = 0.65x - 0.007$; $R^2 = 0.87$) and Terra Nova ($y = 0.90x + 0.0307$; $R^2 = 0.96$), Venture 2009 ($y = 0.89x + 0.600$; $R^2 = 0.98$) starting at 1% and for Venture 2012 starting at 3.2% ($y = 0.59x + 0.082$; $R^2 = 0.82$) (Figure 3). A large variability between data points within individual concentrations was observed for the lower concentrations (≤ 0.1% Hibernia and Terra Nova; ≤ 1% Venture) which were closer to the limit of detection (4 μg/L). Because of high levels of particulates in the Venture PW, modifications to the fluorescence methodology had to be made to quantify this PW. Even with these modifications, levels of TPE were highly variable, for this reason, data points corresponding to lower nominal concentrations (≤ 0.1% Venture 2009 and ≤ 1% Venture 2012) were not used to calculate the linear regression for Venture.

Table 5: Concentrations (ug/L) of total metals (dissolved and particulate phase) in the produced waters tested in the present study. Concentrations were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for all metals except mercury which was quantified by Cold Vapour Atomic Fluorescence Spectroscopy (CV-AFS). Results are from one raw PW sample for each rig.

	Venture 2009	Venture 2012	Terra Nova	Hibernia
Aluminum	100.0	210	< 50	< 50
Antimony	< 2	< 5	< 5	< 5
Arsenic	< 50	< 50	< 50	< 50
Barium	1,240,000	930,000	430	750
Beryllium	1.2	1.9	0.3	0.5
Boron	29,000	32,600	32,100	8,460
Cadmium	2.40	2.97	< 0.02	< 0.02
Calcium	21,800,000	19,600,000	1,050,000	659,000
Chromium	< 10	10	< 10	< 10
Cobalt	< 10	< 10	< 10	< 10

Copper	< 10	20	10	< 10
Iron	137,000	109,000	2,980	9,300
Lead	27	76.1	0.39	0.23
Lithium	36,000	35,100	2,680	993
Magnesium	1,380,000	1,270,000	426,000	992,000
Manganese	24,100	32,200	100	700
Mercury	0.1	0.10	0.011	0.013
Molybdenum	1.0	8	< 5	< 5
Nickel	< 20	< 20	< 20	< 20
Phosphorus	70	270	< 50	5020
Potassium	1,110,000	1,750,000	257,000	351,000
Rubidium	4400	4870	389	296
Selenium	< 50	< 50	< 50	< 50
Silicon	25,600	16,900	21,800	23,200
Silver	0.6	1.1	< 0.2	< 0.2
Sodium	49,500,000	46,500,000	15,600,000	11,600,000
Strontium	2,410,000	2,760,000	50,800	31,400
Sulfur	460	1.360	691,000	799,000
Tellurium	< 2	3	< 2	< 2
Thallium	140	165	< 2	< 2
Tin	< 0.5	0.7	< 0.5	< 0.5
Titanium	< 1	< 1	9	28
Uranium	< 0.005	< 0.005	< 0.005	< 0.005
Vanadium	< 5	14	< 5	< 5
Zinc	2,400	2,910	30	20

Table 6 Concentrations (ug/L) of alkanes, polynuclear aromatic hydrocarbons (PAH), phenols and BTEX in the produced waters tested in the present study. Concentrations were determined by Gas Chromatography-Mass Spectroscopy (GC-MS).

	Venture 2009	Venture 2012	Terra Nova	Hibernia
BTEX	12,952	2,480	8,796	5,005
Alkanes	364.3	52.2	826.1	608
Alkylated PAHs	313.2	190.8	255.5	23.1
Parent PAHs	217.1	190.5	138.2	18.4
Total PAHs	530.3	381.3	393.7	41.5
Phenols	10,527	2,315	4,042	6,068

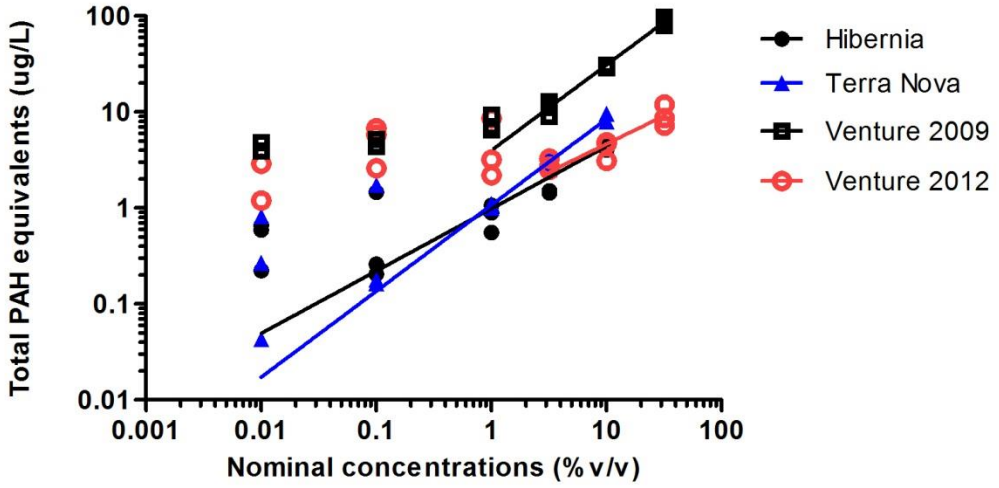

Figure 3 Total polynuclear aromatic hydrocarbons (PAHs) equivalents (ug/L) in Hibernia (y = 0.65x - 0.007; R^2 = 0.87), Terra Nova (y = 0.90x + 0.031; R^2 = 0.96), Venture 2009 (y = 0.89x + 0.600; R^2 = 0.98) and Venture 2012 (y = 0.59x + 0.082; R^2 = 0.82) produced water corresponding to nominal concentrations (% v/v) in test solutions. Data points corresponding to lower nominal concentrations, ≤ 0.1% for Venture 2009 and ≤ 1% for Venture 2012, were not used to calculate the linear regression due to interference from particulates in this produced water.

Comparative Toxicity of Produced Water - Venture 2009, Hibernia and Terra Nova

Heart rates measured in embryos exposed to Venture PW showed no clear dose-response (Tables 7 and 8). For this reason, this end-point was not measured in following bioassays. LC50s could not be calculated for Terra Nova and Hibernia because the highest concentrations tested of 10% resulted in survival rates of 83.5% (±14.5, 95% Confidence Interval) and 83.3% (±14.3) respectively while all embryos died at this concentration in the Venture bioassay. The LC50 for Venture was 3.02% (2.90-3.14%). For all three PW tested, the only treatment to significantly reduce time-to-hatch was the highest concentration in which embryos hatched (3.2% for Venture and 10% for Hibernia and Terra Nova; Tables 8 and 9). Compared to controls, time-to-hatch of embryos exposed to Venture was reduced by approximately 3 days and by approximately 1 day when exposed to either Hibernia or Terra Nova (Table 9). Size-at-hatch was reduced by all three PW. The order of toxicity for size-at-hatch was Venture which produced significantly smaller larvae at only 0.1% compared to 1% for Hibernia and to 3.2% for Terra Nova (Tables 8 and 9). Exposure to Hibernia, Terra Nova and Venture PW resulted in the same types of abnormalities (Figure 3) as are produced following exposure to oils and PAHs. The order of toxicity based on the prevalence of morphological abnormalities was Venture (EC75 = 0.26%; 0.17-0.35%), Terra Nova (0.98%; 0.46-1.51%) and Hibernia (1.25%; 0.97-1.54%) with Venture being significantly more toxic than both Terra Nova and Hibernia but no significant difference between the toxicity of Terra Nova and Hibernia (Figure 5; $F_{2,48} = 29.81$; $p<0.0001$).

Table 7 Average heart-rates of Atlantic herring embryos following chronic exposure to graded concentrations of Venture 2009 (% v/v). Arrows indicate significant differences from control group (ANOVA; Tukey test, p<0.05). N=5 embryos per jar with three jars per concentration.

	CTR	0.001	0.0032	0.01	0.1	0.32	1
Heart rates	57.6±3.7	49.6±3.7↓	55.7±2.9	73.2±3.4↑	67.4±2.6↑	65.6±3.4↑	60.8±3.9

Table 8 Statistical results of 1-factor ANOVA for size-at-hatch, time-to-hatch and heart rate for Atlantic herring embryos following chronic exposure to graded concentrations of Venture 2009, Hibernia and Terra Nova produced water and Venture 2012 at temperature at 7, 10 and 15°C. Heart rates were measured in the Venture 2009 bioassay only.

	Venture 2009	**Hibernia**	**Terra Nova**
Size-at-hatch	$F_{7,392} = 52.405$; $p<0.0001$	$F_{5,320} = 137.482$; $p<0.0001$	$F_{5,312} = 68.307$; $p<0.0001$
Time-to-hatch	$F_{7,442} = 39.624$; $p<0.0001$	$F_{5,322} = 33.854$; $p<0.0001$	$F_{5,318} = 15.831$; $p<0.0001$
Heart rate	$F_{6,81} = 30.770$; $p<0.0001$	NA[a]	NA
	7°C	**10°C**	**15°C**
Size-at-hatch	$F_{3,209} = 58.621$; $p<0.0001$	$F_{4,238} = 361.957$; $p<0.0001$	NS
Time-to-hatch	$F_{3,212} = 31.096$; $p<0.0001$	$F_{4,277} = 27.445$; $p<0.0001$	$F_{2,66} = 12.442$; $p<0.0001$

[a]NA = not available

Table 9 Average time-to-hatch and length-at-hatch (±95%CI) of Atlantic herring embryos following chronic exposure to graded concentrations of Venture 2009, Hibernia and Terra Nova produced water and to Venture 2012 produced water at 7, 10 or 15°C. Individual temperature were analysed separately. Arrows indicate significant differences from control group (ANOVA; Tukey test, p<0.05). N=3 jars per concentration. Blank cells represent concentrations that were not tested in the bioassays.

Time-to-hatch (days)

% (v/v)				Venture 2012		
	Venture 2009	**Hibernia**	**Terra Nova**	**7°C**	**10°C**	**15°C**
CTR	14.1±0.3	13.4±0.1	13.4±0.1	20.7±0.2	13.0±0.2	8.5±0.2
0.001	13.8±0.1					
0.0032	14.0±0.2					
0.01	14.2±0.3	13.6±0.1	13.6±0.2	21.1±0.2↑	13.0±0	9.1±0.4↑
0.1	14.0±0.2	13.7±0.2	13.4±0.2	20.7±0.2	13.0±0	9.9±0.8↑
0.32	14.9±0.1					
1	14.4±0.3	13.5±0.2	13.5±0.2	20.2±0.1↓	13.8±0.4↑	no hatch
3.2	11.1±0.9↓	13.1±0.2	13.2±0.2	no hatch	13.8±0.3↑	no hatch
10	no hatch	12.3±0.2↓	12.5±0.3↓	no hatch	no hatch	no hatch

Length-at-hatch (mm)

% (v/v)				Venture 2012		
	Venture 2009	**Hibernia**	**Terra Nova**	**7°C**	**10°C**	**15°C**
CTR	8.0±0.3	8.1±0.2	8.1±0.2	8.6±0.1	8.4±0.1	6.7±0.2
0.001	8.0±0.2					
0.0032	8.3±0.1					
0.01	8.4±0.1	8.2±0.1	8.1±0.2	8.7±0.1	8.6±0.1	6.4±0.7
0.1	7.5±0.2↓	8.2±0.2	8.0±0.2	8.6±0.1	8.5±0.1	6.3±1.2
0.32	6.9±0.2↓					
1	7.0±0.1↓	7.6±0.3↓	7.7±0.2	7.7±0.2↓	7.0±0.2↓	no hatch
3.2	6.0±0.4↓	6.7±0.3↓	6.8±0.3↓	no hatch	5.7±0.3↓	no hatch

10	no hatch	5.2±0.2↓	5.6±0.2↓	no hatch	no hatch	no hatch

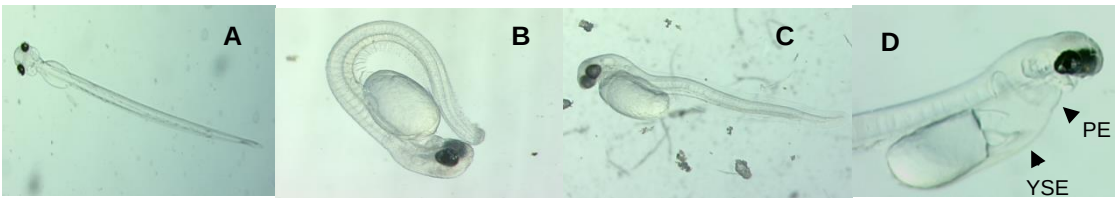

Figure 4 Most common abnormalities observed in Atlantic herring larvae following chronic exposure to graded concentrations of Hibernia, Terra Nova or Venture produced waters. Shown are a normal larva (A), severe spinal deformity (B), slight spinal deformity (C) yolk-sac edema (YSE) and pericardial edema (PE; D). Larvae are approximately 7 mm in length.

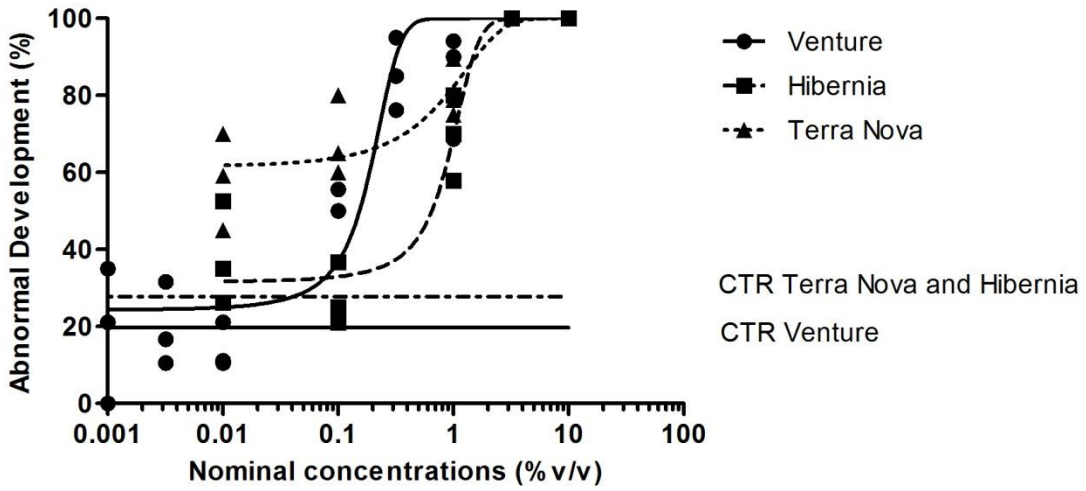

Figure 5 Percent abnormal development for Atlantic herring embryos following chronic exposure to graded concentrations of Venture 2009, Terra Nova or Hibernia produced water. Dose response curves for EC75 were calculated by an ECanything nonlinear regression. N=3 jars/concentration.

Comparative Exposure Regimes - Venture 2009 and Venture 2012

At hatch, the larvae from the Venture PW bioassay were kept and the exposure to the PW continued for one week post-hatch to determine how extending the exposure period in the larval stage could affect survival. This increased exposure resulted in a reduction of the LC50 by two orders of magnitude, 0.07% (0.05-0.10%) for larvae compared to 3.01% (2.88-3.14%) for embryos ($F_{1,38}$ = 862.5; p<0.0001). These results were not surprising because at hatch, larvae in the 0.1% to 3.2% treatment groups were very weak and lay on the bottom of the dish. Consequently, low survival to one week post-hatch could be expected and may not be related to a prolonged exposure to Venture PW as larvae but to exposure to Venture PW as embryos. To test this hypothesis, another bioassay was done which included three groups: 1) Group 1 in which both embryos and larvae were exposed to PW (as was done previously); 2) Group 2 in which embryos were exposed and then reared in clean water at hatch; 3) Group 3 in which embryos were reared in clean water and larvae were exposed to PW. When comparing larval survival at 7 days post-hatch for these three groups, group 1 in which both life stages had been exposed had a significantly lower LC50 of 0.30% (0.17-0.43) compared to 0.82% (0.67-0.96) when only the larvae were exposed, which were both significantly lower than 2.64% (1.79-3.49%) when only the embryonic stage was exposed (Figure 6; $F_{2,48}$ = 40.82; p<0.0001). Therefore, the larval stage of Atlantic herring is more sensitive to PW exposure than the embryonic stage.

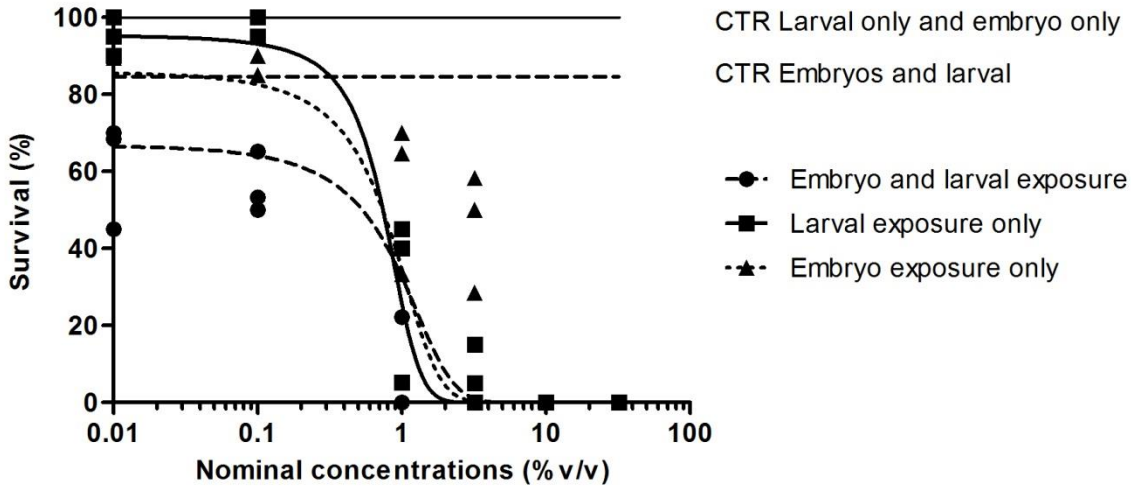

Figure 6 Percent survival for Atlantic herring embryos following chronic exposure to graded concentrations of Venture 2012 during the embryonic period, larval period (7 d post-hatch) or the embryonic and larval period. Dose response curves for LC50 were calculated by a 4-parameter nonlinear regression. N=3 jars/concentration.

Influence of Rearing Temperature on Produced Water Toxicity - Venture 2012

A comparison of the influence of water temperature on the toxicity of PW could not be done for the survival of herring embryos by non-linear regression due to ambiguous fit of the data and very wide confidence intervals. Therefore, the Spearman-Karber method was utilised to calculate an LC50 of 1.72% (1.57-1.88%) at 7°C and 2.93% (2.49-3.45%) at 10°C. Although the Spearman-Karber method does not statistically compare the LC50 values among different groups, the non-overlapping confidence intervals suggest that these values are significantly different (EC, 2005). The LC50 at 15°C could not be calculated because survival rates in the lowest concentration tested were below 50%, therefore the LC50 at 15°C would be < 0.01%, the lowest concentration tested. As expected, an increase in rearing temperature reduced time-to-hatch. Control embryos reared at 15°C took 8.5 (±0.2) days to hatch, compared to 13.0 (±0.2) days for embryos reared at 10°C and 20.7 (±0.2) days for 7°C. When embryos were reared at 15°C, there was a dose-dependent increase in time-to-hatch starting at the lowest concentration tested (Tables 8 and 9). At 10°C there was an increase in time-to-hatch in the two highest concentrations in which embryos hatched. At 7°C, there was an increase at the lowest concentration and a decrease at the highest concentration. For length-at-hatch, embryos exposed to PW at both the 7 and 10°C rearing temperatures were significantly shorter at hatch starting at 1% (Tables 8 and 9). There was no significant reduction in length when embryos were reared at 15°C since embryos only hatch in the two lower concentrations (0.01 and 0.1%). Interestingly, by grouping the control group and lower test concentrations (0.01 and 0.1%), for which there were no significant difference among these groups when testing each temperature separately, there was a significant difference in length-at-hatch between the 15°C and both the 7 and 10°C. The highest rearing temperature produced larvae that were approximately 2mm or app 23% smaller than the 7 and 10°C groups [6.6 ± 0.2 mm (15°C) compared to 8.5 ± 0 mm (10°C) and 8.6 ± 0.1 mm (7°C); $F_{2, 414} = 480.648$, $p<0.0001$]. The EC50 for normal development was significantly lower when embryos were reared at 15°C (0.05%, -0.08-0.17%) compared to 10°C (0.93%, 0.74-1.11%) and 7°C (1.38%, 0.48-2.28%; Figure 7; $F_{2, 47} = 16.34$, $p<0.0001$).

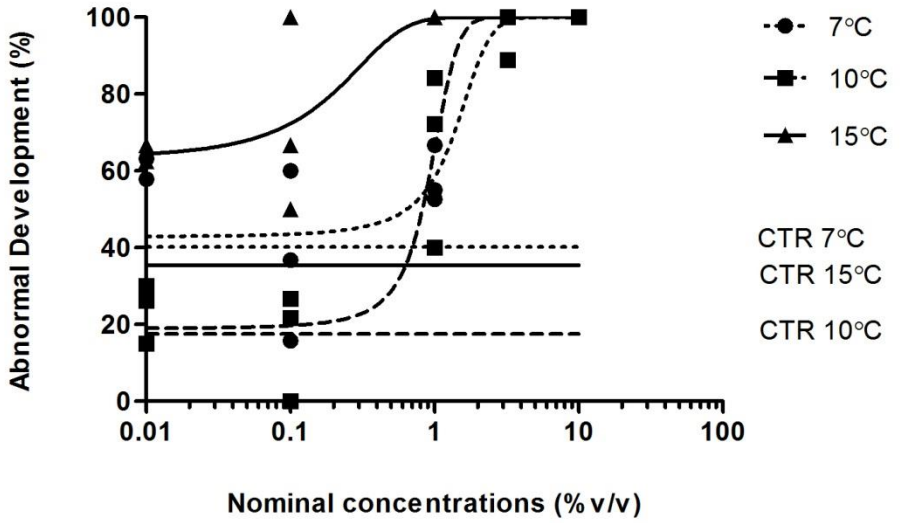

Figure 7 Percent abnormal development for Atlantic herring embryos following chronic exposure to graded concentrations of Venture 2012 produced water at 7, 10 and 15°C. Dose response curves for EC50 were calculated by a 4-parameter nonlinear regression. N=3 jars/concentration.

Discussion

Comparative Chemistry of Produced Waters

Because the Venture platform is a gas production well, while Hibernia and Terra Nova are oil fields, the chemical composition of produced water between the two fields showed some significant differences. For metals, the concentration of sulphur in Hibernia and Terra Nova PW was 2-3 orders of magnitude greater than Venture (Table 5). High concentrations of sulphur could indicate the presence of hydrogen sulphide, a known toxicant in produced waters (Sauer *et al*., 1992). Conversely, Venture had concentrations of barium, iron, lead, manganese, strontium and zinc that were 2-4 orders of magnitude greater than Hibernia and Terra Nova. Despite these high concentrations, dissolved metals likely play a minor role in the overall toxicity of produced water in the natural environment (Neff, 2002). Dissolved metals in anoxic produced water will precipitate on contact with oxygenated seawater (Stephenson *et al*., 1994), which then may settle on bottom sediments in the near-field zone around offshore oil and gas installations (Azetsu-Scott *et al*., 2007).

Compared to metals, the concentrations of organic constituents in the four PW varied by an order of magnitude or less (Table 6). Concentrations of BTEX (Benzene, Tolueue, Ethyl benzene, Xylenes) ranged between 5000 – 12,950 ug/L. Despite these high concentrations, the high volatility of BTEX means that the exposure time would have been limited during this study. Lee *et al*. (2011) reported that aeration of produced water (to simulate mixing with oxygenated seawater) resulted in an almost complete loss of BTEX after 25 hours. In addition, other studies have shown that the loss of BTEX does not significantly change the toxicity of produced water to marine organisms (Flynn *et al*., 1996). Polycyclic aromatic hydrocarbons are the petroleum hydrocarbons of greatest environmental concern in produced water

because of their toxicity and persistence in the environment. Concentrations of PAHs were very similar between the four PW tested, with total PAH (parent + alkylated) concentrations ranging between 41.5 – 530.3 ug/L. The PAH profile in all four produced water samples consisted predominately of low molecular weight compounds (≤3 benzene rings), with 95-99% of the detected PAH compounds falling into this class which include compounds such as naphthalene, fluorene, dibenzothiophene and phenanthrene. High molecular weight PAH with ≥4 benzene rings were detected in the Venture, Hibernia and Terra Nova produced waters with total concentrations up to 30 ug/L. A wide range of phenolic compounds were also detected, which varied considerably between the Venture 2009 and Venture 2012 PW (10,527 vs 2315 ug/L) and similar for the oil production wells (4042 and 6068 ug/L for Terra Nova and Hibernia, respectively). Phenolic compounds, especially when present at high concentrations, may play a significant role in the toxicity of produced water (Neff, 2002; Flynn *et al*., 2002). Straight chain saturated hydrocarbons (alkanes) were also detected between 52.2 and 826.1 ug/L in the four produced waters. Alkanes are generally the result of small dispersed oil droplets in the produced water (Neff, 2002), and are of minimal concern with regard to toxicity to marine organisms.

Comparative Toxicity - Venture 2009, Hibernia and Terra Nova

Of the three PW included in the toxicity comparison of the present study, three of the endpoints analysed, time-to-hatch, survival and morphological development, indicated greater toxicity of Venture 2009 PW, while Terra Nova and Hibernia had similar toxicities. The only end-point in disagreement with this ranking was size-at-hatch which found Hibernia to be more toxic than Terra Nova. However, this difference was due to Terra Nova exposed larvae being only 0.1 mm longer than Hibernia exposed larvae (1% v/v treatment group), a statistically significant difference which may not be ecologically significant. The ranking of the PW toxicity based on the endpoints of the herring early life bioassay correspond well to levels of PAHs in the Venture PW but not those in Hibernia and Terra Nova PWs as levels of PAHs were one order of magnitude lower in the Hibernia PW. In comparison, phenol levels may appear to be a better indicator of the order of toxicity between these PWs since phenols levels were rather similar for Terra Nova and Hibernia PW and approximately 40 -60% more elevated in Venture PW (Table 6). The better agreement between toxicity responses and phenol levels can be observed when comparing the results of the two Venture bioassay, Venture 2009 and Venture 2012, which showed quite different LC50 of 0.07% (Venture 2009) and 0.30% (Venture 2012) when both the embryonic and larval stages were exposed. The LC50 for Venture 2009 was 4.3 times lower than for Venture 2012, which corresponds better to levels of phenols which were 4.5 times lower for Venture 2012 than Venture 2009 compared to PAH levels which were 7 times lower for Venture 2012 than Venture 2009 (Table 6).

The morphological abnormalities observed in the present study following exposure to all four PW tested were the same types of abnormalities as have been observed following exposure to oils, dispersed oils or to individual PAHs (Billiard *et al*., 1999; Carls *et al*., 1999; Incardona *et al*., 2004; Boudreau *et al*., 2009). The most common abnormalities in the present study were yolk-sac edema, vertebral abnormalities, skin lesion, malformation of the fins and some jaw abnormalities. This assemblage of abnormalities, BSD, has been linked to PAH exposure, however, various mechanisms of toxicity have been proposed for PAHs because not all PAHs exert their toxicity in the same manner (Billiard *et al*.,

2008). Consequently, BSD is not always induced following to exposure to PAHs, but is believed to be the results of exposure to three-ring PAHs, such as dibenzothiophene and phenanthrene (Incardona *et al*., 2004). These compounds have a direct effect on cardiac conduction which then disrupts the normal development of the heart, the kidneys and the neural tube and results in morphological abnormalities in the embryo (Incardona *et al*., 2004). In two regions of the North Sea, 2/3-ring PAHs (excluding naphthalenes) represented 40% of PAHs present in PW (Durell *et al*., 2006). In the PW tested in the present study, naphthalenes represented ~65-80% of PAHs, while 3-ring PAHs represented ~15-35% of total PAHs.

Phenols may also be linked to the observed toxicity in the present study. Phenol levels measured in whole PW varied from 2315 (Venture 2012) to 10,527 ug/L (Venture 2009; Table 6). Therefore, concentrations of phenols at the LC50 concentration for Venture 2009 (3.02%) would include total phenol concentrations in the range of 300 ug/L. A study by Birge *et al*. (1979) found that the LC50 to hatch of phenol for various fish species ranged from 300 to 3340 ug/L. Pacific herring are quite sensitive to chemical insult (Rice *et al*., 1979) and recent work in our laboratory has found Atlantic herring to be more sensitive than Pacific herring (Johnson, 2013). Therefore, Atlantic herring could quite conceivably have an LC50 in the lower portion of the range identified by Birge *et al*. (1979). The Birge *et al*. (1979) study also tested a higher range of concentrations, approximately 8000 to 10,000 ug/L, which resulted in 70 to 100% abnormal larvae. These abnormalities, which are similar to those observed in the present study, included vertebral abnormalities and pericardial edema (Birge *et al*., 1979). However, the concentrations of phenol required to produce these abnormalities (range of 8000-10,000 ng/mL) were much higher than those tested in the present study (Table 6). Therefore, the increased lethality to herring embryos following exposure to PW may be related to phenol exposure as well as PAHs but the morphological abnormalities are most likely caused by the exposure to PAHs.

Comparative Exposure Regimes - Venture 2009 and Venture 2012

Early life stages of fish are recognised to be among the most sensitive to chemical insult (von Westernhagen, 1988; Weis and Weis, 1989). In the present study we tested if a prolonged exposure to PW during this critical stage would increase the toxicity of PW. Two assays were included to test this hypothesis and demonstrated that larvae (to 7 d post-hatch) were more sensitive than embryos and exposure during both life stages significantly reduced the LC50 compared to the LC50s of individual life stages. McIntosh *et al*. (2010) also observed increased toxicity with duration and concentration of dispersed weathered crude oil in the early life stages of Atlantic herring. The McIntosh study exposed herring at various stages during early life for exposure periods ranging from 1 to 144 h. The most sensitive stages identified were the gametes and yolk-sac larvae for short exposures of less than 24h but when exposure periods were more than 24h, young embryos were the more sensitive stage. The contradictory finding between the present study and the McIntosh study may be due to the longer exposure period in the present study. In the environment, the three exposure regimes tested in the present study, embryonic exposure, larval exposure and both the embryonic and larval exposure, could conceivably be observed. Herring embryos are demersal and deposited on rocky substrate or marine

vegetation (Blaxter 1985; Alderdice and Hourston, 1985) to limit movement. Therefore, if eggs are deposited within the vicinity of PW discharges, exposure could be more or less constant throughout the embryonic period. Once hatched, the distribution of herring larvae is strongly influenced by wind-induced turbulence (Snauffer, 2013). Therefore, they may stay in the vicinity of the plume (embryonic and larval exposure) or be driven out of the area (embryonic exposure only). This being said, the embryos could also hatch in an undisturbed area and get swept into the plume or patch of PW, exposing the larval stage only. However, to produce the effects describes in this study, exposure to PW was constant whereas concentrations in the environment would be much more variable depending on factors such as winds and currents, which would influence the dispersion of PW. The lower range of concentrations to induce negative impacts in the present study, for example the EC50 of 0.05% in the temperature bioassay for 15°C, would correspond to measured concentrations of approximately 0.2 ug/L total PAH (Table 6). Durell *et al*. (2006) estimated total PAH concentrations of 0.02 to 0.1 ug/L within 1 km of oil and gas platforms based on measured and modeled values for two regions of the North Sea. Therefore, based on concentrations established by Durell *et al*., the concentrations necessary to induce toxicity in the present study would be above those observed in the environment or may only be found in very close proximity to the PW discharge. Hence, even if the exposure regimes tested in the present study may occur in the environment, concentrations of PW may be too low to induce toxicity in the early life stages of fish.

Influence of Rearing Temperature on Produced Water Toxicity – Venture 2012

Results of the present study suggest greater toxicity of PW at warmer temperatures as herring embryos reared at 15°C only survived in the two lowest test concentrations. Different factors may be responsible for the influence of temperature on PW toxicity, but many would indicate that lower temperatures, and not warmer temperatures, would increase the toxicity. For example, embryos reared at warmer temperatures have shorter embryonic period which reduces the exposure period to the toxicant, and therefore could reduce its toxicity. In the present study, herring reared at 15°C took approximately 8.5 d to hatch compared to 20.7 d when reared at 7°C. Embryos reared in the 15°C group not only grew and developed faster than those at 7 and 10°C, but these embryos also grew less because they were approximately 23% smaller at hatch. Therefore, these embryos seemed to hatch at an earlier developmental stage than those reared in colder temperatures. This smaller size-at-hatch may render these larvae more susceptible to PW exposure during the larval stage which we identified as being more sensitive to PW exposure than the embryonic stage. However, exposure to PW during the temperature bioassay did not include the larval stage; therefore the smaller size-at-hatch should not be contributing to the increased toxicity of PW at warmer temperatures. Warmer water temperatures can also increase the biological degradation of PAHs, hence, reducing the exposure to toxic compounds. However, in laboratory experiments in which the test solutions are renewed every two days, the biodegradation levels may not be as influential on toxicity as would be observed in the environment. In contrast, reduced detoxifying enzyme activity, such as ethoxyresorufin-O-deethylase (EROD), in embryos reared at lower temperatures could increase the toxicity of PAHs (Lyons *et al*., 2011). In the present study, although we acknowledge that the factors mentioned previously could explain the influence of

temperature on the toxicity of PW, we hypothesize that the main factor responsible for the difference in toxicity of PW for herring embryos reared at various temperatures is the stock of herring used in the bioassay. Atlantic herring have two spawning stocks, the spring and the fall stocks (Haegele and Schweigert, 1985). The spring stock spawns from April to June at temperatures of approximately 5°C, while the fall stock spawns from August to November at warmer temperatures of 10-15°C (Haegele and Schweigert 1985; Steward and Arnold 1994). We postulate that the adaptation of these stocks to their respective spawning temperatures mediates which rearing temperature will increase the toxicity of PW because of thermal stress. In the present study, we utilised herring from the spring stock which spawn at lower temperatures and we observed greater toxicity at the highest temperature tested of 15°C. In a previous study, we exposed herring from the fall stock, which spawn at higher temperatures, to dispersed oil at the same range of temperatures tested in the present study and found the lower temperature tested of 7°C resulted in greater toxicity (Johnson, 2013). When oil is chemically dispersed to create a chemically-enhanced water accommodated fraction (CEWAF), water temperature can influence the solubility of the PAHs as the oil is initially mixed with the water and dispersant to create the CEWAF, due to increased PAH solubility and/or dispersant effectiveness (Whitehouse 1984; Chandrasekar *et al*., 2005). In the Johnson study, there were 2.2 X more PAHs in the CEWAF stocks at 15°C, although this was lower in the actual test solution, than at 7°C, but toxicity was still greater at 7°C (Johnson, 2013).

Conclusions

The Atlantic herring embryonic bioassay proved to be an effective tool in assessing the toxicity of PW. The ranking of toxicity based on the response of the endpoints measured in the bioassays corresponded to the chemical composition of the PW, especially for phenols. Based on the response of endpoints in the bioassays and phenol levels of the PW, Venture 2009 PW was the most toxic of the three PW included in the toxicity comparison, with Hibernia and Terra Nova having similar toxicities. The herring bioassay can also provide valuable information regarding the relative sensitivity of early life stages. In the present study, the larval stage was identified as the more sensitive stage to PW exposure. The herring bioassay can also provide information regarding the influence of environmental factors on PW toxicity. Our results showed greater toxicity of PW when embryos were reared in warmer temperatures, which is likely caused by the natural adaptation of the spring spawning stock, utilised in this study, to colder rearing temperatures. Because of high dispersion rates of PW in the marine environment, the toxicity observed in the present study following chronic exposure to PW would not likely occur in the environment or would occur only within very close range to the PW discharge.

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APPENDIX 1: Manuscript in preparation (preliminary data).

The effect of chronic exposure to produced water on growth and food consumption of juvenile Atlantic cod (Gadus morhua)

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Abstract
The discharge of produced water (PW) from offshore oil and gas facilities remains an environmental concern due to uncertainty regarding its fate, transport and potential biological effects. Rapid dilution of the oil-related discharge containing poly-aromatic hydrocarbons, phenols, alkylphenols and heavy metals occurs. Marine fish may be chronically exposed to diluted PW at distances far from oil and gas production sites. Juvenile Atlantic cod were exposed intermittently to 0, 100 or 200 ppm of PW from either Sea Rose floating production, storage and offloading vessel (FPSO), Terra Nova, Thebaud or Venture platforms for 14 weeks. Three times weekly the fish were fed and then exposed to PW. Growth and food consumption were monitored. At the end of the exposure significant effects on specific growth rate (SGR) were seen in fish exposed to 200 ppm Sea Rose PW ($P<0.05$) and 100 ppm Thebaud PW ($P< 0.005$). Effects on daily food consumption (FC) were significant ($P< 0.005$) for 100 and 200 ppm Sea Rose PW, 100 and 200 ppm Thebaud PW and 100 ppm Venture PW treated fish at the end of the exposure. The results suggest that chronic exposure to environmentally relevant concentrations of PW from some oil and gas platforms may pose a risk.

Introduction
Produced water (formation and injected water containing production chemicals) represent the largest volume waste stream in oil and gas production operations on most offshore platforms. There is considerable concern about the ocean disposal of produced water, because of the potential danger of chronic ecological harm (Neff *et al*, 2011). With anticipated increases in the number of new offshore platforms, PW discharge has been identified as an issue of concern by both regulators and environmental groups (Zhao *et al*. 2008). The chemical characteristics of PW are different for each production platform or formation from which the oil is extracted. It is typically highly saline and contains elevated levels of heavy metals, hydrocarbons (including polycyclic aromatic hydrocarbons (PAHs)), alkylphenols, ammonia and radionuclides compared to the receiving environment (Lee *et al*., 2005; Burridge *et al*., 2011). The physical and chemical properties of produced water vary widely depending on the geologic age, depth, and geochemistry of the hydrocarbon-bearing formation, as well as the chemical composition of the oil and gas phases in the reservoir, and process chemicals added during production. Because no two produced waters are alike, region specific studies are needed to address the environmental risks from its discharge (Neff *et al*., 2011).

Although the acute toxicity of PW discharges into the environment for marine organisms is probably a threat only within the direct zone of the discharge, the effects of chronic exposure to PW on the biology and/or physiology of organisms are mostly unknown (Pérez-Casanova *et al*., 2010). Continual chronic

exposure may cause sub-lethal changes in populations and communities, including decreased community and genetic diversity, lower reproductive success, decreased growth and fecundity, respiratory problems, behavioral and physiological disorders, decreased developmental success and endocrine disruption (Neff *et al*., 2011).

Fish respond to stressors by eliciting a generalized physiological stress response, which is characterized by an increase in stress hormones and consequent changes that help maintain the animal's normal or homeostatic state. These physiological alterations are grouped as primary responses, which include hormonal changes and secondary responses, which include changes in metabolites, blood ions and hematology (Barton, 2002; Iwama *et al*., 2004). The tertiary response represents whole-animal and population level changes associated with stress. Exposure to stressors, depending on the intensity and duration can lead to decreases in growth, disease resistance, reproductive success, swimming performance and other characteristics of the whole animal or population (Iwama *et al*., 2004).

Food intake and nutritional status of fish can be assessed by using body morphometrics and daily food consumption measurement. Change in weight (mass) is the most commonly used assessment for growth performance. When growth rate is exponential, as it usually is over intervals of a year or less, growth can be expressed as an instantaneous growth rate or as a percentage of instantaneous growth called specific growth rate (Busacker *et al*., 1990).

Atlantic cod (*Gadus morhua*) is a species of considerable ecological and economic importance for Canada. Examining the chronic effects of PW on cod growth and feeding will shed light on the potential long-term impact of oil and gas offshore developments on stocks (Hamoutene *et al*., 2011). Metabolic capacities, feeding and digestive physiology of fish are influenced by environmental parameters. Chronic exposure to PW might affect feeding, which in turn might have consequences on growth/health status of fish populations (Hamoutene *et al*., 2011).

Atlantic cod eggs are pelagic and buoyant. When larvae hatch, they occur from near-surface to depths of 75 m, and they move deeper with growth. Transformation to the juvenile stage occurs at sizes greater than 20 mm and descent from the water column to bottom habitats occurs at sizes of 2.5 - 6 cm. By day, the young juvenile cod remain on the bottom, but at night they rise several meters into the water column and drift in the tidal current while feeding. Early juvenile cod consume more pelagic than benthic invertebrates and medium cod consume more benthic invertebrates and fish (Lough, 2004).

The areas of the northwest Atlantic that have the highest abundance of Atlantic cod are in Canadian waters and include the eastern coast of Labrador south of Cape Harrison, off eastern Newfoundland, the Flemish Cap, the Grand Bank, the Gulf of St. Lawrence, and the Scotian Shelf (Lough, 2004). All four of the PWs used in this study are discharged into Canadian waters where Atlantic cod may be exposed.

In this study we chronically exposed juvenile Atlantic cod to two environmentally relevant concentrations of PW and assessed potential effects on growth parameters and food consumption. Intermittent exposure to PW concentrations of 100 ppm (10 000X dilution) and 200 ppm (5000X dilution) was used in an attempt to mimic the rapid dilution taking place after release in the environment. Rapid dilution of PW by at least 240X has been shown to occur within 50-100 m, 1000X by 4-5 km and up to 9000X at 20 km from the discharge site (Somerville *et al*., 1987; Murray-Smith *et al*., 1996; Pérez-Casanova *et al*., 2010). Factors that affect the rate of dilution of PW include discharge rate and height above or below the sea surface, ambient current speed, turbulent mixing regime, water column stratification, water depth, and differences in density and chemical composition between the

PW and ambient seawater (Neff *et al*., 2011; Reed and Rye, 2011; Niu *et al*., 2011).

Materials and Methods

Produced water

Two of the PWs used in this study were from the gas platforms, Venture and Thebaud. These platforms are off the coast of Nova Scotia, Canada, in the Scotian Shelf region of the Atlantic Ocean (Fig. 1). Water depths at Thebaud and Venture platforms are 29 m and 22 m respectively. Venture platform does preliminary dehydration of the natural gas from its wells and then transports it to Thebaud for further processing. Thebaud platform is the hub of the Sable Offshore Energy Project's activity. Preliminary processing of the gas from the Thebaud wells, as well as gas from the existing satellite platforms – North Triumph and Venture is done at Thebaud and then sent via pipeline to the Goldboro Plant in Guysborough County, Nova Scotia (Sable offshore energy project, 2012). The other two PWs studied were from Sea Rose and Terra Nova oil fields (Fig. 1). SeaRose is a floating production storage and offloading vessel (FPSO) located in the White Rose oil and gas field, off the coast of Newfoundland, Canada on the eastern edge of the Grand Banks in the North Atlantic Ocean. The water depth at the southern part of the White Rose area ranges from 115 to 130 m (Canada-Newfoundland and Labrador Offshore Petroleum Board, 2000). Terra Nova is a FPSO located south of White Rose. Water depths at Terra Nova are between 90 to 100 m (Offshore Technology, 2012).

Acid-washed (1 M HCl) nalgene high-density polyethylene jerricans were provided to platform staff for the collection of PW. Instructions for collection were to fill the jerricans to overflowing to eliminate any headspace. Collection of PW was coordinated so that the samples were returned to scientific staff as soon as logistically possible. The containers were sealed with electrical tape, refrigerated and transported to the Bedford Institute of Oceanography, Dartmouth, Nova Scotia and then on to St. Andrews Biological Station, St. Andrews, New Brunswick where they were refrigerated at 4°C for the duration of the study.

Fish stocks

The juvenile Atlantic cod (Gadus morhua) used in the 2010 experiment arose from research projects at the St. Andrews Biological Station (SABS), St. Andrews, New Brunswick, Canada. Juvenile cod were obtained in 2011 and 2012 from Great Bay Aquaculture, Portsmouth, New Hampshire, USA. The fish were held in flow through filtered sea water at ambient temperature and under simulated natural photoperiod for July and August. Dissolved oxygen and water temperature were recorded daily. The fish were hand fed once daily with Gemma starter feed and then Europa, 2.0 mm feed from Skretting North America (Bayside, New Brunswick, Canada). Groups of forty juvenile cod (9.1 ± 3.9 g) in 2010, (12.7 ± 2.4 g) in 2011 and (13.9 ± 2.2 g) in 2012 were anaesthetized with tricaine methanesulfonate (MS222; Syndel Laboratories Ltd, Vancouver, BC, Canada), tagged with Passive Integrated Transponders (PIT) and placed in 400 L fibreglass tanks with flow through filtered seawater. Fish length and weight were recorded. The fish were allowed to recover in ambient seawater for a few weeks before chronic exposures were started.

Fish exposure

At the end of the acclimation period, tanks were assigned to treatments: control (0 ppm), intermediate dose (100 ppm) and high dose (200 ppm). Three times a week the fish in each tank were slowly hand fed a preweighed amount of food (Specific feeding rate, SFR = 3% to 4%). On the same days, for fourteen weeks, the 100 ppm (10 000X dilution) and the 200 ppm (5000X dilution) groups were exposed to one pulse of PW from either Sea Rose, Terra Nova, Thebaud or Venture platforms. After four hours the standpipes were briefly lifted in each tank and the uneaten food was collected and allowed to

air dry for five days before weighing. The fish were held in 250 L of seawater and the water flow was maintained at 2.5 L min-1 so that the initial pulse dose was gradually diluted over a period of 8 h. Ambient water temperatures during the exposures were 7 to 15°C.

Sampling

At five, ten and fourteen weeks, five fish per tank were sacrificed. Fish were stunned with a sharp blow to the head and blood was collected from the caudal vein with a heparinized disposable syringe and centrifuged at 3500 x g for 10 minutes at 4°C. Plasma was aliquoted and stored at -80°C. Liver and brain tissue were taken and stored for biochemical analyses. Tissues were flash frozen in liquid nitrogen and stored at
-80°C. Length, weight, liver weight, blood volume and sex were recorded for each fish. Remaining fish were anaesthetized and length and weight were recorded. The specific growth rates of each fish based on weight (SGR) were calculated for the three time periods: PIT tag date to five week sampling date, five to ten week sampling date and ten to fourteen week sampling date. The SGRs were calculated using the following formula:
SGR= ((logeY2 - logeY1)/ (t2 - t1)) * 100 where Y2 is the weight at sampling, Y1 is the weight at the previous sampling , t2 is the Julian day at sampling and t1 is the Julian day at the previous sampling.
Food consumption (FC) was calculated as: FC (g•day-1) = g food into tank – g uneaten food.

Water analysis

PW collected from Sea Rose, Terra Nova, Thebaud and Venture platforms was analysed at the Bedford Institute of Oceanography, Dartmouth, Nova Scotia. All produced water samples used in this study were analysed for a suite of organic and inorganic constituents, and the analytical methods used for analysis are summarized elsewhere (Lee *et al*., 2011).

Statistical analysis

Specific growth rates based on weight (SGR) were calculated prior to statistical analysis. All data were analyzed for homogeneity of variance using Levene's test. Dunnett's post hoc comparison test was used to compare the SGRs of the control fish with those of treated fish if equal variances were assumed and Dunnett T3 test was used if equal variances were not assumed. The data sets used for daily food consumption analysis are described in Table 1. The data sets used for the SGR analysis are described in Table 6. SAS version 19.0 (SAS Institute Inc., Cary, NC, USA) was used to conduct all of the statistical analyses.

Results

Comparisons of mean food consumption of control and treated juvenile cod showed that food consumption was affected for 100 and 200 ppm Sea Rose PW, 100 and 200 ppm Thebaud PW and 100 ppm Venture PW treated fish in the final third of the exposure period (weeks 10 to 14) (Fig. 2a, 2b; Table 1) (Dunnett's $P<0.005$). Although the daily food consumption of cod exposed to 200 ppm Venture PW (14.01 g ± 0.27, n = 11) was less than food consumption of control fish (14.77 g ± 0.31, n = 11), the difference was not significant (Dunnett's). Terra Nova PW had no effect on food consumption of juvenile cod when compared to control fish. There were no significant differences in food consumption between control and treated fish in the first 10 weeks of the study (Table 1).

Comparison of the mean SGRs of control and treated juvenile cod showed that mean SGRs of treated fish were significantly different from controls in the final third ofthe exposure period (weeks 10 to 14) for two treatments -100 ppm Thebaud PW (Dunnett's $P<0.005$) and 200 ppm Sea Rose PW (Dunnett's

P<0.05) (Fig. 3; Table 2). There were no significant differences between mean SGR of treated fish and control fish in the first ten weeks of the study.

There was some loss of fish during the study. Juvenile cod are known to be cannibalistic and we saw evidence of this in the gut contents of some fish when sampling in 2010. Greater care was taken in 2011 and 2012 to ensure fish were graded for size at the beginning of the study so as to minimalize cannibalism. Mortality throughout the study was low and unrelated to treatment. Percent mortality ranged between 0% and 5%.

A summary of the PW organic components is presented in Figure 4. Inorganic components are listed in Table 3 with the concentrations of total metals (dissolved + particulate phase) in the produced waters. The concentration of sulphur in Sea Rose and Terra Nova produced water was 3 orders of magnitude greater than Venture and Thebaud. Conversely, both Venture and Thebaud had concentrations of barium, iron, lead, manganese, strontium and zinc that were 2-3 orders of magnitude greater than Sea Rose and Terra Nova. Compared to metals, the concentrations of organic constituents in the four produced waters varied by an order of magnitude or less (Figure 4). Concentrations of BTEX (Benzene, Toluene, Ethyl benzene, Xylenes) ranged between 8.8 – 15.4 mg/L (Figure 4). Concentrations of PAHs were very similar between the four produced water samples, with total PAH (parent + alkylated) concentrations ranging between 0.39 – 0.88 mg/L (Figure 4, Table 4). High molecular weight PAH with ≥4 benzene rings compounds were detected in the Venture, Sea Rose and Terra Nova produced waters with total concentrations up to 0.03 mg/L, while they were not detected in the Thebaud sample (Table 4). A wide range of phenolic compounds were also detected (Table 5), with total phenol concentrations higher in the gas production well produced waters (10.6 – 16.5 mg/L for Venture and Thebaud, respectively) versus the oil production wells (3.5 – 4.0 for Sea Rose and Terra Nova, respectively) (Figure 4, Table 5). Straight chain saturated hydrocarbons (alkanes) were also detected between 0.32 and 1.69 mg/L in the four produced waters (Figure 4).

Table 1. Mean daily food consumption (g) ± SE of Atlantic cod. Asterisks indicate significant differences from control mean daily food consumption (* P < 0.05, **P < 0.005) (Dunnett's test).

Treatment		**Mean Daily Food Consumption (g)**	
2010	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	10.92±0.22 (n=16)	15.81±0.15 (n=15)	14.77±0.31 (n=11)
Venture 100 ppm	10.37±0.38 (n=15)	15.57±0.20 (n=15)	12.71±0.25 ** (n=11)
Venture 200 ppm	10.47±0.42 (n=16)	15.62±0.15 (n=16)	14.01±0.27 (n=11)
2011	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	14.28±0.47 (n=15)	15.96±0.44 (n=12)	16.22±0.40 (n=15)
Sea Rose 100 ppm	13.34±0.71 (n=15)	14.60±0.52 (n=12)	12.90±0.57 ** (n=15)
Sea Rose 200 ppm	14.85±0.49 (n=15)	15.61±0.47 (n=12)	13.12±0.67 ** (n=15)
Thebaud 100 ppm	14.05±0.57 (n=14)	14.98±0.47 (n=12)	11.93±0.53 ** (n=15)
Thebaud 200 ppm	12.52±0.80 (n=14)	14.59±0.54 (n=12)	13.13±0.70 ** (n=15)
2012	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	27.40±2.00 (n=15)	33.35±1.07 (n=15)	32.79±0.69 (n=13)
Terra Nova 100 ppm	28.35±2.14 (n=15)	35.20±0.91 (n=14)	33.95±0.60 (n=13)
Terra Nova 200 ppm	27.42±1.98 (n=15)	32.17±0.94 (n=15)	33.29±0.74 (n=13)

Table 2. Mean specific growth rate ± SE of Atlantic cod. Asterisks indicate significant differences from control mean SGR (* P < 0.05, **P < 0.005) (Dunnett's test).

Treatment		**Mean Specific Growth Rate**	
2010	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	1.285±0.050 (n=68)	0.971±0.054 (n=55)	0.566±0.035 (n=41)
Venture 100 ppm	1.235±0.080 (n=36)	1.041±0.058 (n=26)	0.560±0.054 (n=17)
Venture 200 ppm	1.169±0.092 (n=26)	0.932±0.081 (n=29)	0.485±0.042 (n=23)
2011	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	0.906±0.057 (n=79)	0.912±0.042 (n=69)	0.809 ± 0.035 (n=56)
Sea Rose 100 ppm	0.744±0.078 (n=39)	1.055±0.068 (n=30)	0.833±0.058 (n=23)
Sea Rose 200 ppm	0.921±0.055 (n=38)	0.848±0.051 (n=34)	0.612 ± 0.051* (n=28)
Thebaud 100 ppm	0.758±0.086 (n=40)	0.949±0.057 (n=32)	0.550 ± 0.074** (n=24)
Thebaud 200 ppm	0.881±0.060 (n=37)	0.962±0.055 (n=34)	0.804±0.053 (n=27)
2012	**Weeks 1 to 5**	**Weeks 5 to 10**	**Weeks 10 to 14**
Control	1.352±0.050 (n=76)	0.922±0.043 (n=71)	0.884±0.044 (n=61)
Terra Nova 100 ppm	1.246±0.070 (n=40)	0.967±0.057 (n=39)	0.904±0.060 (n=34)
Terra Nova 200 ppm	1.384±0.078 (n=40)	0.902±0.070 (n=38)	0.968±0.060 (n=33)

Table 3. Summary of metals in produced water.

Parameter	**Venture 09-Jul-09**	**Thebaud23-Jan-11**	**Terra Nova 02-Feb-11**	**Sea Rose 19-Jun-11**
Concentration (µg·L-1)				
Aluminum	100.0	180.0	< 50	< 50
Antimony	< 2	< 5	< 5	< 5
Arsenic	< 50	< 50	< 50	< 50
Barium	1240000	774000	430	860
Beryllium	1.2	1	0.3	0.3
Boron	29000	13300	32100	27000
Cadmium	2.40	1.07	< 0.02	< 0.02
Calcium	21800000	13600000	1050000	757000
Chromium	< 10	20.0	< 10	< 10
Cobalt	< 10	20	< 10	< 10
Copper	< 10	130	10	< 10
Iron	137000	146000	2980	5500
Lead	27	41	0.39	0.46
Lithium	36000	11700	2680	3980

Magnesium	1380000	1040000	426000	398000
Manganese	24100	19400	100	50
Mercury	0.1	0.15	0.011	0.013
Molybdenum	1.0	< 5	< 5	< 5
Nickel	< 20	140	< 20	< 20
Phosphorus	70	< 50	< 50	< 50
Potassium	1110000	685000	257000	287000
Rubidium	4400	1860	389	716
Selenium	< 50	< 50	< 50	< 50
Silicon	25600	14100	21800	24700
Silver	0.6	0.4	< 0.2	< 0.2
Sodium	49500000	43000000	15600000	10900000
Strontium	2410000	1450000	50800	67500
Sulfur	460	770	691000	549000
Tellurium	< 2	< 2	< 2	< 2
Thallium	140	70	< 2	4
Tin	< 0.5	< 0.5	< 0.5	< 0.5
Titanium	< 1	27	9	11
Uranium	< 0.005	< 0.005	< 0.005	< 0.005
Vanadium	< 5	9	< 5	< 5
Zinc	2400	2300	30	< 20

Table 4. Summary of PAHs in produced water.

Compound	Venture	Thebaud	Terra Nova	Sea Rose
	Concentration (ng·mL-1)			
Naphthalene	181.7	403.2	115.3	274.8
C1-N	152.2	290	112.7	229.1
C2-N	64.6	112.2	58.1	114.4
C2-N	19.3	34.8	22.8	45.1
C4-N	6.6	13.7	9.7	16.5
Acenaphthene	0.6	1.2	1	2.6
Acenaphthalene				
Fluorene	15.2	4.4	4.7	13.7
C1-F	10.1	2.7	4.2	12.7

C2-F	5.9	1.6	3.4	9
C3-F	3.2	1.1	2.8	6.4
Dibenzothiophene	0.5	0.3	3.5	2.8
C1-DBT	0.6	0.3	4	2.9
C2-DBT	0.4		3.4	3.3
C3-DBT	0.3		2	2.1
C4-DBT	0.1		1.1	1
Phenanthrene	8.7	2.8	6.8	19.1
C1-Ph	8.9	2.3	6.9	19.1
C2-Ph	5.4	1.4	5.3	13
C3-Ph	2.8	0.6	3.4	7.6
C4-Ph	1.2	0.3	2.1	4.1
Anthracene			5.6	
Fluoranthene	0.2		0.1	0.4
Pyrene	0.2		0.2	0.5
C1-Pyr	1.3	0.1	0.7	2.4
C2-Pyr	1.3		1	2.8
C3-Pyr	1		1	2.6
C4-Pyr	0.8		1.3	2.8
Naphthobenzothiophene			0.3	0.5
C1-NBT	0.3	1	1	1.2
C2-NBT	0.7	2.8	2.8	3.6
C3-NBT			0.9	1
C4-NBT			0.5	0.6
Chrysene	0.1	0.5	0.5	1.4
C1-Chry	0.4	0.8	0.8	1.9
C2-Chry	1.5	2.2	2.2	2.9
C3-Chry	0.2	0.8	0.8	1.5
C4-Chry			0.7	1.2
Benz[a]anthracene	0.2			0.6
Benzo[b]fluoranthene	0.1	0.1	0/1	0.8
Benzo[k]fluoranthene				
Benzo[e]pyrene				0.7
Benzo[a]pyrene				0.4
Perylene				
Indeno[1,2,3-cd]pyrene			0.2	
Dibenz[a,h]anthracene			0.1	
Benzo[ghi]perylene				
Alkylated PAH	304.5	465.6	260.4	525.1
Parent PAH	192.2	407.6	133.7	304.2
Total PAH	496.7	873.2	394	829.2

Table 5. Summary of phenols in produced water.

Compound	Venture	Thebaud	Terra Nova	Sea Rose
	Concentration (ng·mL-1)			
Phenol	7609.2	4145.1	911.9	446.2
o-cresol	1694.3	5377.5	916.3	851.4
m & p-cresol	914.4	3157.3	1010.6	901.7
2,6-dimethylphenol	12.4	294.5	48.6	62.3
2-ethylphenol	74.8	650.6	101.6	90
2,4 & 2,5-dimethylphenol	51	1026.8	242.7	307.9
3 & 4-ethylphenol	173.5	746	374.5	406.7
2,3-dimethylphenol	13	132.8	43.3	65.8
2-isoproplyphenol	9.7	190.9	71.5	55.6
2-proplyphenol	10	98.4	26.6	22.6
3 & 4-isopropylphenol	27.2	514.9	166.3	171.6
2-sec-butylphenol	6.2	32.7	24.6	21.8
3 & 4-tert butylphenol	10.7	82.3	47.6	55.4
4-sec-butylphenol	8.6	62.4	34.1	36.6
4-isopropyl-3-methylphenol	<1	28.7	21.9	26.9
4-nonylphenol	9.2	<1	<1	<1
Total Phenols	10624	16541	4042	3522.4

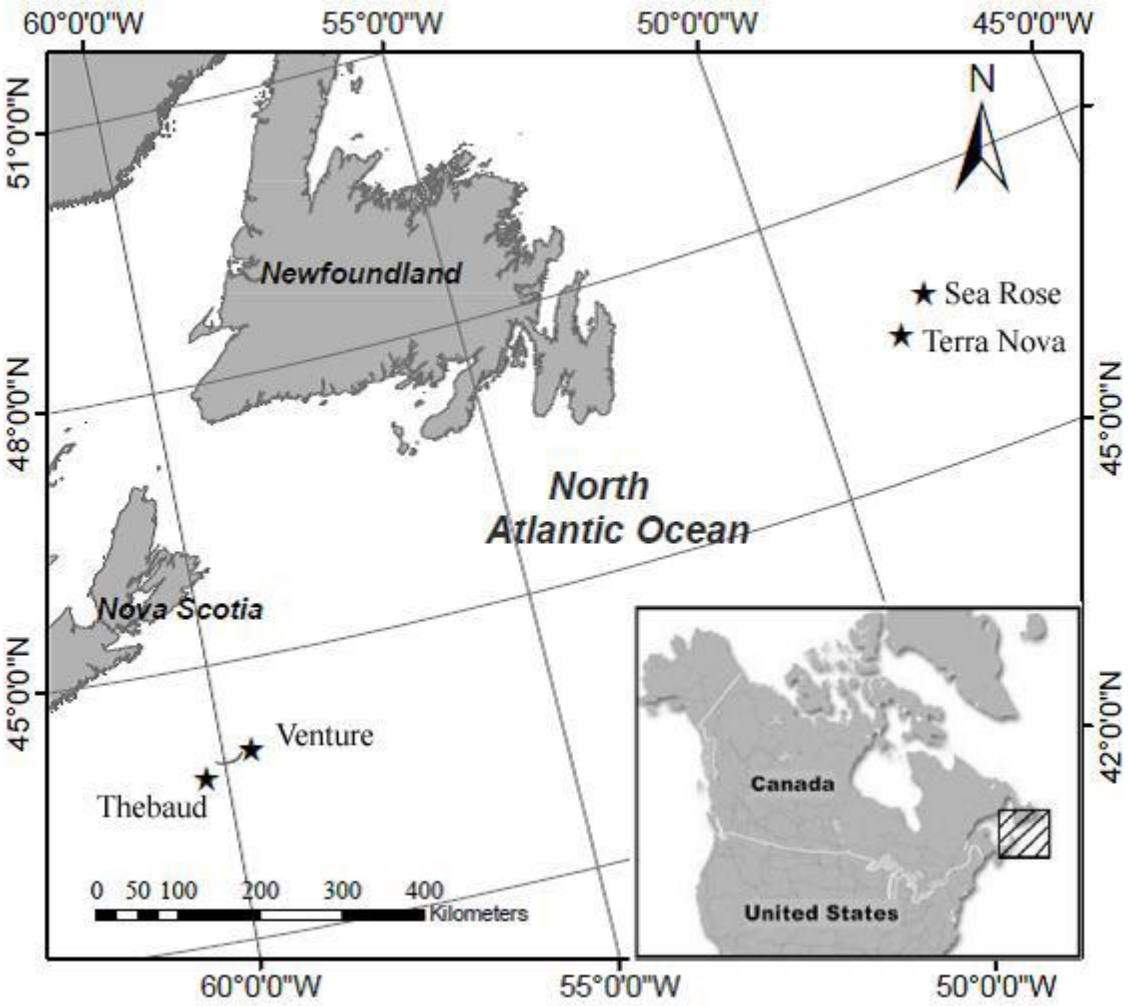

Fig.1. Locations of offshore oil and gas production sites for produced water collection off the east coast of Canada. Stars indicate production sites.

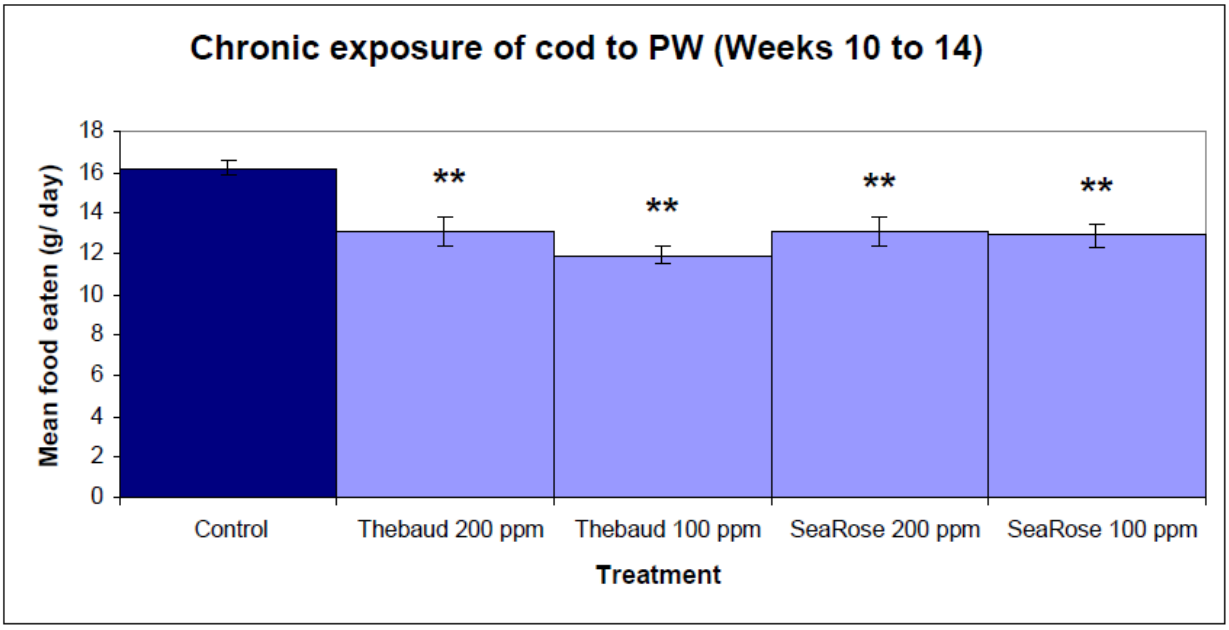

Fig. 2a. Histogram of mean daily food consumption (g) ± SE of Atlantic cod for Thebaud and Sea Rose PW treatments. Asterisks indicate significant differences from control mean daily food consumption (* P < 0.05, **P < 0.005) (Dunnett's test).

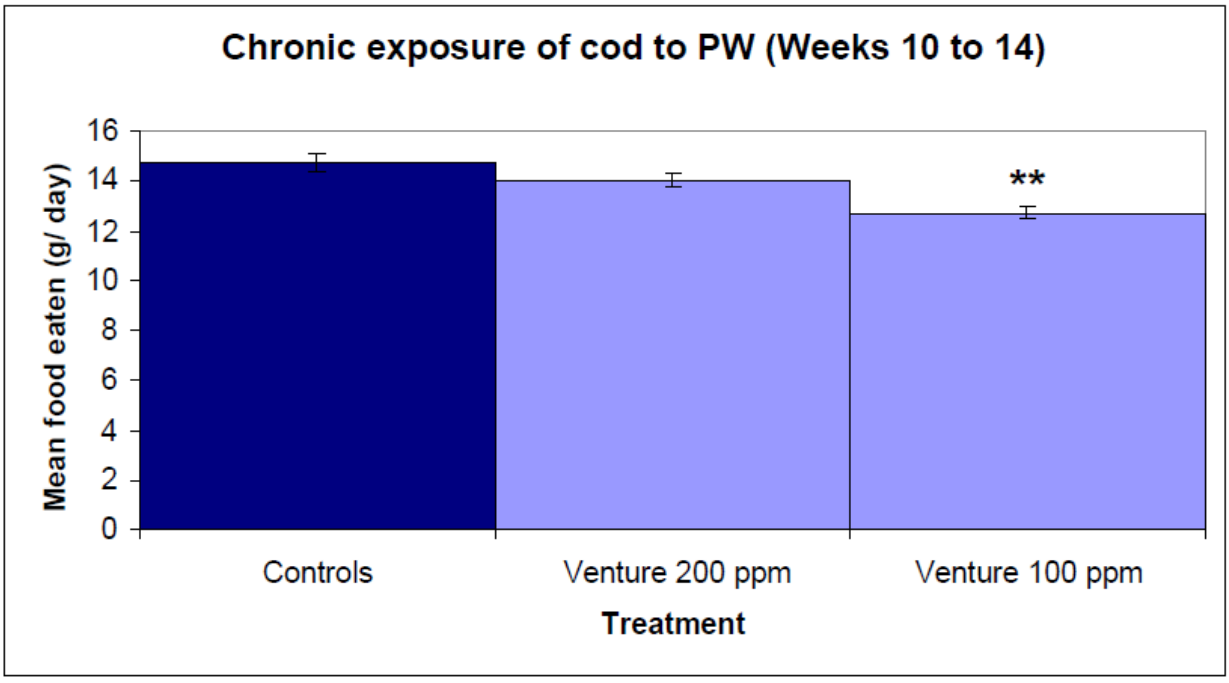

Fig. 2b. Histogram of mean daily food consumption (g) ± SE of Atlantic cod for Venture PW treatments. Asterisks indicate significant differences from control mean daily food consumption (* P < 0.05, **P < 0.005) (Dunnett's test).

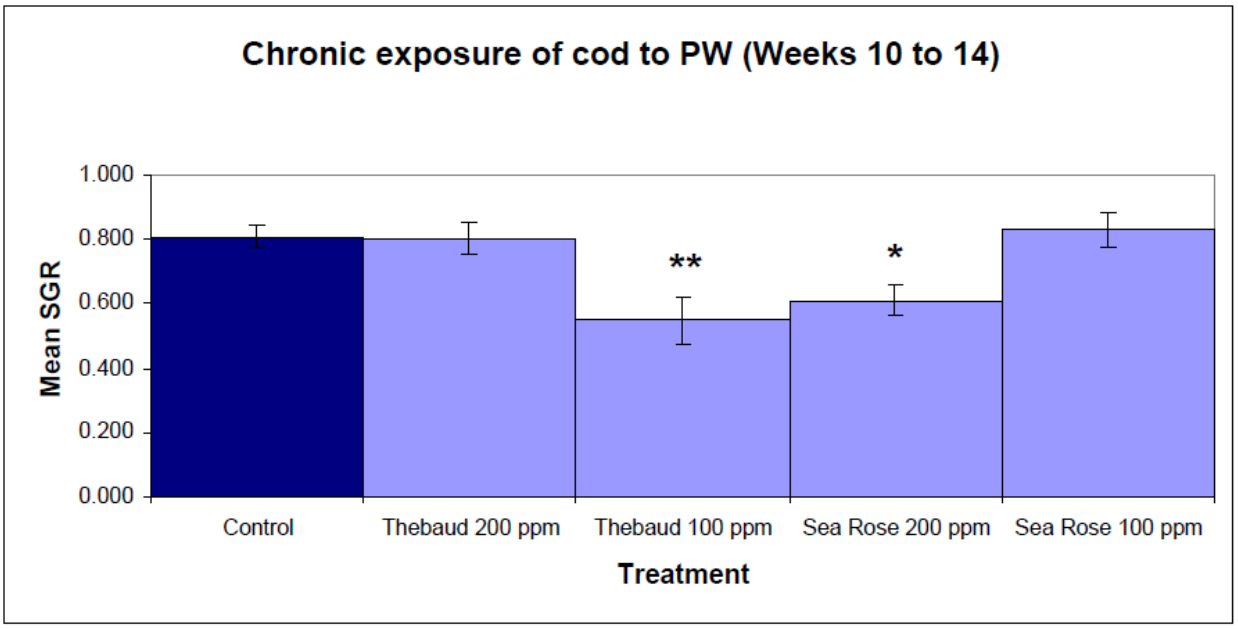

Fig.3. Histogram of specific growth rate ± SE of Atlantic cod for Thebaud and Sea Rose PW treatments. Asterisks indicate significant differences from control SGR (* $P < 0.05$, **$P < 0.005$) (Dunnett's test).

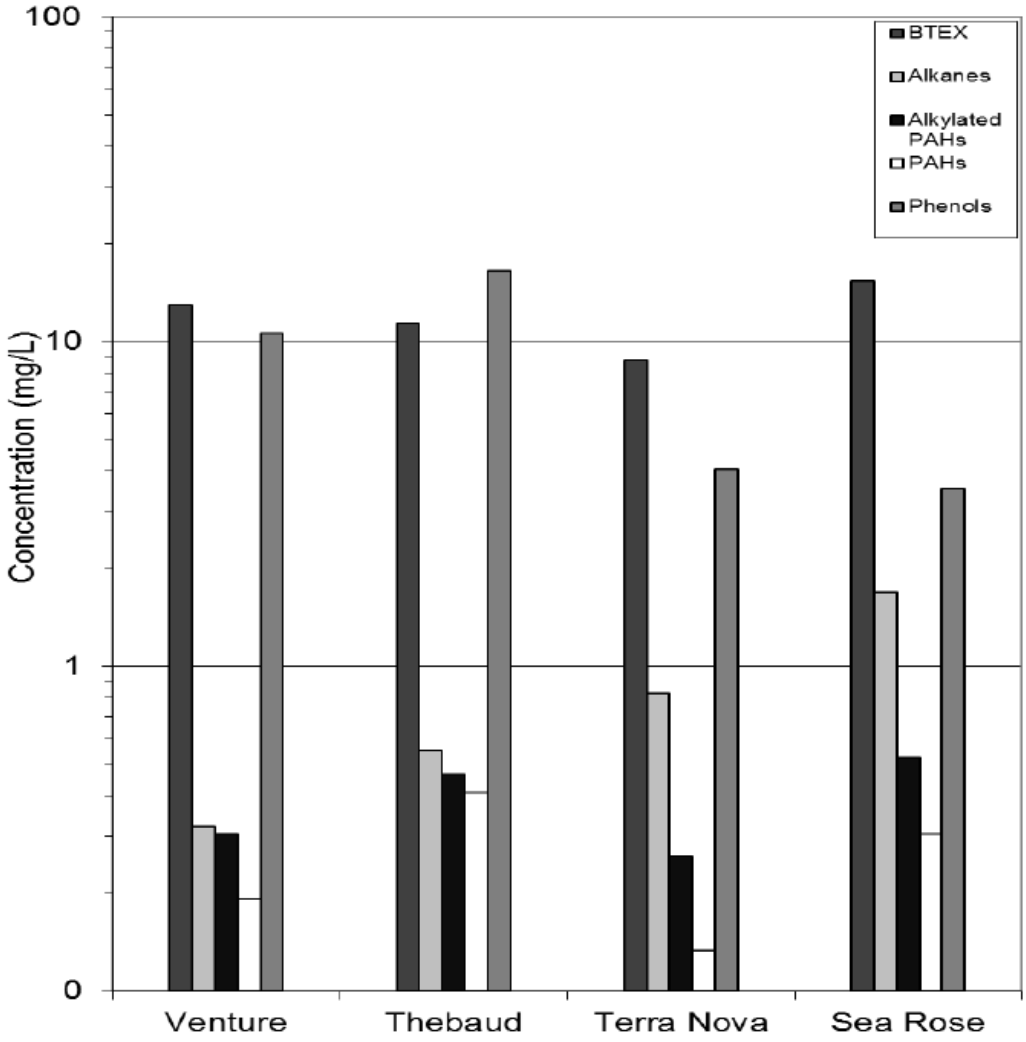

Fig.4. Summary of organic components in produced water.

Discussion

In this study effects were seen on mean SGR of juvenile cod exposed intermittently to 100 ppm (10 000 X dilution) Thebaud PW and 200 ppm (5000 X dilution) Sea Rose PW when compared to controls. These effects were only seen in the final third of the 14 week study. However, cod exposed to 200 ppm (5000 X dilution) Thebaud PW and 100 ppm (10 000 X dilution) Sea Rose PW did not have significantly lower mean SGRs at any time in the study. The treatments used in this study are environmentally relevant concentrations as rapid dilution of PW occurs by at least 1000 X by 4-5 km and up to 9000X at 20 km from the discharge site (Somerville *et al*., 1987; Murray-Smith *et al*., 1996; Pérez-Casanova *et al*., 2010).

There are few studies that address the potential effect of PW on the growth parameters of economically important finfish species. Pérez-Casanova *et al*. (2010) exposed juvenile cod intermittently

for 22 weeks to Hibernia platform PW at the same environmentally relevant concentrations as used in our study and saw no effects on growth or food intake. Hibernia offshore oil platform is off the coast of Newfoundland, Canada on the eastern edge of the Grand Banks in the North Atlantic Ocean. Hamoutene *et al*. (2011) exposed larger cod (average weight 774 g) intermittently for 11 weeks to Hibernia platform PW at the same environmentally relevant concentrations as used in our study and saw no effects on growth or food intake. Burridge *et al*. (2011) chronically exposed juvenile cod (mean weight 23 g) to 0.05% (2000X dilution) for 45 days and saw no growth effects. Stephen *et al*. (2000) used a semi-static exposure of post-metamorphic juvenile turbot, Scophthalmus maximus to PW from a North Sea oil platform. They saw no effects on SGR after 1 or 3 week exposures but did see significant effects in the growth of turbot exposed to 0.001% PW (100 000X dilution) after 6 weeks which the authors attributed to an increase in energy expenditure due to increased swimming activity. Increased swimming activity in the wild may be an avoidance response leading to movement out of an effluent plume in the near-field or pocket in the far-field. This was their lowest exposure concentration and they did not see significant growth effects at any of the higher exposure concentrations. In contrast, they saw reduced swimming activity in the fish exposed to 1% PW (100X dilution) and suggested narcotic action of either the external hydrocarbons or those accumulated internally. Stephens *et al*. (2000) also suggested that they could not presume growth would be unaffected in the wild. Reduced physical activity can be predicted to reduce food-searching time and capture resulting in negative impact on growth.

Food consumption was significantly affected for cod in some treatments compared to controls in our study. The effect of exposure to PW on SGR were only seen for some dilutions of PW yet most treated fish except those exposed to Terra Nova PW had a lower daily food consumption compared to control fish. The daily food consumption effect was not observed until the final third of the 14 week study which may indicate that the intermittent exposures had a cumulative effect. Perhaps an intermittent exposure period longer than 14 weeks would have resulted in a continued reduction in daily food consumption or growth rate for the cod in the other exposure groups. Fish were provided with a limited body-weight related ration of food in this study to ensure that they were hungry and increase the chances that they would feed. The resultant effects on growth are unlikely to be identical to those occurring in the wild. The narcotic effect of hydrocarbons suggested by Stephens *et al*. (2000) may have contributed to lower food consumption by the cod in some treatments. Affected fish that have a limited opportunity to feed may not be up to the task and may become susceptible to predation.

The negative effects on growth observed in this study at environmentally relevant concentrations of PW are ecologically significant. These results suggest that juvenile cod are affected by intermittent exposure. In the wild, depressed growth could result in size-dependent predation (Werner *et al*., 1983; Stephens *et al*., 2000).

Studies indicate that the PWs discharged from gas/ condensate platforms are about 10 times more toxic than the PWs discharged from oil platforms (Veil *et al*., 2004). However, for PW discharged offshore, the volumes from gas production are much lower, so the total impact may be less (Veil *et al*., 2004). Preliminary exposures of Atlantic cod eggs and larvae to PW from gas platforms (Thebaud and Venture) have shown greater toxicity than with exposures to PW from oil platforms (Sea Rose, Terra Nova and Hibernia) (Burridge *et al*., unpublished).

Produced water is a highly complex mixture containing thousands of compounds with widely varying chemical properties. The complex nature of produced water makes it challenging to try and relate biological effects to one or more specific compounds, and instead the toxicity of whole mixtures of chemicals should be considered. The chemical composition of produced water varies depending on the age, depth, and geochemistry of the hydrocarbon-bearing formation, as well as the chemical composition of the oil and gas phases found in the reservoir (Fisheries and Oceans Canada, 2011). The Venture and Thebaud platforms are both gas production wells in the Sable gas fields offshore of Nova Scotia, while Sea Rose and Terra Nova are oil fields in the Grand Banks offshore Newfoundland. As such,

the chemical composition of produced water between the two fields showed some significant differences.

High concentrations of sulphur in Sea Rose and Terra Nova produced water could indicate the presence of hydrogen sulphide, a known toxicant in produced waters (Sauer *et al*., 1992). Venture and Thebaud had concentrations of barium, iron, lead, manganese, strontium and zinc that were 2-3 orders of magnitude greater than Sea Rose and Terra Nova. Despite these high concentrations, dissolved metals likely play a minor role in the overall toxicity of produced water in the natural environment (Neff, 2002). Dissolved metals in anoxic produced water will precipitate on contact with oxygenated seawater (Stephenson *et al*., 1994), which then may settle on bottom sediments in the near-field zone around offshore oil and gas installations (Azetsu-Scott *et al*., 2007).

Concentrations of BTEX (Benzene, Toluene, Ethyl benzene, Xylenes) ranged between 8.8 – 15.4 mg/L. Despite these high concentrations, the high volatility of BTEX means that the exposure time would have been limited during this study. Lee *et al*. (2011) reported that aeration of produced water (to simulate mixing with oxygenated seawater) resulted in an almost complete loss of BTEX after 25 hours. In addition, other studies have shown that the loss of BTEX does not significantly change the toxicity of produced water to marine organisms (Flynn *et al*., 1996). Polycyclic aromatic hydrocarbons (PAH) are the petroleum hydrocarbons of greatest environmental concern in produced water because of their toxicity and persistence in the environment. Concentrations of PAHs were very similar between the four produced water samples, with total PAH (parent + alkylated) concentrations ranging between 0.39 – 0.88 mg/L. The PAH profile in all four produced water samples consisted predominately of low molecular weight compounds (≤3 benzene rings), with 95-99% of the detected PAH compounds falling into this class. These compounds, such as naphthalene, fluorene, dibenzothiophene and phenanthrene are generally considered less toxic than high molecular weight PAH (Neff, 2002). In addition, aeration of the exposure water would have reduced the concentration of these semi-volatile compounds (Lee *et al*., 2011). Of greater concern with regard to toxicological effects is the high molecular weight PAH with ≥4 benzene rings. These compounds were detected in the Venture, Sea Rose and Terra Nova produced waters with total concentrations up to 0.03 mg/L, while they were not detected in the Thebaud sample. A wide range of phenolic compounds was also detected, with total phenol concentrations higher in the gas production well produced waters (10.6 – 16.5 mg/L for Venture and Thebaud, respectively) versus the oil production wells (3.5 – 4.0 for Sea Rose and Terra Nova, respectively). Phenolic compounds, especially when present at high concentrations, may play a significant role in the toxicity of produced water (Neff, 2002, Flynn *et al*., 2002). Straight chain saturated hydrocarbons (alkanes) were also detected between 0.32 and 1.69 mg/L in the four produced waters. Alkanes are generally the result of small dispersed oil droplets in the produced water (Neff, 2002), and are of minimal concern with regard to toxicity to marine organisms.

Conclusion

Our results indicate that intermittent exposure to environmentally relevant doses of PW from both oil and gas platforms can reduce daily food consumption and can affect growth of juvenile cod. The exposures of PW mimicked the rapid dilutions that occur following discharge into the environment. Discharge of PW to the waters of the Scotian Shelf and edge of the Grand Banks, two highly productive areas that have some of the highest abundance of cod in Canadian waters could pose a risk to juvenile cod. The effects of chronic, low level exposures of PW on important marine species such as cod may become evident only after monitoring several life stages. Earlier life stages (egg, larval and juvenile) of cod are vulnerable as they have little control over their movement in the ocean currents and may be unable to avoid being caught in a plume or pocket of PW.

The data presented here indicates that effects can occur and need to be considered in a risk

assessment context but generalizations about the effects of PW cannot be made. The complex nature of PW makes it challenging to try and relate biological effects to one or more specific compounds, and instead the toxicity of whole mixtures of chemicals should be considered. Chemical analysis of PW may be of little predictive value. The consequences of long-term effects on cod populations from offshore oil and gas facilities that have a 15-20 year life-cycle need to be better understood.

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APPENDIX 2: Poster presented at ATW 2012

The effect of chronic exposure to produced water on growth and food consumption of juvenile Atlantic cod (*Gadus morhua*)

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Introduction

Produced water (PW) is waste generated during production of oil and gas from offshore oil and gas wells. The discharge of PW remains an environmental concern due to uncertainty regarding its fate, transport and potential biological effects. Dilution of the oil-related discharge containing poly-aromatic hydrocarbons, phenols, alkylphenols and heavy metals occurs rapidly. However, marine fish may be chronically exposed to diluted PW at distances far from oil and gas production sites. Atlantic cod is an important commercial groundfish species found throughout the North Atlantic. Early life stages of Atlantic cod (eggs, larvae and juveniles) have little control over their movement in the ocean currents and may be unable to avoid being caught in a plume of PW. In this study juvenile Atlantic cod were exposed intermittently to environmentally relevant concentrations of PW from either Sea Rose floating production, storage and offloading vessel (FPSO), Thebaud or Venture platforms for 14 weeks. Sea Rose is an oil production facility while Thebaud and Venture are gas production facilities.

Methods

Juvenile Atlantic cod were exposed three times a week to 0, 100 or 200 ppm of PW from either Sea Rose FPSO, Thebaud or Venture platforms for 14 weeks. The fish were fed prior to each exposure to PW. Growth and food consumption were monitored. Produced water samples were collected and analyzed (Table1).

Results

At the end of the exposure significant effects on specific growth rate (SGR) were seen in fish exposed to 200 ppm Sea Rose PW ($P<0.05$) and 100 ppm Thebaud PW ($P< 0.005$). Effects on daily food consumption (FC) were significant ($P< 0.005$) for all but one group of treated fish at the end of the exposure.

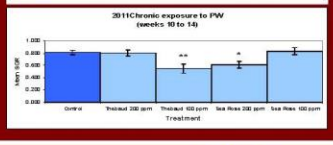
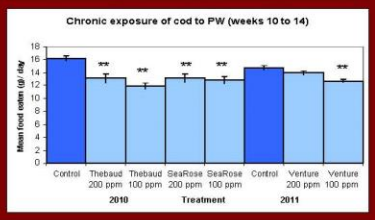


Table 1	Sea Rose PW (collected 2011)	Thebaud PW (collected 2011)	Venture PW (collected 2009)
PW component	Concentration (ppb)		
Alkanes	1695	301	364
Methylated PAHs	511	20	313
Total PAHs	829	60	530
Total phenols	3076	16039	10530
Alkylphenols	2065	11894	3209
Total BTEX	15416	2291	12952



Conclusions

- The results suggest that chronic exposure to environmentally- relevant concentrations of PW (5000X and 10000X dilution) from both oil and gas platforms may affect juvenile cod. Dilution of 10000X can occur at greater than 20 km from a PW discharge site indicating risk for adverse effects at considerable distances from production facilities.
- PW contains compounds of environmental concern such as aromatic and aliphatic hydrocarbons and phenols (in particular, alkylphenols).
- The chemical characteristics of PW are different for each production platform or formation from which the oil is extracted.
- The chronic effects of PW on growth and feeding shed light on the potential long-term impact of oil and gas offshore developments on Atlantic cod stocks.

APPENDIX 3: Poster presented at ATW 2010

Fertilisation and Hatching Success of Atlantic Cod (*Gadus morhua*) Eggs When Exposed to Various Concentrations of Produced Water

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Introduction

Fertilisation and hatching success of marine fish eggs in the vicinity of offshore oil and gas installations may be affected due to the overboard dumping of produced water. This is due in part to the cocktail of chemicals found in the produced water, which can contain, but not limited to PAHs, BTEX, phenols and alkylated phenols.

This study examined the fertilisation and hatching success of Atlantic cod (*Gadus morhua*) eggs when exposed to varying concentrations of produced water sourced from an offshore gas platform.

Methodology

- Atlantic cod eggs and milt obtained from in-house broodstock.
- Fertilisation took place in various concentrations of produced water (20 mL) using eggs (1.5 mL) and milt (200 µL) which were then allowed to water harden for 5 minutes.
- Fertilised eggs were rinsed then transferred to their respective produced water dilutions (200 mL). These were held at *ca* +6°C for 24h then the fertilisation success was determined.
- Viable fertilised eggs transferred to 96-well plates containing fresh sea water and held at *ca* +6°C.
- Eggs were monitored until hatch.
- Produced water samples were extracted and analysed for total PAH content using synchronous scanning fluorescence spectroscopy.

Results

- For fertilisation success, no significant difference was noted for the 0.32% v/v, 1.08% v/v and 3.6% v/v exposure groups compared to the Control group.
- Zero fertilisation success for the 12% v/v group.
- Majority of eggs hatched by 18 days post fertilisation.
- For hatching success, there was no significant difference for the 0.32% v/v and 1.08% v/v exposure groups compared to the Control group.
- A significant difference was noted for the 3.6% v/v exposure group compared to the Control group for hatching success.

Conclusions

- Fertilisation and hatching success of cod eggs was not affected at a dilution up to 1.08% v/v.
- Fertilisation and hatching success of Atlantic cod eggs was not affected by environmentally relevant dilutions of produced water used in this study.
- Fertilisation success may be influenced by the salinity of the exposure water. This area of research is ongoing.

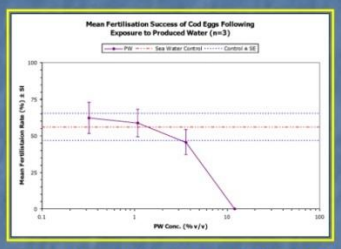

Figure 1. Mean Fertilisation Rate of Atlantic Cod Eggs Following Exposure to Produced Water

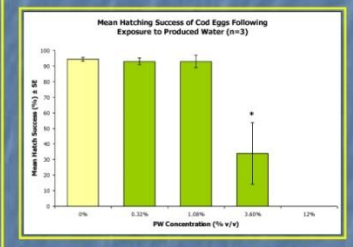

Figure 2. Mean Hatching Success of Cod Eggs Following Exposure to Produced Water
(* = p<0.05; ANOVA Followed by Tukey Multiple Comparison Test)

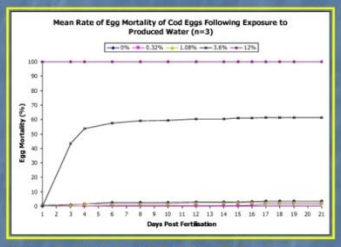

Figure 3. Mortality of Cod Eggs Following Exposure to Produced Water

Distance From Discharge Site	Dilution of Produced Water	Equivalent Dilution (%)
50 to 100 m	240x	0.42
4 to 5 km	1000x	0.10
20 km	9000x	0.011

Table 1. Dilution of Produced Water Based on Distance from Discharge Site (Somerville *et al.*, 1987; Murray-Smith *et al.*, 1996)

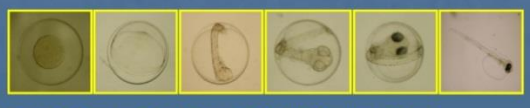

Figure 4. Representative Pictures of Cod Egg Development Post Fertilisation (Day 1, 4, 8, 12, 16-unhatched and 16-hatched)

Produced Water Concentration (% v/v)	Determined Total PAH Concentration (ng/mL)	Determined Salinity (ppt)
0	-	33
0.32	2.021	33
1.08	8.564	35
3.6	20.550	40
12	50.927	55

Table 2. Determined Salinity and Total PAH of Produced Water Dilutions Used for Exposures

APPENDIX 4: P. 353-373 In: Lee, K. and J. Neff (eds.) Produced water: environmental risks and advances in mitigation technologies. Springer, New York.

Application of Microbiological Methods to Assess the Potential Impact of Produced Water Discharges

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Abstract Microbial production and activity in produced water directly recovered from the discharge stream of offshore oil and gas production facilities off the east coast of Canada were examined before and after aeration in a series of concentrations to determine the effect of dilution at sea. Aeration and dilution resulted in reduced toxicity due to volatilization and oxidation of the lighter hydrocarbons including polycyclic aromatic hydrocarbons (PAHs), alkylated PAHs, benzene, toluene, ethylbenzene, xylene and short-chain alkanes (C_{10}-C_{14}). A fraction of the detrimental effects on microbial productivity and activity could also be attributed to the elevated salinity associated with produced water. These results suggest that caution should be given to the manipulation of produced water samples used for toxicity/risk assessment studies.

1 Introduction

Produced water represents the largest volume waste stream in oil and gas production operations on most offshore platforms (Neff et al., 2011; Krause, 1995) It typically contains inorganic compounds (trace metals, nutrients), volatile aromatic compounds such as BTEX (benzene, toluene, ethylbenzene, xylenes), polycyclic aromatic hydrocarbons (PAHs such as naphthalene), phenols, organic acids and additives (Manfra et al. 2007). There is concern that the ocean discharge of produced water with its associated manufactured and naturally occurring chemicals may pose adverse impacts to the marine ecosystem (Din and Abu, 1992; Krause et al, 1992; Stromgren et al., 1995; Stagg and McIntosh 1996; Holdway 2002; Querbach et al., 2005; Hamoutene et al, 2010; Perz-Casanova et al., 2010). Despite predicted and measured high rates of dilution for the produced water plume following discharge (Neff, 1987), a level of concern over its discharge at sea still remains for several reasons including: 1) the volume of produced water discharge typically increases as reservoirs become depleted, and 2) recent studies have suggested that some components in produced water such as metals, high molecular weight aromatic hydrocarbons and saturated hydrocarbons may accumulate in the sediments and the surface micro-layer as a result of natural physico-chemical processes (Neff, 2002; Durell et al., 2004; Lee et al., 2005; Azetsu-Scott et al., 2007).

Site specific studies are needed to assess the impact of produced waters as its chemical constituents can vary, depending on the location of its extraction within various parts of the oil and gas reserve and the waste treatment technologies used prior to its discharge (Collins, 1975). To determine the environmental risk of produced water discharges in waters off the east coast of Canada, changes in the microbial production and activity before and after aeration were investigated in samples of produced water collected from the Hibernia Gravity Based Structure (GBS), the Terra Nova Floating Production, Storage and Offloading (FPSO) unit on the Grand Banks, and the Venture platform on the Scotian Shelf of Canada (Figure 1, Table 1). These experiments were done to elucidate the effect of dilution on the toxicity of produced water when discharge occurs at sea.

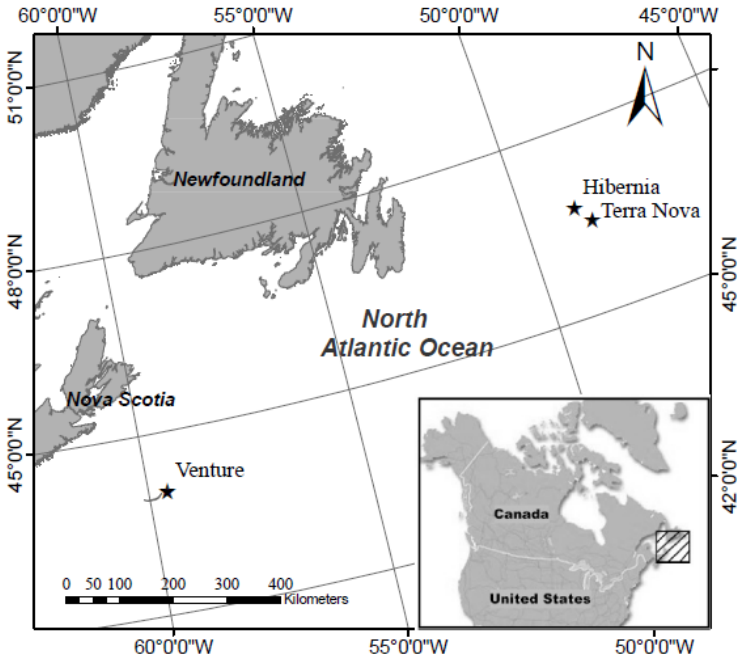

Figure 1: Locations of offshore oil and gas production sites for sample collection off the east coast of Canada

Table 1: Volume (m^3) of produced water discharged by platforms during the sample collection period (C-NLOPB, 2011; CNSOPB, 2011)

Platform **Month**	Hibernia April	Hibernia July	Terra Nova July	Venture July
Year	2004	2008	2008	2009
For Month	312,493	592,101	374,783	8,449

For Year	4,882,460	7,175,520	3,692,715	135,730
Cumulative as of 31 Aug 2010	45,075,904		20,325,220	1,551,264

2 Methods

2.1 Preparation of Produced Water Sample Containers

Produced water was collected in new, high density polyethylene (HDPE) 4 L wide mouth bottles, 10 L HDPE Nalgene® jerricans, and 4 L amber glass bottles (Fisher Scientific). The wide mouth plastic bottles, which were specifically designed to study the influence of chemical kinetics on produced water toxicity, were fitted with conical ultra high molecular weight polyethylene lid inserts to eliminate a headspace with the closure of the bottles. The bottles and jerricans were cleaned by filling with 1M HCl for 48-72 h, rinsing 5 times with de-ionized, distilled water, air drying and sealing. Amber glass bottles (4 L) were solvent rinsed with acetone, hexane and dichloromethane and air dried prior to closure.

2.2 Collection of Produced Water

Produced water sample collection was performed onboard Hibernia, Terra Nova and Venture by personnel associated with each production facility. Samples were drawn from a point in the process stream after treatment for regulatory compliance and immediately prior to discharge. The bottles were filled to overflowing to eliminate any headspace and returned to the laboratory as soon as logistically possible. Samples were maintained at 4°C in the dark prior to testing. Dilution experiments commenced from the day of collection to as long as one week after collection. Details of collection dates and experimental commencements are provided in Table 2.

Table 2: Produced water collection, date of experimental manipulation and test conditions

Source	**Sampling Date**	**Salinity (ppt)**	**pH**	**Expt. Start Date – Fresh PW**	**Expt. Start Date –Aerated PW**	**Aeration (h)**	**Incubation Temp (°C)**
Hibernia	16 Apr 04	46.5	7.4	20 Apr 04	3 May 04	68	2.4 – 3.2
Hibernia	13 Jul 08	45	8	13 Jul 08	14 Jul 08	25	15.2 – 16.5
Terra	7 Jul 08	45	8	8 Jul 08	11 Jul 08	70.75	15.2 –

Nova							16.5
Venture	9 Jul 09	201.3	6.5	16 Jul 09	18 Jul 09	46	15 - 16

2.3 Sub-Sampling of Produced Water

Sealed samples from the field were opened under nitrogen within a glove box (Hibernia 2004) or within a fume hood (Hibernia 2008, Terra Nova and Venture) for the distribution of subsamples immediately prior to analysis for

- microbiology dilution experiments (500 mL Nalgene® bottles),
- nutrients (2 × 60 mL acid rinsed plastic bottles, frozen at -20ºC),
- salinity (WTWLF 197 conductivity meter),
- pH (Orion® 230A pH meter or Colorphast EM Reagents test strips),
- dissolved metals (1 × 1 L Teflon bottle),
- organics (1 L for PAH and hydrocarbons, 1 L for phenols, in a 2.3 L solvent rinsed amber glass bottle, acidified to pH <2 with 6N HCl),
- benzene, toluene, ethylbenzene and xylene (BTEX) analysis (2 × 40 mL purge and trap vials, refrigerated at 4°C and analyzed within 14 days of collection).

2.4 Microbiological Experiments

2.4.1 Aeration Experiments

Upon contact with oxygenated seawater, produced water is oxidized, resulting in the transformation of complex hydrocarbons into simpler molecules and the precipitation of metals out of solution, forming flocs (Azetsu-Scott et al., 2007). To examine the differences between 'fresh' un-oxidized produced water and that which has reached equilibrium at sea (i.e. on mixing with oxygenated seawater during discharge), subsamples of the produced water were aerated by the addition of compressed air through an air-stone (table 2). Measurements of pH and salinity were made immediately following the aeration period and subsamples were collected for bacterial productivity (^{3}H thymidine) and activity (^{14}C glutamic acid) experiments.

2.4.2 Dilution Experiments

Dilution experiments were conducted using the freshly collected produced water recovered from the Hibernia, Terra Nova and Venture production sites to examine the effects on bacterial productivity (^{3}H thymidine) and activity (^{14}C glutamic acid). Experiments were conducted in the laboratory (2004, 2009) and at sea (2008).

Freshly dispensed produced water was mixed with fresh, unfiltered seawater collected immediately prior to the start of each experiment, to prepare the following concentration series (as % produced

water) in 2004: 0, 0.25, 0.5, 1, and 10%. In 2008 and 2009, concentrations of 2.5, 5, and 20% were added to the test series. Due to the high dilution rate following discharge (SOEP, 1996; Neff, 2002), these concentrations represented worst-case conditions expected to be encountered in close proximity to the platform.

2.4.3 Assessing the Influence of Salinity

Salinity has been recognized as a component of produced water that can cause or contribute to toxic effects on marine organisms (Neff, 2002) including indigenous bacteria. The salinity of Venture produced water was over 200 parts per thousand (ppt) – almost 7 times greater than that of the surrounding seawater. To determine the effect of salinity on the bacterial response, a dilution experiment was run concurrently with Venture produced water, whereby sodium chloride was added to the seawater as an additional treatment to match the salinity of each dilution of produced water.

2.4.4 Assessing Bacterial Productivity

Bacteria contain one chromosome. When a cell grows and divides, every new chromosome that is synthesized represents a new bacterial cell; hence, there is a direct correlation between rates of DNA synthesis and cell division. Bacterial productivity was measured by quantifying the rates of ^{3}H-thymidine incorporation into DNA, (Fuhrman and Azam, 1982) using protocols adapted from Bell (1993) and Li et al. (1993).

For each produced water test concentration, 4 replicates of 32 mL were measured into 50 mL culture tubes and high specific activity ^{3}H thymidine (Methyl-^{3}H, Perkin Elmer, 72.2-87.3 Ci/mmol, cat # NET-027Z) was immediately added for a final concentration of 5 nM. Tubes were capped and vortexed.

Triplicate aliquots of 10 mL from 2 replicate samples were filtered immediately (“zero time filtration blanks”) onto 0.2 µm Nuclepore® polycarbonate membrane filters, rinsed with freshly filtered (0.2 µm Millipore®) seawater, then folded in half, placed in a glassine envelope and immediately frozen at -20°C. After 3 hours, the filtration process was repeated for the remaining 2 tubes which had been incubated in the dark at ambient water temperature (table 2). All filters in glassine envelopes were maintained frozen until extraction of total radio labeled macromolecules.

Individual frozen polycarbonate filters were subsequently placed into an 18 × 150 mm glass test tube in an ice bath at 0°C to thaw for 15 minutes and then re-suspended in 5 mL of ice cold 5% (v/v) trichloroacetic acid (TCA) for 20 minutes to precipitate DNA. Then the contents were filtered onto a Whatman® GF/f filter, rinsed twice with 5% TCA followed by 1 mL of 95% ethanol to remove thymidine adsorbed to cell wall lipids, but not incorporated into DNA. Both the GF/f and polycarbonate filters were placed into 20 mL glass scintillation vials and were digested by addition of 0.25 mL of hyamine hydroxide, for 30 minutes at 55°C. After cooling, the pH was adjusted by adding 50 µL of glacial acetic acid. This was followed by the addition of 10 mL of liquid scintillation cocktail (Beckman Ready Safe). Samples were maintained in the dark until radioactivity was measured using a Beckman-Coulter LS 6500 liquid scintillation counter and data expressed as disintegrations per minute (DPM).

Equations from Bell (1993) were used to calculate productivity as the rate of thymidine uptake in moles/L/h. Conversion factors provided by Ducklow and Carlson (1992) and Li et al. (1993) were used to calculate this rate in terms of cells (cells/L/h) and grams of carbon (gC/L/h). Data was normalized and expressed as a percent of the control value (unfiltered seawater collected on the day of the experiment taken to be 100%).

2.4.5 Assessing Bacterial Activity

Since produced water contains organic acids, changes in heterotrophic uptake may be a suitable means to monitor the potential biological effect of produced water discharges. In the current study, the relative rates of heterotrophic activity in seawater were determined by the amount of radio-labeled organic substrate (^{14}C-glutamic acid) added at one substrate concentration, and taken up in a unit of time (Griffiths et al., 1977). For each dilution of produced water, 8 replicates of 10 mL were dispensed into glass test tubes (8 mm × 150 mm), and 0.1 µmol of ^{14}C(U)-glutamic acid (Perkin Elmer, Specific activity 250-278 mCi/mmol, cat # NEC-290E) was added to each tube. Blanks were prepared by adding 1 mL of 6N H_2SO_4 to 2 of the replicates prior to isotope addition. Tubes were capped with respiration traps consisting of a rubber stopper fitted with a plastic center well cup (Kontes) containing a Whatman® 25 mm GF/c filter folded in an accordion-like manner. All samples were incubated in the dark for 4 hours at ambient seawater temperatures (Table 2). The incubation period was terminated and $^{14}CO_2$ expelled by injecting 1 mL of 6N H_2SO_4 into each tube using a disposable 23G needle fitted to a 1 mL syringe. Another 1 mL syringe fitted with a 23G needle was used to inject 0.1 mL of ß-phenethylamine onto the GF/c filter in order to absorb the expelled $^{14}CO_2$.

After 12 h, the rubber stopper with the well was removed from the test tube, and the GF/c respiration filter taken with forceps and placed in a glass scintillation vial along with 10 mL of liquid scintillation cocktail (Beckman Ready Safe). The remaining sample in the tube was used to measure uptake. It was filtered onto Millipore® nitrocellulose MF filters (0.22 µm, 25 mm), rinsed 3 times with 5 mL fresh, filtered (0.22 µm Millipore®) seawater and placed in a glass scintillation vial along with 1 mL of ethyl acetate to dissolve the filter before addition of 10 mL of cocktail. Radioactivity of these uptake and respiration samples was measured on a Beckman-Coulter LS 6500 liquid scintillation counter, and results expressed in DPM.

Uptake and respiration rates were calculated as ng/L/h (Parsons et al., 1984) and summed to determine gross uptake. Data was normalized and expressed as a percent of the control value (unfiltered seawater collected on the day of the experiment taken to be 100%).

2.5 Chemistry Analysis

2.5.1 PAH and Aliphatic Hydrocarbons

PAH and aliphatic hydrocarbons were analyzed using a modified version of EPA Method 8270. The 1 L produced water sample was spiked with a surrogate standard containing a series of deuterated aliphatic and aromatic hydrocarbons, and extracted with 3 x 50 mL of dichloromethane in a separatory funnel. The solvent was concentrated on a TurboVap and the extract purified on a silica gel column. The purified extract was exchanged into isooctane and spiked with internal standards. Samples were analyzed using an Agilent 6890 Gas Chromatograph (GC) coupled to a 5975 Mass Spectrometer (MS). The column was a Supelco MDN-5s of 30 m length × 250 µm internal diameter × 0.25 µm film thickness, with a 1 m retention gap of deactivated fused silica. A 1 µL aliquot was injected using the oven track mode. Helium was the carrier gas with a flow rate of 1.0 mL/min. The oven temperature program was set to hold at 85ºC for 2 min, followed by a ramp to 280ºC at 4ºC/min held for 20 min. Total run time was 70.75 min. The MS was operated in the selected ion monitoring (SIM) mode with specific ions and retention windows applied for each compound. Samples were calibrated against a seven-point curve containing a mixture of aliphatic hydrocarbons as well as parent and alkyl PAH. For some of the alkyl PAH where standards were not available, the response of the parent PAH was used for quantification.

2.5.2 Alkylated and Nonyl Phenols

Phenols were processed according to a modified version of EPA Method 8041. The 1 L produced water sample was acidified with 6N HCl to a pH <2 and extracted in a separatory funnel with dichloromethane, concentrated on a TurboVap, the extract exchanged into hexane and spiked with an internal standard of deuterated phenol. Samples were analyzed with an Agilent 6890 GC set up as before (2.5.1), except that the oven temperature was held at 55ºC for 2 min followed by a 10ºC/min ramp to 100ºC held for 2 min, a 1ºC/min ramp to 115ºC held 2 min, and a 20ºC/min ramp to 220ºC held 4 min for 34.75 min total run time. The MS was set up as before (2.5.1) and calibration was done with a ten-point curve.

2.5.3 BTEX

The 40 mL purge and trap samples for BTEX were analyzed using a modified version of EPA Method 8240. The analytical system consisted of a Teledyne Tekmar purge and trap system, coupled to an Agilent 6890 GC and a 5973N MS. An auto sampler was used to dispense 5 mL into the purge chamber. The sample was purged with helium for 11 minutes, followed by a desorption time of two minutes. The GC column was set up (2.5.1) and run in split/splitless mode with a ratio of 50:1. Helium was the carrier gas at 1.0 mL/min. The oven was set to hold 50ºC (8 min) followed by a 4ºC/min ramp to 60ºC and then a 40ºC/min ramp to 280ºC (total run = 18 min). The MS was run in SIM mode and samples were calibrated against a nine-point curve.

2.5.4 Inorganics

Inorganics in fresh produced water were quantified by RPC Science and Engineering (Fredericton, NB, Canada) by inductively coupled plasma mass spectrometry analysis of a highly diluted sample using

Standards Council of Canada (SCC) approved procedures 4.M01 and 4.M29. Mercury was analyzed using cold vapour atomic fluorescence spectroscopy (SCC approved procedure 4.M52).

Nutrients (ammonia, silicate, nitrate and nitrite) were analyzed by segmented flow analysis (Technicon II). The determination of soluble silicates (Technicon Industrial Method No. 186-72W released March 1973, adapted from Strickland and Parsons, 1972) in seawater was based on the reduction of a silicomolybdate in acidic solution to 'molybdenum blue' by ascorbic acid which is read colorimetrically at 660nm. Oxalic acid is introduced to the sample stream before the addition of ascorbic acid to eliminate interference from phosphate.

The determination of nitrate/nitrite followed Technicon Industrial Method No. 158-71W released December 1972 (adapted from Armstrong et al., 1967; Grasshoff, 1969; U.S. Department of the Interior, 1969). The method was based on the principle of nitrate reduction to nitrite by a copper-cadmium reductor column. The nitrite ion then reacts with sulphanilamide under acidic conditions to form a diazo compound. This compound then couples with N-1-naphlylethylenediamine dihydrochloride to form a reddish-purple azo dye, which is read colorimetrically at 550 nm. Nitrite is determined with identical chemistry but omitting the copper-cadmium column from the sample stream.

The method for ammonia (Kerouel and Aminot, 1997) was based on the reaction of ammonia with orthophtaldialdehyde (OPA) and sulphite to form an intense fluorescent product. Determination is done fluorometrically with excitation at 370 nm and emission at 418-700 nm.

3 Results

3.1 Microbiology

3.1.1 Aeration and Dilution Experiments

In the 2004 Hibernia sample, microbial productivity rates at 85-100% of control values were only slightly depressed in the aerated samples over a concentration range of 0.25-1% produced water. However, activity was significantly suppressed to 20% of the control value in fresh and aerated test samples at a 10% produced water concentration level. (a). Despite higher rates of bacterial productivity in the aerated samples at produced water concentrations <1%, analysis of the data showed that very little glutamic acid was metabolized. This might have been because of the abundance of alternative or preferred substrate present in the produced water.

In terms of relative heterotrophic activity, while significant suppression was also observed at the higher experimental concentration (10% produced water), at lower concentrations (<1% produced water) there was little effect on microbial response (Figure 2)

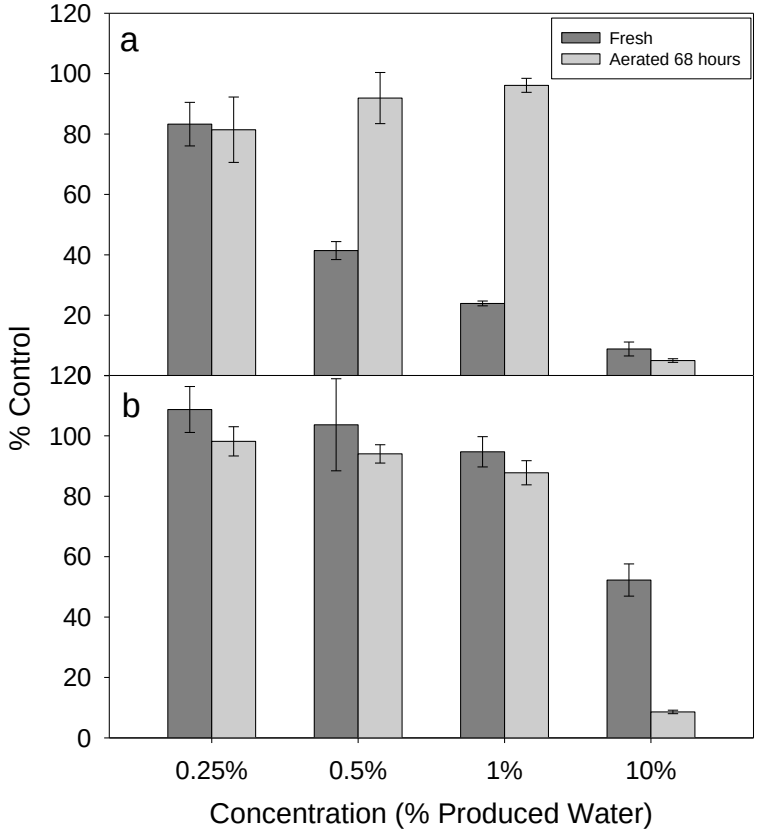

Figure 2: Bacterial productivity (a) and activity (b) in fresh and aerated Hibernia 2004 produced water

In the 2008 Hibernia sample, both bacterial productivity and relative hetrotrophic activity rates remained close to control levels or were positively stimulated in the aerated samples in produced water concentrations up to 10% (Figure 3)

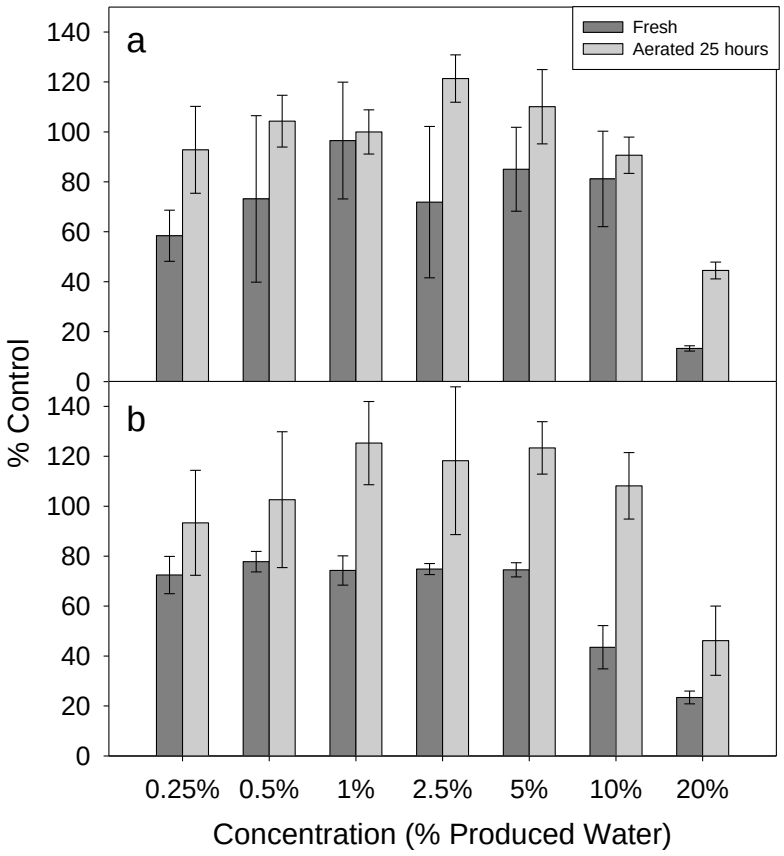

Figure 3: Bacterial productivity (a) and relative heterotrophic activity (b) in fresh and aerated Hibernia 2008 produced water

For fresh produced water, the average microbial productivity rates fluctuated between about 55 and 95% of the control values (Fig. 3a)while relative heterotrophic activity rates remained at about 75% of the control value in the 0.25-5% concentration range and declined at 10 and 20% (Fig. 3b).

In the aerated samples there was no significant suppression in bacterial productivity and glutamic acid metabolism rates at concentrations below 20% produced water.

The trend was even clearer and more striking in the Terra Nova 2008 fresh sample compared with the aerated (Figure 4).

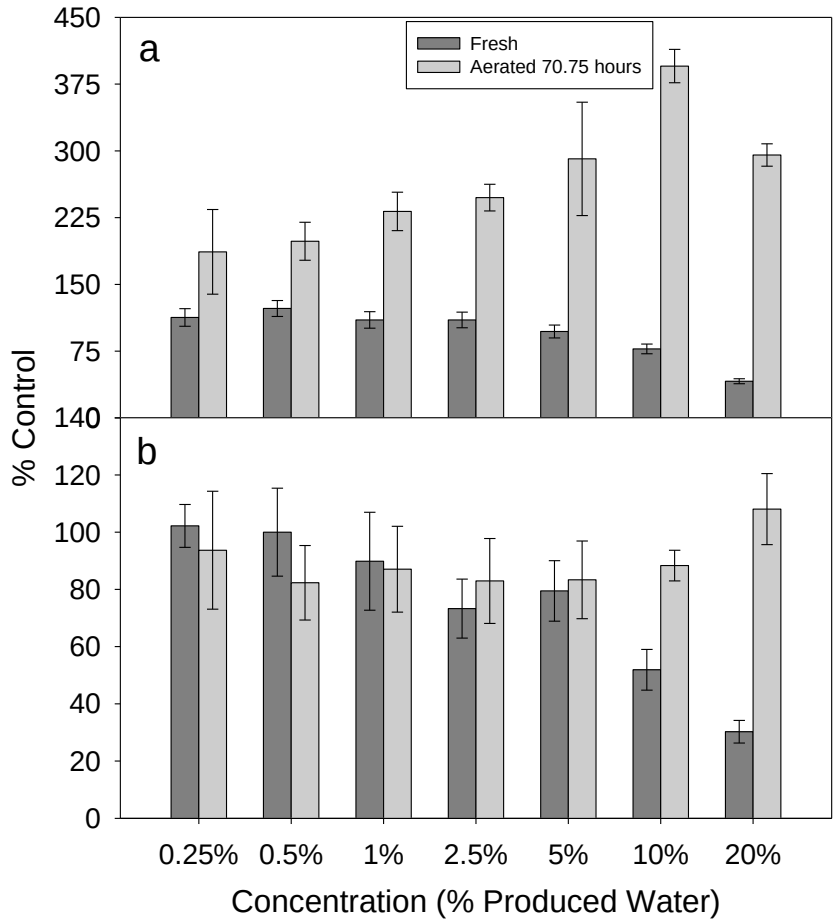

Figure 4: Bacterial productivity (a) and activity (b) in fresh and aerated Terra Nova 2008 produced water

Bacterial productivity rates were sustained at 90-120% of the control values for seawater samples containing fresh produced water at a concentration of 0.25-5%. While an inhibitory effect was observed in microbial productivity and heterotrophic activity at higher concentrations (10-20%) of fresh produced water, in the aerated samples microbial productivity was clearly stimulated and continued to increase to a maximum average of about 380% of the control value in the 10% concentration, then declined to about 280% of the control value at 20% produced water (Fig. 4a).

Activity in the fresh sample was maintained at an average of about 75-105% of the control value over a concentration range of 0.25-5% produced water before showing an inhibitory effect (Fig, 4b).

In contrast, activity in the aerated sample remained at a value within 80% of the control rates (Fig 4b).

For the 2009 Venture sample, dilution experiments showed an inhibitory effect on bacterial productivity and heterotrophic uptake potential in seawater containing fresh produced water at concentrations as low as 0.5% (Figure 5).

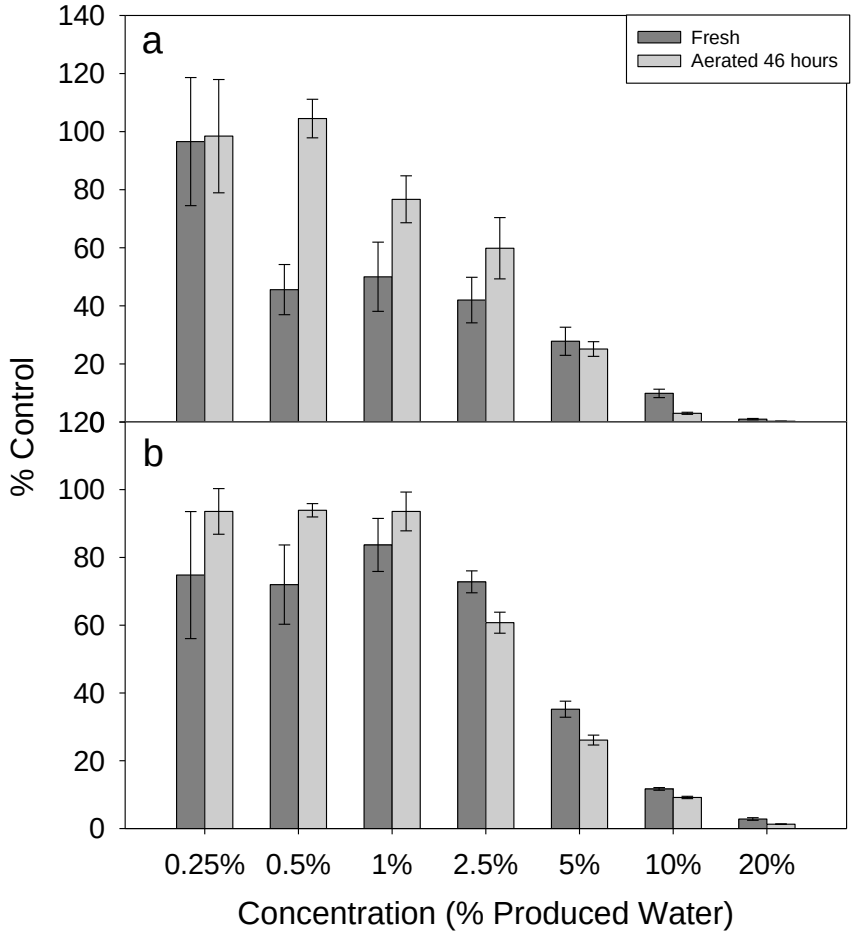

Figure 5: Bacterial productivity (a) and activity (b) in fresh and aerated Venture 2009 produced water

Over the lower experimental concentration range (up to 2.5% produced water), aeration significantly reduced the inhibitory effect.

3.1.2 Assessing the Influence of Salinity

As the salinity values for produced water from the Venture platform were extremely high at 200 ppt, an effort was made to elucidate the influence of salinity on the results of microbial assays. This was accomplished by adding additional treatments to the dilution experiment (a sample set with NaCl additions only) to account for the salinity toxicity associated with produced water.

Under the experimental test conditions (Figure 6) salinity associated with produced water from Venture could account for a fraction of the observed inhibition in bacterial productivity and activity at concentrations >2.5%.

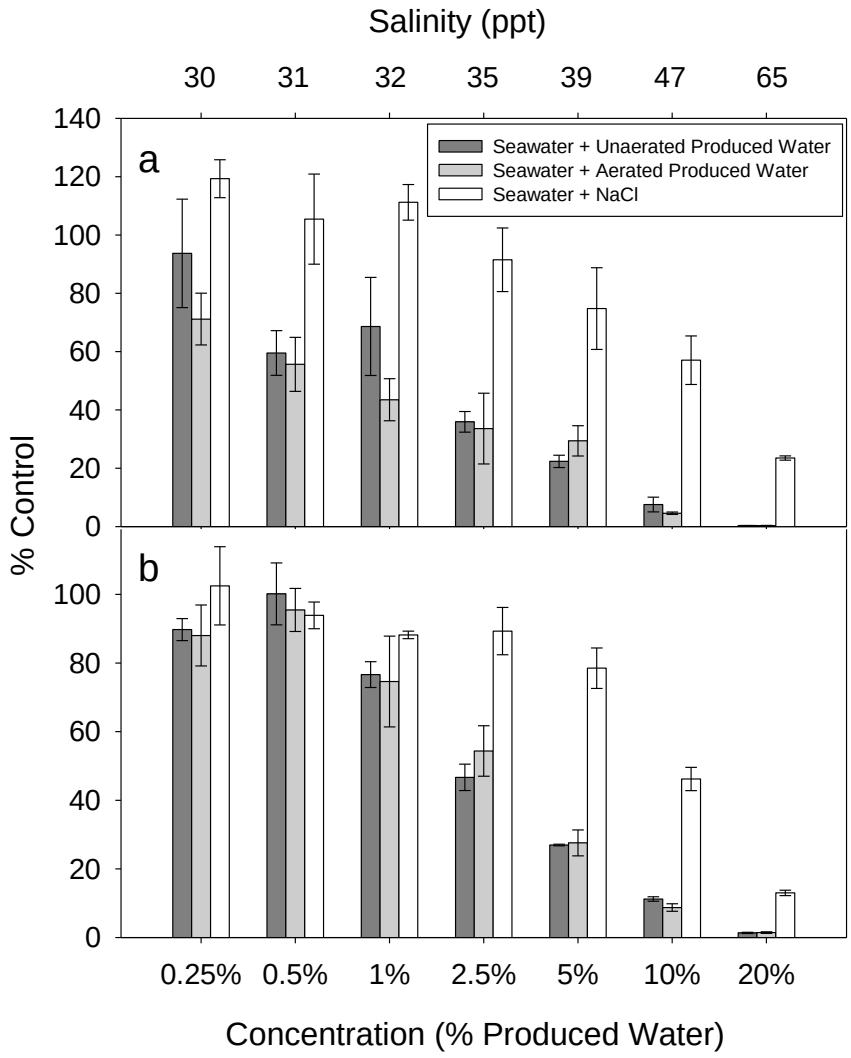

Figure 6: Influence of salinity on bacterial productivity (a) and activity (b) in fresh and aerated Venture 2009 produced water

3.2 Chemistry

3.2.1 Organics

The organics composition of the produced water collected from the three different rigs was similar (Figure 7), with BTEX and phenols dominating. Alkane concentrations were also similar among the three locations, while PAH concentrations followed the order of Venture > Terra Nova > Hibernia. The PAH

content of Terra Nova and Hibernia produced water consisted of 50-75% naphthalene (parent + alkylated), and Venture was 85%.

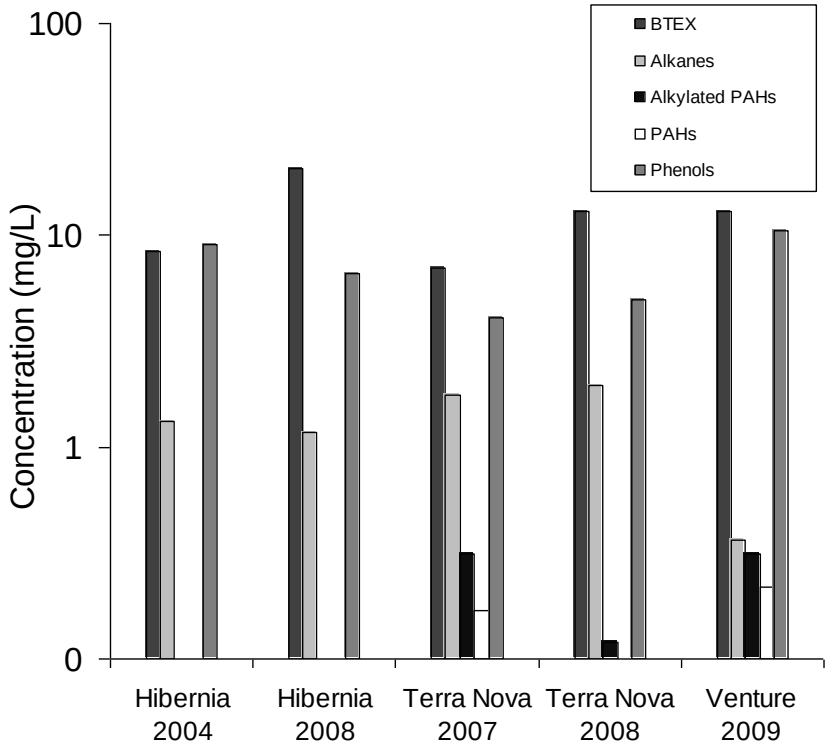

Figure 7: Comparison of organic composition of all fresh produced water samples

Samples were aerated to simulate the effects of chemical kinetic reactions after produced water discharged into the open sea reaches equilibrium. The loss of organics from aeration (Figure 8) followed the order of their relative vapour pressures with BTEX almost completely removed, followed by PAHs (dominated by highly volatile naphthalene) and their alkylated homologs, phenols, and longer-chain alkanes (C_{15}-C_{30}).

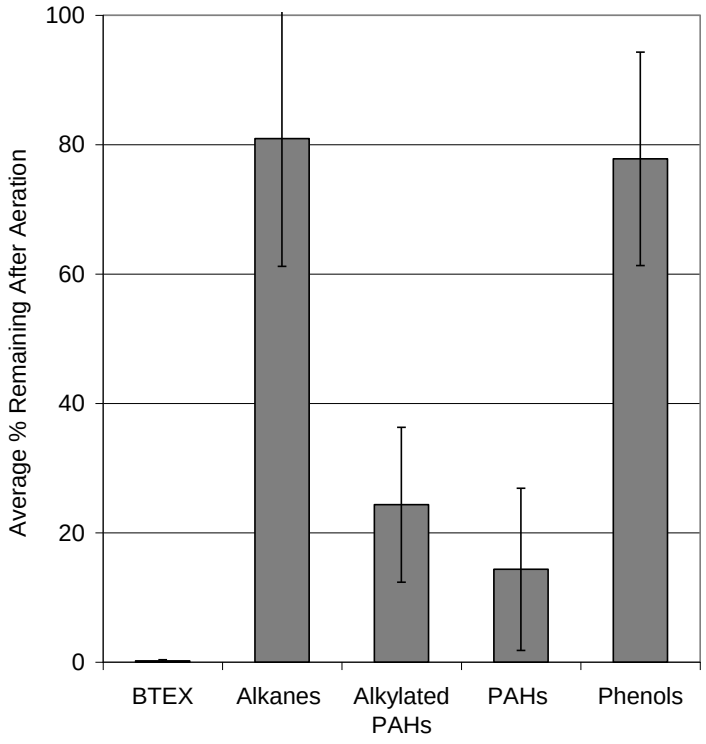

Figure 8: Effect of aeration on organic constituents of produced water from the three platforms

3.2.2 Inorganics

Inorganic constituents were relatively similar among the three platforms (Table 3), except that Venture produced water had concentrations of ammonia that were an order of magnitude greater than Hibernia and Terra Nova. Venture produced water was also much more saline than Hibernia or Terra Nova.

Table 3: Salinity, and nutrients (silicate, phosphate, nitrate, nitrite and ammonia) for fresh and aerated produced water samples.

Parameter	Hibernia				Terra Nova				Venture	
	2004		2008		2007		2008		2009	
	Raw	Aerated	Raw	Aerated	Raw	Aerated	Raw	Aerated	Raw	Aerated
Salinity	46.4	46.3	37.3	--	47.9	--	45.7	--	203.5	198.0

(ppt)										
Silicate (µM)	742.9	861.6	526.5	785.9	539.3	916.0	694.9	823.5	349.7	455.5
Phosphate (µM)	37.5	34.2	1.1	3.0	0.5	1.0	0.6	0.6	--	--
Nitrate (µM)	0.4	0.4	0.5	0.6	0.1	0.2	0.3	0.5	1.3	1.6
Ammonia (µM)	2033	2055	1152	1315	3444	2647	2497	2115	20828	21790
Nitrite (µM)	0.30	0.25	0.14	0.14	0.15	0.17	0.27	0.30	1.72	2.13

Concentrations of dissolved metals (Table 4) were also similar between Hibernia and Terra Nova, while Venture had levels of barium, manganese, iron, strontium and zinc elevated by 1-4 orders of magnitude. Compared to Hibernia and Terra Nova, the concentration of sulphur at Venture was approximately 4 orders of magnitude lower. As expected, aeration had little or no effect on the concentration of dissolved metals.

Table 4 Analysis of metals and other inorganics for fresh and aerated produced water

Parameter (µg/L)	**Hibernia**		**Venture**	
	2004		**2009**	
	Fresh	**Aerated**	**Fresh**	**Aerated**
Aluminum	2.0	4.3	100	120
Antimony	0.1	0.1	< 2	< 2
Arsenic	< 20	< 20	< 50	< 50
Barium	478	481	1240000	1260000
Beryllium	0.1	0.1	1	1.4
Bismuth	--	--	< 0.5	< 0.5
Boron	15800	15850	29000	29000
Cadmium	0.04	0.02	2.00	2.30

Calcium	885000	889000	21800000	22200000
Chromium	< 1	< 1	< 10	< 10
Cobalt	< 1	< 1	< 10	< 10
Copper	2.5	1.3	< 10	< 10
Iron	925	1310	137000	137000
Lanthanum	0.0	0.0	16	17
Lead	1.9	0.2	27	35
Lithium	1870	1865	36000	35000
Magnesium	815000	832500	1380000	1410000
Manganese	447	459	24100	24500
Mercury	--	--	0.1	--
Molybdenum	0.8	0.7	1	1.1
Nickel	76.0	80.5	< 20	< 20
Phosphorus	5340	5030	70	< 50
Potassium	274000	276000	1110000	1050000
Rubidium	315	310	4400	4300
Selenium	< 10	< 10	< 50	< 50
Silicon	21700	21850	25600	25900
Silver	--	--	1	0.5
Sodium	15350000	15300000	49500000	50700000
Strontium	62750	63100	2410000	2460000
Sulfur	670000	673500	460	300
Tellurium	--	--	< 2	< 2
Thallium	2.7	2.8	140	150
Thorium	< 0.005	< 0.005	< 0.2	< 0.2
Tin	< 0.05	< 0.05	< 0.5	< 0.5
Titanium	< 20	< 20	< 1	2

Uranium	< 0.002	< 0.002	< 0.005	0.058
Vanadium	0.9	1.6	< 5	< 5
Zinc	7.5	6.6	2400	2400

5 Discussion

Microorganisms were chosen for toxicity test analysis in this study as they react very quickly to their surrounding environment and are responsible for basic metabolic processes of environmental significance in the ocean such as nutrient regeneration, carbon fixation, contaminant biodegradation and biotransformation.

The results of the dilution experiments showed a decline in the rates of bacterial production and relative heterotrophic activity at concentrations >5-10% produced water. This is in general agreement with a reported effective toxicity concentration (EC50) for the marine bacterium, *Vibrio fischeri* (formerly *Photobacterium phosphoreum*) of 3.5-6.3% produced water from the North Sea oil platforms, Clyde, Forties Charlie, Brent Delta and Brae Alpha (Stagg et al., 1996).

Chemical kinetic reactions that occur following the release of anoxic produced water and its subsequent dilution in the open ocean upon discharge, have been found to alter its toxicity over time (Lee et al., 2005). The significance of this process was clearly illustrated in the controlled dose-response aeration-dilution experiments using natural microbial populations as the test organisms. A typical toxicity dose-response curve, with initial increase in productivity at low concentrations of produced water due to addition of nutrients followed by inhibition above a threshold value, was observed with fresh produced water. Following aeration for 44 h, to simulate equilibration in the ocean following discharge, produced water of the same concentration gradient elicited a stimulatory response. The difference associated with sample aeration can be attributed to the loss of predominantly volatile low molecular weight hydrocarbons and sequestering of hydrolysis metals as precipitates or in suspended organic matter, thereby reducing the toxicity of these constituents. Under favorable environmental conditions where nutrients are not limited and toxicity is not an issue, indigenous bacteria have the capacity to metabolize the labile organic compounds associated with produced water.

In this study, phenol was one of the major constituents of toxic concern found in all the produced water samples. This compound is a known toxicant to the marine bacterium, *V. fischeri*, and is, in fact, used as a reference toxicant in the Microtox assay (Microbics, 1991). Dehydrogenase catalyses the oxidation of organic chemicals, and its activity is a measure of bacterial growth and respiration. At about 300 mg/L, phenol has been shown to cause a 50% reduction in bacterial community dehydrogenase activity after 24 h exposure, and dehydrogenase activity for *Bacillus* sp. and *Escherichia* sp. was cut 50% within 24 h at phenol concentrations of 700-1400 mg/L (Nweke and Okpokwasili, 2010). Numerical modeling studies have suggested that the stimulatory effect of nutrients associated with produced water discharges on plankton growth may alter ecosystem trophic level dynamics (Rivkin et al, 2000; Khelifa et al., 2003). However, high concentrations of ammonia (>400 mM) are also known to be toxic to bacteria (Sprott and Patel, 1986). Our experiments showed that ammonia concentration (about 2-20 mM) was not reduced by aeration, and it is possible that its high concentration in the produced water from the Venture facility could account for the consistent inhibitory response generated for growth and metabolism both before and after aeration.

Two distinct strategies can be employed to discern the ecological effects of produced water effluents: predictive and observational (Middleditch, 1984). The predictive approach, as described in our field sampling and laboratory experiments, is based on determining the identification and concentrations of components within the produced water and their toxicity. The observational approach, although site specific, will provide direct and unequivocal information relating to "real world" effects. In this regard, field observations have also been made within the study region. Lee et al. (2005) noted no significant differences for ^{3}H-thymidine uptake rates in water column depth profiles in transect lines from 0.5 to 20 km away from the Hibernia offshore platform. Using advanced techniques in genomics to assess changes in microbial community structure and function, Yeung et al. (2011) determined that the impact zone from produced water discharge from the Hibernia offshore platform (with a much higher produced water discharge rate than the Venture offshore platform on the Scotian Shelf) was limited to within 500 m of the discharge point. On the Scotian Shelf, the analysis of potential environmental effects of produced water discharges using an integrated modeling approach indicated that the soluble benzene and naphthalene components reach chronic no-effect concentration levels at a distance of only 1.0 m from the discharge point (Berry and Wells, 2004). The limited persistence of these compounds that resulted in a low rank for the risk of adverse environmental effects, was attributed to advection processes linked to tidal currents within the region.

6 Conclusions

The concentrations of organic compounds in the Hibernia, Terra Nova and Venture fresh produced water samples under study were well within the range of values reported worldwide (Neff, 2002). The lower molecular weight compounds, BTEX and naphthalene, are less influenced by the efficiency of the oil-water separation process during cleanup before discharge than the higher molecular weight PAHs, and are not measured by standard regulatory oil and grease analytical methods (Argonne National Laboratories, 2004). However, as shown in the dilution studies which simulated natural weathering processes, these compounds have limited environmental persistence. Under current regulatory practices, the hydrocarbons in produced water (especially the PAHs) represent the organic compounds of greatest environmental concern (Neff, 2002). Nevertheless, as suggested by the results of our aeration and dilution studies, natural chemical kinetic reactions following the discharge of produced water can effectively reduce the toxicity to a point at which indigenous microbes are capable of biodegrading or biotransforming the remaining residual organic compounds.

The results of this scientific study clearly highlight the importance of standardizing protocols for sample collection, treatment and storage for environmental impact assessment of produced water discharged at sea. Furthermore, as the composition of produced water may vary from one geological formation to another, consideration must be made on a case-to-case basis. While the characteristics of the produced water samples in this study from offshore oil and gas production sites were similar to other produced waters worldwide, specific anomalies must be taken into account when interpreting toxicity data. For

example, the Venture produced water had a higher salinity and was higher in ammonia, but lower in sulphur than the other samples.

Ecological relevance must also be considered. Toxic responses are directly linked to both concentration and exposure time. When produced water is discharged at sea, numerous scientific studies have suggested that natural physical oceanographic processes would result in rapid dilution by factors of 10 to 100 fold within tens of meters of the platform (Brandsma and Smith, 1996; Flynn et al., 1996; Smith, 1993; Stromgren et al., 1995; Terrens and Tait, 1993; Somerville et al., 1987). In a comprehensive North Sea study with field monitoring data from mussels and semi-permeable membrane devices (SPMDs) and the application of a dose-related risk and effect assessment model (DREAM), Durell et al. (2006) reported that surface water total PAH concentrations ranged from 25 to 350 ng/L within 1 km of the platform discharge point, and reached background levels of 4-8 ng/L within 5-10 km of the discharge – a 100,000-fold dilution of the PAH in the discharge water. In terms of the impact zone from produced water discharges, Burns et al. (1999) used data on bioaccumulation from bivalves and microbial growth inhibition studies from a shallow tropical marine ecosystem on the Northwest Shelf of Australia, to validate chemistry and model predictions of a potential biological impact area which extended 900 m from the point of discharge. Yet they also concluded that dispersion and degradation processes were fast enough to mitigate any long-term build up of contaminants within the system.

Our controlled dilution studies in the laboratory with aerated produced water (to simulate equilibrium conditions following discharge into the sea) showed that toxic effects were only elicited at concentrations exceeding 0.5% produced water. Analysis of the data clearly showed the reduction of toxicity attributed to chemical kinetic reactions. In addition, as noted by Flynn et al. (1996), a large proportion of the organic material in produced water samples are highly biodegradable, as standard laboratory tests showed the biodegradation of >90% of phenols and PAHs in addition to the reduction of BTEX to concentrations below the detection limit (<0.5 ppb). Considering the limited volume of produced water released, and the high rates of dilution following discharge, the results of our microbiological studies showed that deleterious effects occur only within the immediate vicinity of the discharge point. Based on these findings, there would be minimal environmental risks associated with the discharge of produced water from oil and gas activities off the east coast of Canada.

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APPENDIX 5: P. 329-344 In: Lee, K. and J. Neff (eds) Produced water: environmental risks and advances in mitigation techniques. Spring, New York.

Effects of Production Water from the Hibernia Platform on the Survival, Growth and Biochemistry of Juvenile Atlantic Cod (*Gadus morhua*) and Mummichog (*Fundulus heteroclitus macrolepidotus*)

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Abstract Juvenile Atlantic cod and mummichog were exposed to a range of concentrations of produced water (PW) collected from the Hibernia oil production platform in 2005 and 2006. PAH exposure was measured by induction of cytochrome P450 (CYP1A) as indicated by ethoxyresorufin-*O*-deethylase (EROD) activity. In short-term exposures mummichog exposed to PW collected near the produced water discharge of the platform showed no change in EROD response compared to controls. EROD activity in livers collected from juvenile cod was significantly elevated in response to exposure to dilutions of PW but only at concentrations greater than 1.67% by volume. When juvenile cod were exposed to 0.05% PW (by volume) for 45 days there was no significant change in EROD activity, growth or plasma vitellogenin compared to unexposed fish. Embryo development and hatching in mummichog were slowed by exposure to dilutions of raw PW as low as 1%. However, mortality and developmental abnormalities were only observed at high concentrations (10% and 66%). PW from the Hibernia platform poses a low risk to cod and mummichog for the endpoints tested in this study.

1 Introduction

Produced water (PW) is the waste usually generated in largest volume during production of oil and gas from offshore oil and gas wells (Neff et al. 2006). The Canada-Newfoundland and Labrador Offshore Petroleum Board (CNLOPB) report that 5.72×10^6 and 7.18×10^6 tonnes of PW were released from the Hibernia platform in 2007 and 2008, respectively (CNLOPB 2009). Most PW is fossil water (formation water) that has accumulated over millions of years with fossil fuels in geologic formations deep in the earth. PW also may contain some surface water that has been injected into the formation for enhanced oil recovery. PW reaches the surface from natural oil seeps world-wide and during production of oil and gas from a well (Neff et al. 2006). The chemical characteristics of PW are different for each production platform or formation from which the oil is extracted. It is typically highly saline and contains elevated levels of heavy metals, hydrocarbons (including polycyclic aromatic hydrocarbons (PAHs)), alkylphenols,

ammonia and radionuclides compared to the receiving environment (Lee et al., 2005; Sturve et al., 2006).

With anticipated increases in the number of new offshore platforms, PW discharge has been identified as an issue of concern by both regulators and environmental groups (Zhao et al., 2008). While both monitoring and preliminary modeling efforts have been reported over the past decades, the possible long-term adverse effects of PW discharge into localized marine environments require further study.

The environmental impact of PW has been assessed by monitoring the chemical characteristics of freshly discharged PW and by ecotoxicological measurements (Azetsu-Scott et al. 2007). Because the chemical make-up of PW varies with its source the results of these studies are variable.

One of the most toxic groups of compounds found in PW, PAHs, have been shown to have cytotoxic, immunotoxic, and mutagenic or carcinogenic effects in marine organisms (Sturve et al., 2006). PAH exposure can be estimated by a standardized laboratory assay of cytochrome P-450 (CYP1A) induction (Hodson et al., 1996). The terminal oxidase enzyme of the mixed-function oxygenase (MFO) system is the iron-containing hemoprotein CYP1A. Ethoxyresorufin *O*-deethylase (EROD) is part of the family of cytochrome enzymes and is induced in the presence of certain xenobiotic compounds including PAHs (Hodson et al., 1991). The CYP1A enzyme catalyzes the hydroxylation of PAH to a more soluble and excretable form in the bile, and assays of liver CYP1A activity provide a good biomarker of PAH exposure in fish (McCarty et al., 2002).

PAHs and alkylphenols are estrogenic compounds (Kime, 1998). Therefore, PW exposure may adversely affect the hormonal system of fish. It is accepted that exposure to estrogen-like compounds in the aquatic environment may result in elevation of the yolk protein, vitellogenin (Vtg) in male fish (see for example Allen et al., 1999). Scott et al. (2006) have developed an enzyme linked immunosorbant assay (ELISA) for VTG in cod and have shown it to be sensitive to environmentally-relevant concentrations of contaminants.

Atlantic cod (*Gadus morhua*) is an important commercial species of groundfish. They are found throughout the North Atlantic including the Grand Banks of Newfoundland. This area is also the site of an expanding oil exploration industry. Mummichog (*Fundulus heteroclitus*) is one of the most common fish species in shallow coastal and estuarine waters of the Northwest Atlantic. Considerable work has been done on their responses to anthropogenic contaminants and they have been proposed as a model species for detecting physiological responses to environmental change (Burnett et al., 2007). In this paper we describe the results of lab-based studies wherein Atlantic cod, mummichog and early life stages of mummichog were exposed to PW from the Hibernia production platform. The endpoints studied include biomarkers of exposure (EROD) and of effects (growth and reproductive abnormalities).

2 Materials and Methods

2.1 Collection of Hibernia Produced Water

PW is discharged 40 m below the water surface at the Hibernia platform. Seawater samples were collected at the surface of the PW plume approximately 20 m from the platform on 2 July 2005. Approximately 70 L of seawater was collected in acid-washed (1 M HCl) Nalgene jerricans, transported to the Gulf Fisheries Centre (GFC), Moncton, NB, transferred to hexane / acetone-washed 20L glass bottles, sealed and refrigerated at 4-10ºC until use in mummichog experiments on July 19th.

In addition, raw PW was collected on the Hibernia platform 25 June 2006 in Teflon-sealed, acid-washed Nalgene jerricans. The containers were held at 4-10ºC, transported to the GFC or the St. Andrews Biological Station (SABS) and stored at 4ºC until used in bioassays. Mummichog embryo bioassays were conducted with 2006 PW in July, 2006. Growth and EROD induction experiments with cod were conducted with 2006 PW at SABS from December 2006 through March 2007.

2.2 Fish

Mature northern mummichog (*Fundulus heteroclitus macrolepidotus*) were collected by beach seine in July, 2005 from the Kouchibouguac River estuary in Grand Barachois, New Brunswick. Kouchibouguac estuary has limited development (a few cottages along the shores and some recreational boating) and was used as a reference site in a recent study of anthropogenic effects on local populations of mummichog and Atlantic silverside (*Menidia menidia*; Theriault et al., 2007).

Mummichog were held for 1 week at the GFC aquarium facility prior to EROD assay. Fish were held at densities of less than 2 g/L in recirculated artificial seawater (Kent Sea Salt in RO-treated municipal (Moncton, NB) water) at 15 parts per thousand, 22-23ºC and a photoperiod of 16h light: 8h dark. Fish were fed twice daily with Aquatox flake food (Zeigler Bros., Gardners, PA, USA) supplemented once a week with frozen, vitamin-enriched bloodworms (chironomid larvae; Bio-pure, Hikari Sales, Hayward CA, USA).

Atlantic cod (*Gadus morhua*) were obtained from the Genome Canada, Cod Genome Project hatchery at SABS. The fish were held in filtered sea water with simulated natural photoperiod and ambient temperature for seven months. Oxygen and temperature were recorded daily. The fish were hand fed once daily with a dry pellet mixture of Gemma starter feed and Europa, 2.0 mm feed from Skretting North America (Bayside, New Brunswick, Canada) and a cod marine, 1.5 mm feed from Ewos Canada, Ltd. (Surrey, British Columbia, Canada).

2.3 Exposures and Endpoints

2.3.1 Mummichog Embryonic Development Experiments

Mummichog broodstock were beach seined from the Kouchibouguac estuary (early July, 2006) and held in 1200-L recirculating aquaria (22-23°C, 15 ppt) at the GFC until the assay began later the same month. Eggs were artificially fertilized after being collected from females by ventral pressure. Males were sacrificed by spinal severance to obtain the gonads because it was impossible to draw enough milt by ventral pressure. The testes were ground then stirred with the eggs and left to sit for approximately 10 min to allow fertilisation to occur (Boudreau et al., 2004a; Boudreau et al., 2004b; Boudreau et al., 2005). Eggs were then rinsed with artificial seawater and placed in covered Pyrex Petri dishes (100 mm diameter; 20 eggs/dish) with 50 mL of test solution. Eggs were examined for development after 24 hours and unfertilised eggs were removed leaving 10 to 18 live embryos per dish. There were six replicates (Petri dishes) per treatment group. Treatments were 1, 10 and 66% PW collected from the Hibernia platform in June 2006. As the salinity of raw Hibernia PW is ~48 ppt, dilution to 66% is required to reach a salinity of 30-34 ppt. All dilutions were prepared with artificial seawater. Test solutions were renewed 3 times per week with freshly mixed solutions. Experiments were conducted at 25°C with no aeration. Photoperiod was 16 h light: 8 h dark.

Mortalities were monitored daily and dead animals were removed. Live embryos were observed by microscope to detect morphological abnormalities commonly found in toxicological studies (von Westernhagen, 1988; Boudreau et al., 2004a; Boudreau et al., 2004b; Boudreau et al., 2005). Specifically, embryos were monitored for abnormalities related to blue sac disease associated with PAH exposure (Brinkworth et al., 2003). Heart rates were measured from 5 randomly selected embryos per dish on day 7 post-fertilisation. Time-to-hatch was noted and larvae were measured at hatch (nearest 0.01 mm). Observations and measurements were made with a computer-based image analysis system (Matrox Inspector, version 3.0, Matrox Imaging, Dorval, QC) linked to a microscope (Leitz, Wild Photomakroskop M400, Leica Microsystems Inc., Willowdale ON) (16× to 90×) through a video camera (Hitachi, HV-D25, Fisher Scientific Ltd, Nepean ON).

2.3.2 Mummichog EROD Induction Experiments

Adult mummichog were held in 1200-L recirculating aquaria (22-23°C, 15 ppt) at the GFC until 48 h prior to initiation of bioassays. They were then acclimated to full seawater over a 48 h period. Mummichog were exposed for 24 h to decimal dilutions (0.01% to 100%) of receiving environment seawater collected off the Hibernia platform in July 2005. In order to test effects of 100% receiving water all treatment groups including the negative control were tested at a salinity of full seawater (30-34ppt salinity). Also included in the bioassay was a positive control of 10 $\mu g \cdot L^{-1}$ beta-naphthoflavone (BNF; dissolved in 100% methanol). In order to achieve the target concentration of BNF, 1 ml of the stock solution (in methanol) was added to 10 L water resulting in a methanol concentration of 0.01%. There were 2 replicates per treatment group, each with 10L of aerated test solution and 5 fish (mummichog: 5.52 g ± 0.46) Temperature was maintained at 22-23°C, the photoperiod was 16h light: 8h dark.

At the end of the exposure period fish were sacrificed by anaesthetic overdose [320 mg/L of tricaine methanesulfonate (Sigma-Aldrich, Oakville, ON, Canada) and livers were extracted, weighed, placed in cryovials (Fisher Scientific Company, Ottawa, ON, Canada), frozen in liquid nitrogen and stored in a -80ºC freezer until analysis. After removal of the fish from their exposure containers, salinity, pH, dissolved oxygen and ammonia concentration were noted for both replicates.

2.3.3 Juvenile Cod EROD Induction Experiments

Juvenile cod (mean weight = 24.8g; mean length = 14.5 cm)were exposed for 48 hours to one of five concentrations (0.06%, 0.19%, 0.56%, 1.67% and 5%) of PW, a seawater control or a positive control, 10 $\mu g \cdot L^{-1}$ of ß- naphthoflavone (BNF, prepared in methanol). In order to achieve the target concentration of BNF, 10 ml of the stock solution (in methanol) was added to 25L water resulting in a methanol concentration of 0.04%. Five fish per concentration were exposed at ambient light and water temperature of 10°C ± 1°C in 10 or 25 L glass aquaria fitted with clear acrylic lids. Loading rates were 5-10 g of $fish \cdot L^{-1}$ test solution. Half of the exposure water was replaced with fresh solution of PW after 24 h. The 48-hour exposure was repeated 2 times for a total of three bioassays. Dissolved oxygen (DO) was monitored throughout the test and in the first 48-h exposure was maintained at 70-80% except for two measurements made at T = 24 prior to the water change when the DO level dropped to 65-70%. There was no treatment-related trend in the DO.

A 500 ml water sample was taken from the aquarium spiked with 0.56% PW at 0, 24 and 48 h and preserved with 0.5 ml 6 N HCl. The water samples were held at 4 °C for chemical analysis. At the end of 48 hours, all the fish were sacrificed by a blow to the head, blood was collected from the caudal vein followed by cervical transection. Liver and muscle samples were collected. Length, weight and gender were recorded. Tissue samples were quick frozen in liquid nitrogen. Blood samples were centrifuged at 6000 rpm for 10 minutes and the plasma collected. Blood and tissue were frozen at -80°C.

2.3.4 Juvenile cod growth experiments

One hundred and forty juvenile cod (mean weight = 23.0g; mean length = 13.7cm) were tagged with Passive Integrated Transponders (PIT) and allowed to recover for several weeks in ambient (SABS) seawater. Seventy PIT tagged juvenile cod were exposed continuously for 45 days to 0.05% PW and 70 fish were held in running seawater and served as controls. Full-strength PW was delivered by controlled, gravity flow using a mariotte bottle. The flow rate was maintained at 1 $ml \cdot min^{-1}$ and was mixed with flowing sea water (2.5 $L \cdot min^{-1}$) prior to entering the 400 L exposure tank. The fish were held in a simulated natural photoperiod and 10 ± 2°C sea water. Water temperature was maintained with the addition of hot salt water into a header tank. DO and temperature were monitored throughout the bioassay. The fish were hand fed once a day a dry pellet of EWOS (EWOS Canada, Ltd. Surrey BC) marine sinking cod diet. Five hundred mL water samples were collected periodically, preserved with 0.5 ml 6 N HCl and stored at 4˚C.

At days 3, 7, 14, 28 and 45 of the exposure and days 14 and 60 post-exposure 8 cod were sacrificed by a quick blow to the head, blood was collected from the caudal vein followed by cervical transection. Liver, gill and muscle were collected for biochemical analysis. In addition, gender, length and weight data were recorded. Specific growth rates were determined using changes in fish weights according to Busacker et al. (1990). Tissue samples were quick frozen in liquid nitrogen.

2.4 Chemical and Biochemical analyses

2.4.1 EROD Assay

Frozen livers were thawed, a sub-sample taken, put in a tared micro-centrifuge tube and weight recorded. Sub-samples of liver were homogenized and diluted with HEPES-KCl buffer (pH 7.5, 0.15 M KCl, 0.02 M HEPES) using a hand-held motor driven Kontes pestle. All steps were carried out on ice. The homogenates were centrifuged at 9850 rpm for 20 minutes at 2°C. The post-mitochondrial supernatant (S-9 fraction) was collected with a Pasteur pipette, taking care to avoid the pellet and the floating lipid layer (Hodson et al.1991). S-9 fractions were frozen at -80°C in micro-centrifuge tubes until analysis.

Liver samples collected from mummichog were analysed at Queen's University, ON, Canada for ethoxyresorufin-*O*-deethylase (EROD) activity. Samples of cod liver were analysed at SABS. The kinetic microplate assay used is described in Hodson et al. (1996). EROD activity in cod liver S9 fractions was measured using a BioTek FLx 800 spectrofluorometer using excitation and emission wavelengths of 530 nm and 590 nm respectively. The protein levels in the same S-9 samples were quantified with a Bio-Rad assay (Bradford) dye reagent using a BioTek Powerwave XS UV/Vis spectrophotometer with absorbance set at 600 nm. BioTek Gen 5 software was used to view triplicate results for each sample.

EROD activity was calculated from the slope of the curve over the selected time. Activity was converted to picomoles resorufin per minute per milligram protein in the S9 fraction.

2.4.2 Plasma Vitellogenin

Blood samples were centrifuged at 6000 rpm for 10 minutes and the plasma collected. Blood and tissue were frozen at -80°C. Plasma samples were analysed for plasma vitellogenin (Vtg) using Biosense cod Vtg kit (Biosense Laboratories, Bergin, Norway) by an enzyme linked immunosorbent assay (ELISA). The sensitivity of the kit was verified by Dr. Robert Roy (Fisheries and Oceans Canada, Mont Joli QC) using plasma collected from Atlantic cod that had received estrogen injections at SABS.

2.4.3 Water Analysis

PW collected at the Hibernia platform was analysed at the Bedford Institute of Oceanography. Water samples were extracted and analysed for organic compounds according to King and Lee (2004) and for inorganic compounds according to Azetsu-Scott et al. (2007). The water samples collected during cod bioassays were analysed for organic compounds according to King and Lee (2004).

2.5 Data Analysis

Effects of treatments on mummichog embryo survival and incidence of morphological abnormalities were tested by probit regression. EROD activity, heart rates, time-to-hatch and size-at-hatch were tested for normality (probability plot) and homoscedastisticity (Fmax test) and transformed where necessary. Treatment effects were tested by parametric Analysis of Variance (ANOVA) models followed by Tukey's multiple ranges test, or by the non-parametric Kruskal-Wallis test followed by Nemenyi or Noether non-parametric multiple range tests if transformations did not correct non-normal distributions. Nested ANOVA removed the variability added by replicates to test for influence of treatment only. Because size-at-hatch can be affected by the duration of the embryo stage (i.e., longer incubation producing longer larvae; von Westernhagen, 1988), Analysis of Covariance (ANCOVA) was used to remove effects of incubation duration from comparisons of size-at-hatch. All analyses were performed with Systat version 11.0 (SPSS Inc., Chicago, IL, USA). The level of significance was $P<0.05$. Back-transformed means are accompanied by their 95% confidence interval.

Specific EROD activities were Log- transformed to establish normal distribution and homogenous variances. Log-transformed data were treated by analysis of variance (level of significance was $P<0.05$) to determine if differences were present between treated and untreated fish. Where significant differences were indicated, log means were compared by Tukey's multiple comparisons test.

3 Results and Discussion

3.1 Mummichog Embryonic Development

Survival of mummichog embryos exposed to Hibernia PW was reduced by the two highest concentrations tested, 10% and 66% ($P<0.05$, Table 1). Morphological development of embryos was also significantly affected by PW but only at the highest concentration of 66% ($P<0.05$, Table 1). Common morphological abnormalities observed during the study included cardio-vascular lesions such as pericardial edema (accumulation of fluids in the pericardial sac; Fig. 1B), haemorrhaging (accumulation of blood outside the blood vessel; Fig. 1B) and hemostasis most commonly along the tail (accumulation of blood inside the blood vessel; Fig. 1C) and general retarded development of the embryo (Fig. 1D). Other end-points tested proved more sensitive to PW and were all significantly impacted by the lowest concentration tested of 1% PW (Table 1). Heart rates were significantly reduced by treatment with 1 and 66% but not 10% ($P<0.05$). Embryos exposed to 1 and 10% PW took longer to hatch than controls ($P<0.05$) and none of the embryos hatched in the highest concentration of 66%. At hatch, embryos in the 1 and 10% treatment groups were smaller than control fish ($P < 0.05$). This suite of abnormalities and reduced growth was described originally in fish exposed to dioxins and was referred to as blue sac

disease (Helder, 1980; Walker et al., 1991). This condition has also been reported in fish exposed to PAHs (Billiard et al., 1999; Brinkworth et al., 2003). PAHs are among the most toxic compounds in oils and are believed to be the causative agents for blue sac disease in fish exposed to oils. In mummichog, these lesions have been reported following exposure to Alaska North Slope crude oil and Mesa light crude oil (Couillard, 2002) and we have recently reported blue sac disease in mummichog exposed to Orimulsion (Boudreau et al., 2009). The results of the present study show that a high concentration of Hibernia PW is also capable of producing morphological abnormalities, categorised as blue sac disease.

3.2 EROD Induction

3.2.1 Mummichog

Twenty four hour exposure to dilutions of seawater taken in the plume of PW from the Hibernia platform did not significantly elevate EROD activity in mummichog, though fish exposed to the positive control (10 $\mu g \cdot L^{-1}$ BNF) did show a significant increase in EROD activity ($P < 0.05$; Fig. 2A).

Consistently high basal levels of EROD have been observed in previous studies with mummichog (Whyte et al., 2000). Mummichog collected in three other New-Brunswick estuaries (Horton's Creek tributary of the Miramichi River, Shediac Bridge and Saint-Louis) have also shown high basal levels of EROD activity (S. Ramachandran, Queen's University, Kinston ON, personal communication). Nevertheless, even though basal levels were high in the present assay, significant induction was seen in response to the BNF positive control consistent with Whyte et al. (2000). This suggests that the high basal levels did not preclude response to a strong inducer but they may have rendered the assay insensitive to a weak inducer.

These results suggest that PW collected from the Hibernia platform contains biologically insignificant concentrations of specific Ah-R active compounds or the threshold for activation of the CYP1A system in this species fish is not achieved under our experimental conditions. Hodson et al. (2008) have shown that, in rainbow trout (*Oncorhynchus mykiss*), the alkylphenanthrenes fluorenes and napthobenzothiophenes are the main components of oil that induce CYP1A. Analysis of PW from Hibernia (2005) showed an average concentration of fluorene in the dissolved phase of 2205 $ng \cdot L^{-1}$ and methylated phenanthrenes at average concentrations of 277 $ng \cdot L^{-1}$, 501 $ng \cdot L^{-1}$ and 1182 $ng \cdot L^{-1}$ for 2-methlyphenanthrene, dimethylphenanthrene and methylphenanthrene respectively. It is not known if the compounds identified by Hodson et al. (2008) have the same potency in mummichog.

3.2.2 Juvenile Cod Dose-Response

The level of EROD activity in livers collected from cod exposed to a range of PW concentrations ranged from 0.2 to 12.6 $pmol \cdot min^{-1} \cdot mg^{-1}$. A comparison of treatment effects is shown in Figure 3. Only in PW

concentrations of 1.67% and 5% was EROD activity significantly ($P<0.05$) elevated compared to controls. BNF exposure resulted in elevated EROD activity in cod livers but the increase was not significant compared to controls. EROD activity in BNF-exposed cod was significantly higher than that observed in cod exposed to 0.06%, 0.19 % or 0.56% PW. The compounds identified by Hodson et al. (2008) as having CYP1A inducing qualities in trout were detectable in raw PW from Hibernia collected in 2006 and in the PW used in the cod bioassays. Raw PW contained an average of 743 $ng \cdot L^{-1}$, 260 $ng \cdot L^{-1}$ and 507 $ng \cdot L^{-1}$ of methylphenanthrene, 2-methylphenanthrene and dimethylphenanthrene respectively in the dissolved phase (Table 2). Of the four PW water samples analysed, one had significantly higher concentrations of the methylated phenanthrenes than the other three thereby increasing the average. Fluorene was present at an average concentration of 1902 $ng \cdot L^{-1}$ and napthobenzothiophene was not detectable in the dissolved phase (Table 2). In the acute (48h) exposures water samples were collected from the 0.56% exposure tanks. Only methylphenanthrene was detected in the water samples and then only in one test (Table 2). Although it is not known if these compounds have the same effect in cod as they do in trout, the lack of EROD induction at 0.56% PW is consistent with no measurable alkylated phenanthrenes, fluorine or napthobenzothiophene. It is clear from the EROD induction experiment that PW from Hibernia will activate the CYP1A system in cod at higher concentrations (above 1.67%, Figure 3).

3.2.4 Juvenile Cod Chronic Exposure

During the 45-day exposure of juvenile cod to 0.05% PW from Hibernia there was no significant difference ($P<0.05$) in EROD activity between treated and untreated fish (Figure 4). EROD activity was low throughout the study. The maximum measured value was approximately 5 $pmol \cdot min^{-1} \cdot mg^{-1}$. The EROD activities reported are lower than those reported (in this study) for cod exposed to higher concentrations in an acute exposure and values reported for mummichog. Hepatic EROD induction is also lower than values reported for Atlantic cod collected from areas of known contamination (Aas and Klungsøyr, 1998; Lee and Anderson, 2005; Schnell et al., 2008). Abrahamson et al. (2008) have reported that there is no difference between gill and liver EROD activity in cod caged near PW outflows and cod from reference sites. During the chronic exposure experiment, the compounds identified by Hodson et al. (2008) as being responsible for EROD induction in trout (methylphenanthrene, 2-methylphenanthrene, dimethylphenanthrene and fluorine) were not detected in samples collected from the exposure tank. Holth et al. (2009) exposed cod to low molecular weight PAHs and alkylphenols for 44 weeks. They found that EROD induction peaked after 4 weeks but was back to control levels at 32 weeks.

Sturve et al. (2006) reported that chronic exposure of juvenile cod to North Sea oil (0.5 ppm) resulted in a significant elevation of EROD activity in livers. When alkylphenols were added to the mixture the level of EROD induction was significantly reduced. These authors suggest alkylphenols present in PW are capable of interfering with the oil-mediated CYP1A response in cod. PW contains significant amounts of alkylphenols (Hasselburg et al., 2004). Analysis of raw PW from Hibernia in 2005 and 2006 showed dramatically different levels of phenols. The average total phenol concentration in 2005 PW was 11.934 $mg \cdot L^{-1}$ of which nonylphenol represented 276 $ng \cdot L^{-1}$ (data not shown). In 2006 the raw PW had an

average phenol concentration of 3.962 $mg \cdot L^{-1}$ and no detectable levels of nonylphenol. Alkylphenols represented about 50% of the total phenols and were made up of methyl, ethyl and propyl phenols (Table 2). It is possible, therefore, that the higher concentrations of alkylphenols in the 2005 samples may have had an inhibitory effect on EROD activity in the mummichog. This remains speculative as experiments were not conducted with mummichog in 2006 and no cod were tested in 2005.

Growth of cod, as indicated by the specific growth rate, was unaffected ($P>0.05$) by exposure to Hibernia PW for 45 days (Figure 6).

3.3 Juvenile Cod VTG

There was no significant difference ($P>0.05$) in plasma Vtg between control male cod and male cod exposed to PW for 45 days (data not shown). This result is consistent with the low level of alkylphenols in Hibernia PW collected in 2006. McLeese et al. (1981) reported that Atlantic salmon (*Salmo salar*) bioaccumulate alkylphenols. While this study was conducted with freshwater fish it shows the potential for fish to accumulate alkylphenols and suggests the potential for direct endocrine effects within the organism and for bio-magnification in the food chain. Scott et al. (2006) reported a strong relationship between Vtg concentration in male cod and size. They suggest larger (>5 kg) fish are picking up endocrine disrupting compounds in their diet (other fish) as opposed to benthic invertebrates which constitute the main diet items for smaller cod or from water.

4 Conclusion

It is clear from the data presented that PW from the Hibernia platform has no effect on the species tested at environmentally relevant concentrations. The endpoints investigated: EROD induction, growth, and embryological abnormalities were only observed at PW concentrations of 1% or greater. These data show that indicators of exposure and of effects are only observed at high concentrations relative to expected environmental exposure. Hamoutene et al. (2007) state that dispersion models predict that PW will be diluted by at least 240 times within 50-100 m of the PW discharge and by 9000 times 20 km from the discharge. Other authors suggest dilution factors of 1000:1 within 50 m of the discharge (Furuholt, 1996). Regardless of the models used, it is clear that the concentrations of PW that produced measurable effects in the endpoints investigated in this paper would only be observed very close to the PW discharge meaning that the risks associated with these endpoints are very small. While our results indicate low risk for the endpoints investigated, other biomarkers have been identified that do appear to be affected by exposure to PW or to representative compounds. Holth et al. (2009) have shown that DNA adducts are present in bile of cod chronically exposed exposed to low levels of PAHs and alkylphenols. Bohne-Kjersem et al. (2009) exposed cod to 0.01% PW from the North Sea and using proteomics found that a large number of proteins were affected. These proteins play a role in immune systems, fertility, metabolism, morphology, and perception.

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Table 1 Responses of mummichog embryos to Hibernia PW; arrow indicates direction of effect; threshold concentration indicates lowest concentration at which a significantly different response was seen from water control

End-point	Direction	Threshold concentration (% v/v)
Survival	↓	10%
Normal development	↓	66%
Heart rate	↓	1% (not significant at 10%)
Time-to-hatch	↑	1%
Size-at-hatch	↓	1%

Table 2 Concentration ($ng \cdot L^{-1}$) of alkylphenanthrenes, fluorenes, napthobenzothiophene and (in $mg \cdot L^{-1}$) total phenols and alkylphenols in PW collected from the Hibernia platform in 2006 and in water samples collected from bioassays with Atlantic cod conducted with dilutions of the same PW

Analyte (dissolved)	Source of water sample(s)		
	Raw PW Hibernia 2006	48-h dose-response (0.56%) T=0	Chronic exposure (0.05% PW)
Total methylated PAH	70090 (n=4)	266 (n=3)	ND
methylphenanthrene	743 (n=4)*	100 **	ND
2-methylphenanthrene	260 (n=4)*	ND	ND
dimethylphenanthrene	507 (n=4)*	ND	ND
fluorene	1902 (n=4)	ND	ND
napthobenzothiophene	ND	ND	ND
Σ Phenols	3.962 $mg \cdot L^{-1}$	0.012 $mg \cdot L^{-1}$	0.009 $mg \cdot L^{-1}$

Σ alkylphenols (methyl to butyl phenols, no nonylphenols present)	1.519 $mg \cdot L^{-1}$	0.008 $mg \cdot L^{-1}$	0.004 $mg \cdot L^{-1}$

*one PW sample with significantly higher concentrations compared to the other three

**compound only detectable in one sample from three tests

Figure Captions

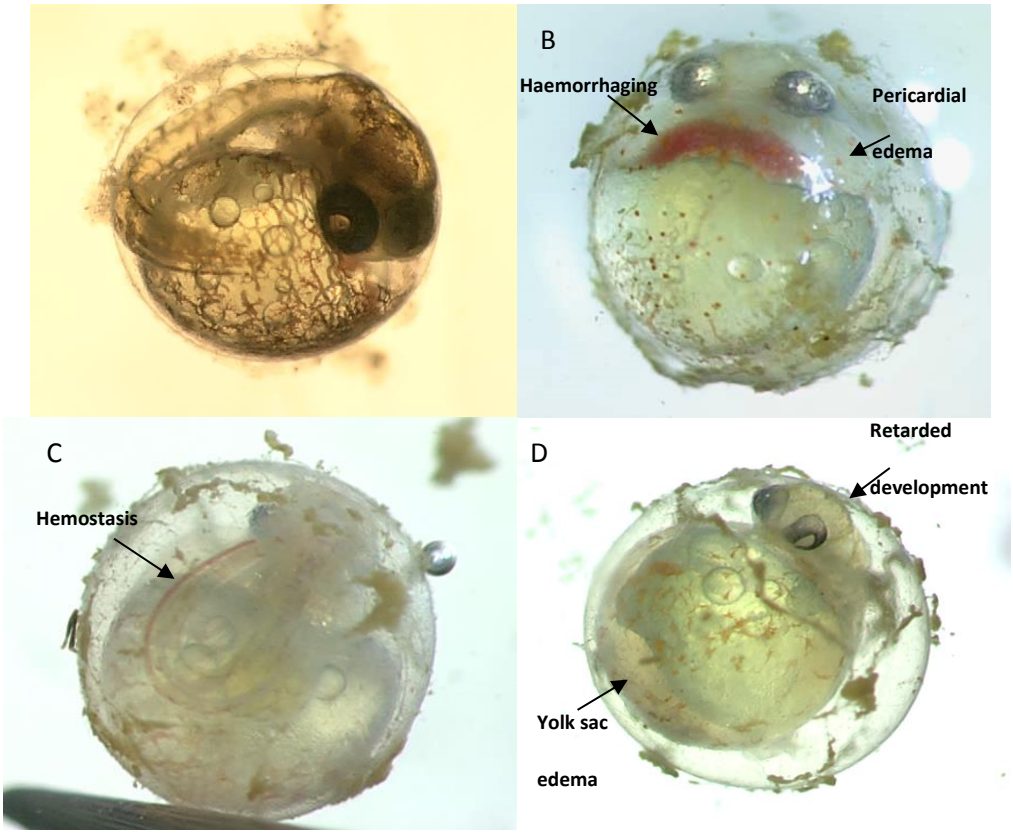

Fig. 1 Most common abnormalities observed in mummichog embryos exposed to graded doses of pure Hibernia PW from fertilisation: (A) normal embryo; (B) haemorrhaging and pericardial edema, left and right arrows respectively; (C) hemostasis along the tail; (D) retarded development and yolk sac edema, upper and lower arrows respectively; egg diameter is approximately 2 mm

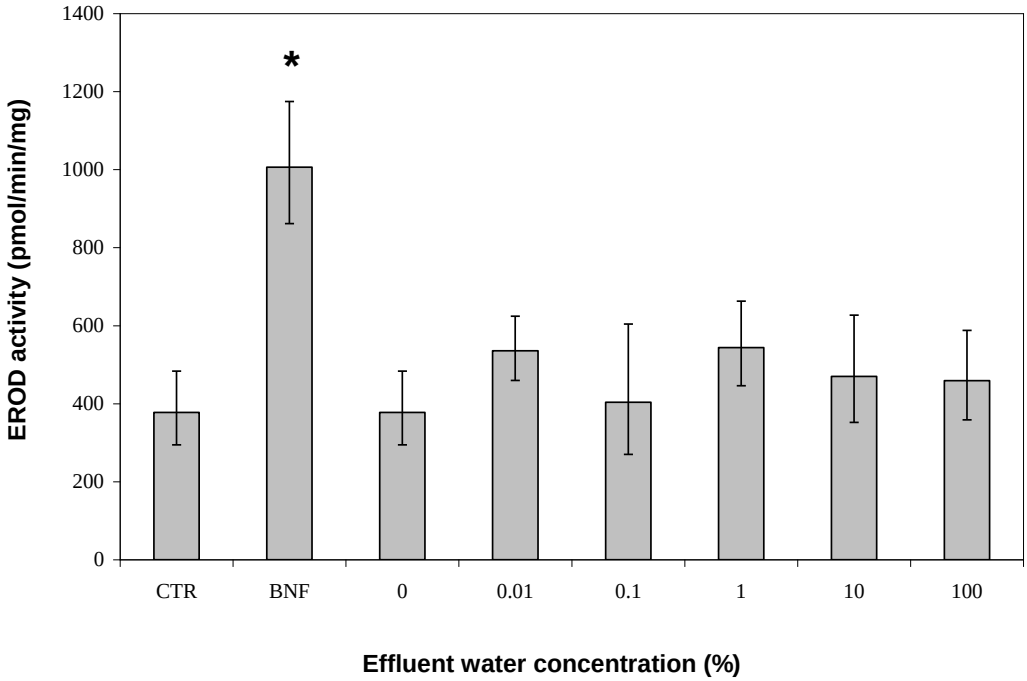

Fig. 2 Mean EROD activity (±95% confidence intervals) in mummichog (*Fundulus heteroclitus*) after a 24 h exposure to field collected water samples in the receiving environment of the Hibernia oil platform; asterisk indicates significant difference from water control (Nested ANOVA, replicates nested within treatments followed by Tukey's multiple range test); each treatment had 2 replicates of 5 fish each

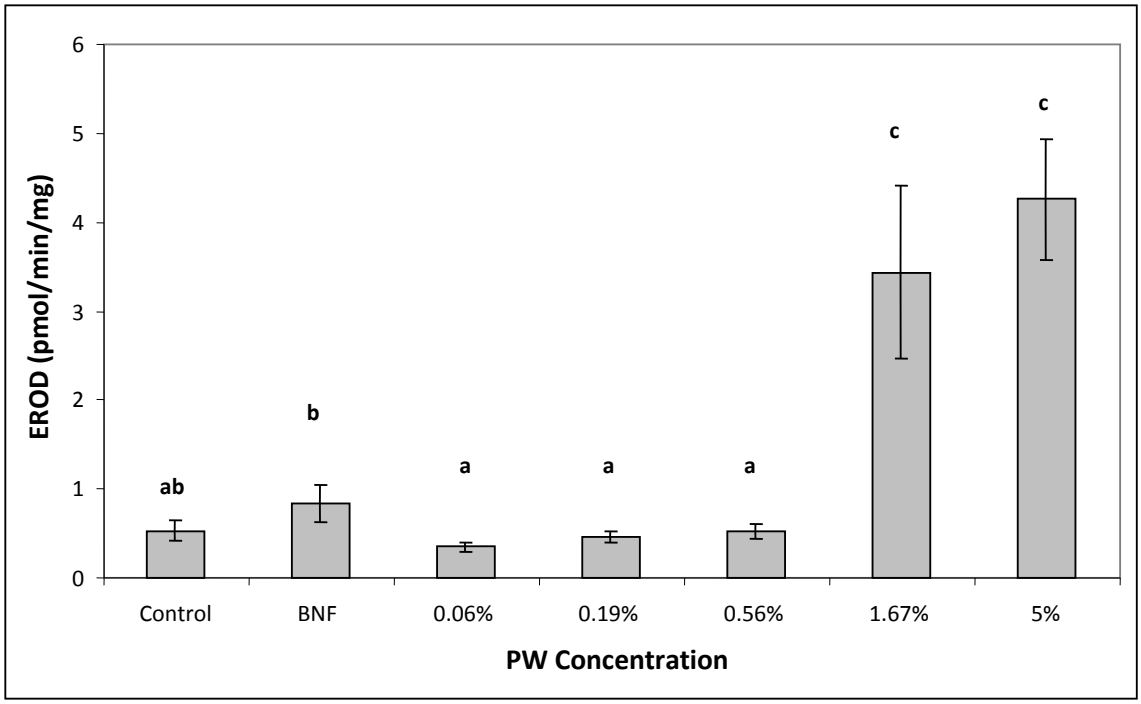

Fig. 3 EROD activity in livers collected from Atlantic cod exposed for 48 hr to produced water from the Hibernia platform (2006); each bar represents the mean value for a sample of 5 fish; bars identified with the same letter are not significantly different from each other (ANOVA, P<0.05)

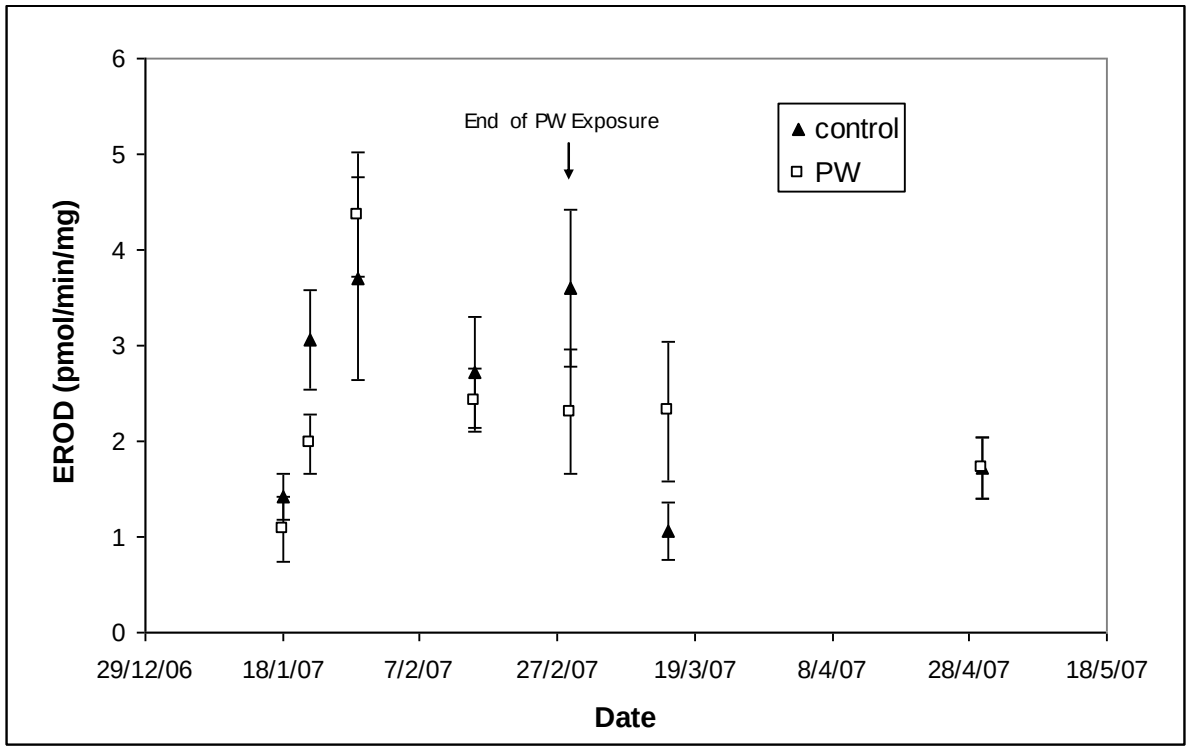

Fig. 4 EROD activity in livers collected from Atlantic cod chronically exposed for 45 days to 0.05% to PW from Hibernia (2006), then transferred to untreated water for 60 days; each data point represents the mean value for a sample of 8 fish

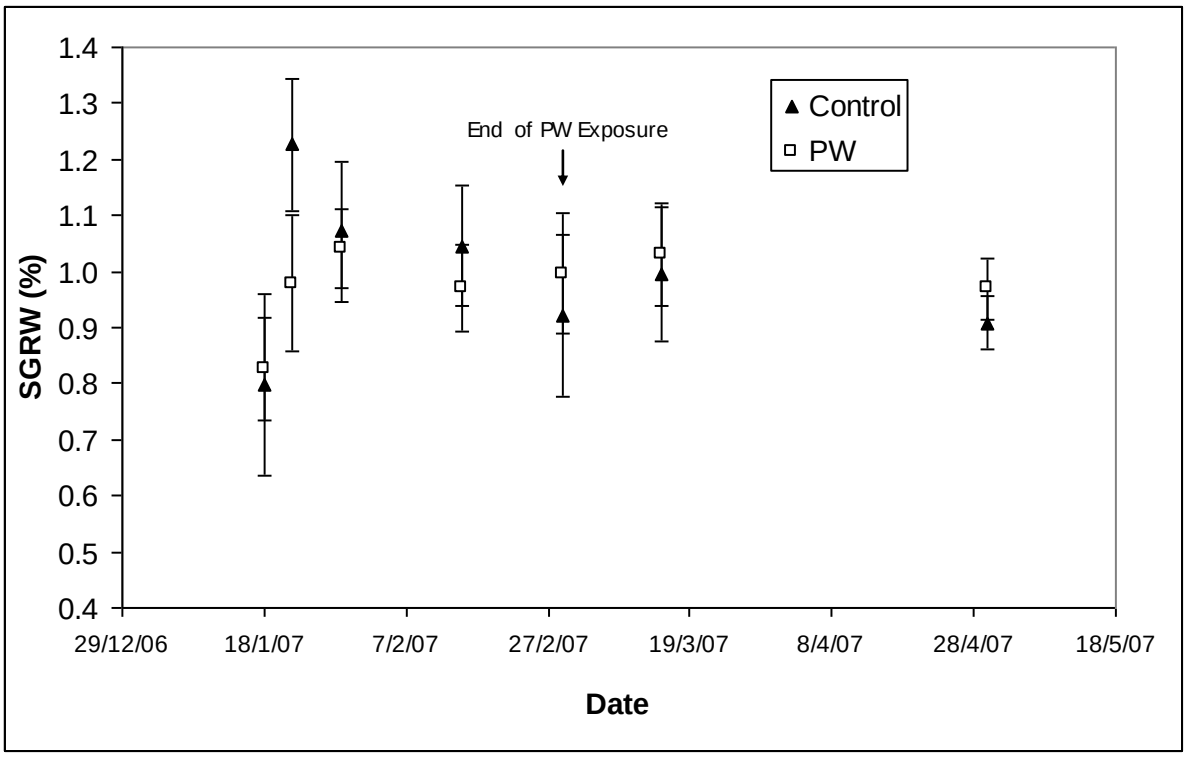

Fig. 5 Specific growth rate by weight (%) of juvenile cod exposed for 45 days to 0.05% PW from Hibernia (2006), then transferred to untreated water for 60 days; each data point represents the mean value from a sample of 8 fish