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In vitro Effect of Produced Water on Cod, *Gadus morhua*, Sperm Cells and Fertilization

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Abstract The in vitro effect of produced water released by oil and gas platforms was assessed by exposing cod sperm cells to realistic concentrations of this mixture (100, 200, 500 ppm). We investigated produced water impact on enzymes of the aerobic (citrate synthase) and glycolytic metabolism (lactate dehydrogenase), lipid catabolism (lipase), as well as an anti-oxidant enzyme (catalase). Fertilization rates, viability, respiration, ATP, and total motility duration were also evaluated. To explore correlations between these parameters, we have also tested the effect of conserving sperm for 24 h at 4°C. After conservation, fertilization success was decreased but other parameters were not affected. Produced water did not result in a significant change in fertilization; a significant increase in sperm protein amounts and citrate synthase activity can be observed. No correlations are found between parameters showing that sperm viability and unchanged energy levels do not translate into equivalent fertilization capacity. To conclude, exposure of sperm to produced water resulted only in subtle effects on cells. These findings bring information on the effect of produced water on sperm itself rather than on spermatogenesis or testis development of an exposed fish.

Keywords Produced water · In vitro · Sperm · Cod

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Produced water (PW) is the largest volume waste stream in the offshore oil exploration and production processes. PW dispersion modeling shows that dilution by at least 240 times occurs within 50–100 m, and up to 9,000 times by 20 km from the discharge (Somerville et al. 1987). Because Atlantic cod fertilization occurs in the water and cod spermatozoa can stay motile and fertile for a long time (e.g., Trippel and Morgan 1994), sperm cells may enter in contact with PW and be affected by it. Previous studies on catfish (*Claria gariepinus*) spermatozoa (Mansour et al. 2003), as well as on cyprinid and salmonid sperm cells (Lahnsteiner et al. 1999), have demonstrated that the sperm plasma membrane is highly permeable to different types of metabolites. PW contains compounds of environmental concern such as aromatic and aliphatic hydrocarbons, phenols (in particular alkylphenols that can cause endocrine disruption), metals, and traces of chemicals added during production (Roe 1998). Pollutants can cause reproductive dysfunction either by direct action (toxicity) on the gametes, or indirectly by modulation of the endocrine system. For example, studies on alkylphenols (APs) endocrine effects in fish, including APs present in PW (Meier et al. 2007), have shown a direct cytotoxic effect of these compounds on gametes (Kinnberg et al. 2000). Thus, it is of importance to examine both the possibility that environmental estrogens disrupt spermatogenesis as well as the possibility of direct effects on the motility and fertilizing ability of mature sperm after ejaculation (Hara et al. 2007).

In this study, we have focused on the effect of PW on spermatozoa by exposing sperm cells in vitro to environmentally relevant concentrations of PW. Despite their simple morphological construction, spermatozoa have a complex capacity for glycolysis, oxidative phosphorylation, as well as lipid anabolism and catabolism (e.g.,

Lahnsteiner et al. 1999). We have investigated PW effects on sperm enzymes of the aerobic (citrate synthase) and glycolytic metabolism (lactate dehydrogenase), as well as an enzyme involved in lipid catabolism (lipase). Fertilization rates, viability, respiration, ATP levels, and total motility times were also assessed. In addition, the activity of an anti-oxidant enzyme (catalase) was measured. Some of the parameters studied here are still totally unknown in cod sperm. Therefore, to explore correlations between these parameters, we have also tested the effect of a "treatment" on these same biomarkers of sperm quality. The treatment is conserving samples for 24 h at 4°C to help identify correlations and understand how some of these parameters may change together. If strong correlations are found among parameters, this will allow us to better explain the effect of PW on more than one biomarker.

Materials and Methods

Sperm samples were collected by stripping 10 adult Atlantic cod males (5–7 years old) originally from Bay Bulls (Newfoundland, Canada). Fish were held in 35 m^3 tanks supplied with flow-through seawater at a density of approximately 20 kg/m^3 at the Joe Brown Aquatics Research Building (Memorial University of Newfoundland) for the purpose of a broodstock development project. These broodfish were fed to satiation bi-weekly on a diet of baitfish (herring, mackerel and squid) supplemented with a marine broodstock vitamin and mineral premix pellet. PW was sampled in July 2008 and kept in Nalgene high density polyethylene jerricans provided to the Hibernia platform (Newfoundland, Canada) staff for collection. 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 on board the oceanographic vessel CCGS Hudson (Fisheries and Oceans Canada) as soon as logistically possible. PW was conserved in the fridge and transferred to the Northwest Atlantic Fisheries Center and used in less than week (at 4°C) to initiate in vitro sperm incubations. PW was analyzed at the Bedford Institute of Oceanography (Halifax, Canada) by GC-MSD (Perez-Casanova et al. 2010). The analytical procedure used for chemical analyses is based on a modified version of US EPA Method 8270C (1996) (for phenols and hydrocarbons). The method detection limits are <50 part-per-billion for PAHs and alkylated PAHs and <5 part-per-billion for phenols and alkylphenols. All sperm samples were diluted 50-fold in either seawater (control) or three different PW dilutions: 10,000× dilution (100 ppm), 5,000× dilution (200 ppm), and 2,000× dilution (500 ppm). Evaluation of motility duration was initiated as soon as sperm cells were in contact with seawater/PW while other measurements were performed after 1 h incubation in seawater/PW at 4°C. Respiration, motility, viability and fertilization percentage evaluation were performed on the day of sampling (after 1 h of incubation). Samples for ATP and enzymatic measurements were stored at −70°C until further analysis. Sperm samples were also collected to test the effect of fridge conservation. After 24 h in the fridge, viability, fertilization, respiration and motility duration were evaluated again (using the same female as the day before) while the remaining samples were frozen at −70°C for the other measurements.

Respiration was monitored for approximately 15 min using a YSI 5300A biological oxygen monitor as described by other authors (Lahnsteiner et al. 1999; Mansour et al. 2003). Results are expressed in % O_2/min/10^7 cells (cell counts were performed using a hemocytometer). To minimize egg quality effect, fertilization rates were determined by obtaining eggs from one female only. 100 µl of eggs were dry fertilized with 5 µl of sperm, and seawater or PW dilutions were added according to the protocol described by Lush (2005). Dry fertilization is a method of fish egg fertilization performed in vitro, whereby eggs are artificially fertilized in the laboratory after removing the gametes from the fish manually. This is accomplished by placing sperm directly on the eggs and subsequently adding seawater to activate (mobilize) the sperm. Fertilization occurs in an artificial vessel such as a petri dish or beaker. Fertilization rates (in percentages) were evaluated after 24 h at 4°C by counting the number of eggs with visible cell division. Motility duration was determined with the use of a compound light microscope and recorded as the time from activation to when 100% of sperm cells showed only vibratory movement. Sperm viability was evaluated by flow cytometry using a Live/Dead® Sperm Viability kit (L-7011) by molecular probes. For enzymatic analyses, frozen sperm samples were thawed and gently homogenized and centrifuged at 14,000*g* for 10 min (Gronczewska et al. 2003). The supernatants were used for enzymatic determination. All assays were done in duplicate and at room temperature. Lactate dehydrogenase (LDH) was assessed using the method of Mitchell et al. (1980). Citrate synthase (CS) was analyzed using SIGMA Kit CS 0720, while lipase (LIP) was analyzed by the use of a colorimetric Pointe Scientific kit P803-L7503-01. Catalase (CAT) levels were determined according to the method described by Chance and Herbert (1950). Total protein concentration was determined using Lowry's method (1951). All enzymatic activities are expressed in moles/min/10^9cells or units/10^9 or 10^{12}cells. ATP was determined with the use of a SIGMA kit FL-AA, with sample preparation before freezing as described in Perchec et al. (1995). All statistical analysis was performed using Sigma Stat

software. For PW exposures, data was compared using a one way ANOVA. When applicable, pair-wise multiple comparisons were performed with the use of the Holm–Sidak procedure. All data pertaining to the effect of fridge conservation were compared using a *t* test. Percentages (fertilization, viability) were transformed using Arcsin square root transformation. To better understand if a change in one of the sperm parameters would result in changes in the other parameters we chose to impose a "treatment" to the sperm samples. The treatment chosen is conservation of sperm for 24 h at 4°C. We explored potential correlations between sperm parameters within one data set (fresh or fridge) as well as both data sets together (fresh and fridge). This may allow us to identify correlations that might be masked by the natural individual variability in the data. Correlations between sperm parameters were assessed by applying the Pearson Product Moment Correlation procedure with subsequent Bonferroni adjustment of probabilities.

Results and Discussion

Chemical analysis of PW sample is summarized in Table 1 [results already cited in Perez-Casanova et al. (2010). The detailed analyses of individual polyaromatic hydrocarbons (PAHs) and alkanes that are part of the composition of PW are not presented to maintain reasonable content. Only main classes of known toxic components of PW are presented here to provide information for the interpretation of potential effects.

After exposure to PW, a significant increase is observed in sperm protein amounts and CS activity (Table 2). Holm–Sidak comparisons of CS and protein values show that significant differences are found between control values and the highest dose of PW (500 ppm). In a study on Artic char (*Salvelinus alpinus*) sperm conservation, Mansour et al. (2008) demonstrated that the addition of seminal plasma protein to a semen extender had a deleterious effect on post thaw sperm viability and fertility. Fluorometry results indicated that sperm membrane damage was higher in semen frozen in extender containing seminal plasma protein than in semen frozen in extender only. Some catalytic enzymes (especially proteases), which might be included in protein fractions, might have been activated, thus inducing a deleterious effect on sperm cells (Mansour et al. 2008). The increase in protein amounts seen after exposure to PW may provoke a potential deleterious effect of PW in cells. Nonetheless, none of the other endpoints investigated show a toxic effect on sperm cells. CS is traditionally localized in the mitochondrial matrix of cells and may be considered as a marker for mitochondrial volume (Phillips et al. 2000). Burness et al. (2005) also showed that in bluegill (*Lepomis macrochirus*) ATP levels early in motility were related to the activity of creatine phosphokinase and CS. In a study on the newt (*Cynops pyrrhogaster*) Harada et al. (2007) have shown that CS may play dual roles: as the mitochondrial enzyme initializing the tricarboxylic acid cycle and as a sperm factor for egg activation. Some CS proteins are localized in a region where no mitochondria are detected indicating that CS is also present outside the mitochondria potentially taking on other roles (Harada et al. 2007). The fact that the observed CS increase in this study did not translate into a change in respiration or ATP may infer that CS could be involved in mechanisms other than its known mitochondrial role. More studies on the roles of CS in fish are necessary to conclude on that point.

This study also shows that biochemical and motility parameters investigated are not affected by storage at 4°C but that fertilization success is decreased (Table 3). These results confirm knowledge on the longevity of cod sperm (e.g., Trippel and Morgan 1994) but show that sperm viability and unchanged energy levels do not translate into equivalent fertilization capacity. It is also worth noting that even if eggs are taken from the same female, a batch effect between day 1 and day 2 (Hamoutene et al. 2009) may still have taken place and explain part of the observed difference in fertilization.

Potential correlations between all parameters were explored with the use of Pearson Product Moment Correlation and showed no significant correlations. Finding correlations (positive or negative) between sperm enzymatic determinations and fertilization has proven to be difficult despite the fact that both are involved in similar processes (Lahnsteiner et al. 1999). In a study on genistein (a phyto-estrogen) in catfish (*Ictalurus punctatus*) and walleye (*Sander vitreus*), Green and Kelly (2008) have found that observed declines in ATP content and motility time reductions with increasing genistein concentrations resulted in decreased fertilization rates in channel catfish only. A species-dependent pattern may exist in how parameters are correlated with fertilization rates rendering generalizations on correlations difficult to do. Moreover, there is considerable variation among species in the

Table 1 Composition of produced water sampled in July 2008 from Hibernia offshore platform

PW component	Concentration (ppb)
Alkanes	1175.54
PAHs	69.46
Methylated PAHs	38.41
Total phenols	6558.96
Alkylphenols	3207.30

Analysis was done by GC–MSD. Values are mean of n = 2 samples

Table 2 Sperm parameters after produced water exposure (values are mean ± standard deviation, n = 10)

	Control	100 ppm	200 ppm	500 ppm
VIAB (%)	88.73 ± 9.65	88.29 ± 6.47	86.25 ± 12.68	92.27 ± 5.59
RESP (%O_2/10^9cells)	0.04 ± 0.03	0.04 ± 0.02	0.03 ± 0.02	0.06 ± 0.05
MOT (min)	19.00 ± 5.68	20.00 ± 8.16	20.00 ± 8.16	19.00 ± 8.76
Fertilization (%), $n = 6$	78.00 ± 11.93	69.00 ± 11.98		74.33 ± 7.94
PROT (mg/10^9cells)	**0.48 ± 0.27**	**0.36 ± 0.20**	**0.59 ± 0.23**	**0.88 ± 0.25***
ATP (pmol/10^9cells)	5.06 ± 4.41	5.96 ± 7.07	5.76 ± 4.47	5.83 ± 4.78
CS (mmol/min/10^9cells)	**20.40 ± 9.50**	**19.10 ± 12.00**	**23.20 ± 6.68**	**30.30 ± 11.60***
CAT (unit/10^9cells)	9.13 ± 5.92	9.15 ± 7.95	11.72 ± 9.50	10.16 ± 6.28
LDH (nmol/min/10^9cells)	1.04 ± 0.76	1.13 ± 0.86	1.20 ± 0.57	0.82 ± 0.56
LIP (unit/10^{12}cells)	2.90 ± 1.64	3.66 ± 2.12	4.02 ± 3.28	3.97 ± 2.72

Values in bold indicate significant differences ($p < 0.05$) after one way ANOVA

VIAB viability, *RESP* respiration, *MOT* motility duration, *PROT* proteins, *CS* citrate synthase, *CAT* catalase, *LDH* lactate dehydrogenase, *LIP* lipase

* Significant differences ($p < 0.05$) after application of the Holm–Sidak method for multiple comparison (when compared to control value)

Table 3 Sperm parameters after conservation at 4°C for 24 h (values are mean ± standard deviation)

	Day 1	Day 2 (after fridge conservation)
VIAB (%), n = 6	90.70 ± 4.13	96.30 ± 2.40
RESP (%O_2/10^7 cells), n = 6	0.015 ± 0.007	0.018 ± 0.020
MOT (min), n = 6	19.0 ± 5.7	34.0 ± 30.6
Fertilization (%), n = 6	78.00 ± 11.93	52.67 ± 10.17*
PROT (mg/10^9 cells), n = 16	0.55 ± 0.23	0.66 ± 0.26
ATP (pmol/10^9 cells), n = 16	11.52 ± 13.41	7.68 ± 4.77
CS (mmol/min/10^9cells), n = 16	7.31 ± 3.65	11.97 ± 9.86
CAT (unit/10^9cells), n = 16	3.75 ± 2.49	3.77 ± 3.02
LDH (nmol/min/10^9 cells), n = 16	2.35 ± 1.62	2.25 ± 1.62

VIAB viability, *RESP* respiration, *MOT* motility duration, *PROT* proteins, *CS* citrate synthase, *CAT* catalase, *LDH* lactate dehydrogenase

* Significant differences ($p < 0.05$) after *t* test application

identity of fuels used to support motility (Mansour et al. 2003). As stated earlier, the significant decrease found in fertilization rates after conservation at 4°C was not accompanied by changes in any of the other parameters. Hara et al. (2007) have shown an effect of nonylphenol (NP) on the average swimming speed of spermatozoa. Nonetheless, no evaluation of fertilization was done as part of their study. Moreover, the NP concentrations used were higher than the NP concentrations found in natural aquatic environments (from 0.23 to 96 nmol/L) (Hara et al. 2007). The PW dilutions used in our experiment are environmentally relevant and translate to an exposure range of 0.2–1.6 ppb of APs (1.36–7.28 nmol/L). No comprehensive evaluation of motility was completed as part of our study suggesting that an effect on velocity still needs to be investigated. To conclude, exposure of sperm cells to environmentally relevant concentrations of PW does not result in a strong toxicity to cells but some subtle effects can be seen. The apparent inconsistency of the recorded changes as well the lack of correlation between parameters renders the formulation of a hypothesis on the effect of PW on sperm metabolism difficult. Nonetheless, these findings bring information on the effect of PW on the sperm itself rather than on spermatogenesis or testis development of an exposed fish. This study highlights the importance of further exploring the effect of PW on sperm motility and metabolism.

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