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RESEARCH ARTICLE

Impact of dibutyl phthalate and benzyl butyl phthalate on motility parameters of sperm from the European pikeperch *Sander lucioperca* (L.)

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Abstract. The present study was the first attempt to evaluate the impact of dibutyl phthalate (DBP) and benzyl butyl phthalate (BBP) at concentrations of 100, 1000, and 10000 μg DBP dm^{-3} and 20, 200, and 2000 μg BBP dm^{-3} on the motility parameters of the sperm of European pikeperch, *Sander lucioperca* (L.), using computer assisted sperm analysis (CASA). The *in vitro* studies were conducted in the seminal plasma, which is the natural environment of the sperm, using two methods. The first was with the immediate activation of sperm movement in the presence of phthalates (0 h), while the second utilized phthalate incubation for 4, 24, and 48 h. It was confirmed that none of the compounds analyzed had a significant impact on the percentage of motile sperm (MOT), average path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), or amplitude of lateral sperm head displacement (ALH) ($P > 0.05$). It is plausible that in natural aquatic ecosystems, neither dibutyl phthalate nor benzyl butyl phthalate will affect motility of fish sperm at external fertilization event. It is necessary to track *in vivo* the impact these compounds have in concentrations similar to existing in the natural environment on the fish sperm quality.

Keywords: xenobiotic, dibutyl phthalate, benzyl butyl phthalate, sperm, CASA, *Sander lucioperca*

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Introduction

Plasticizers, which are used to modify the properties of polymers, are the largest group of compounds used in the manufacture and processing of synthetic materials (with an approximate 55% share of the global market; Bortel 2008). The most commonly used plasticizers are diester phthalates such as dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), and di-2-ethylhexyl phthalate (DEHP), diethyl phthalate (DEP), and diisononyl phthalate (DINP) (Koch et al. 2003). These compounds do not bind chemically with other polymers, and since they only dissipate through their matrixes, they migrate easily into the natural environment. Concentrations of DBP in the surface waters of the United States and Europe range from 0.01 to 622.9 μg dm^{-3}, while those of BBP range from 0.01 to 49.0 μg dm^{-3} (Fatoki and Vernon 1990, Wypych 2004). The solubility of the two phthalates in water (at a temperature of 25°C) is relatively low at 11.2 mg dm^{-3} for DBP (Staples et al. 1997) and 2.7 mg dm^{-3} for BBP (CMA 1999). At their

respective solubility limits in water, both of these compounds are highly toxic to fish. The DBP lethal concentration (that which results in 50% fish death (LC50)) was determined in 96-hour static tests to range from 0.48 (for bluegill, *Lepomis macrochirus* Raf.) to 1.54 mg dm^{-3} (for fathead minnow, *Pimephales promelas* Raf.). However, the LC50 for BBP ranged from 0.68 (for sheepshead minnow, *Cyprinodon variegatus variegatus* Lacepéde) to 1.70 mg dm^{-3} (for bluegill) (Adams et al. 1995).

The sperm of teleost fish are extraordinarily sensitive to a range of environmental factors. This is due to external fertilization process in which sperm are released outside the animal body and remain unprotected in surrounded environment during all steps of fertilization. Their metabolic capabilities are limited to the production of energy and processes regulating movement (Lahnsteiner et al. 1999). Foreign substances that pollute aquatic basins can have a direct impact on the ability of the sperm to fertilize egg cells. Additionally, fish that are exposed to xenobiotics chronically can store significant quantities of them in their bodies. It has been demonstrated that phthalates can reach the ejaculate of mammals from the nuclear or supranuclear plasma, the vas defernentia, the seminal vesicles, or from associated glands (Ong et al. 2002). Consequently, xenobiotics present in the gonads can impact the quality of sperm during maturation and when they are stored in the seminal vesicles.

Evaluations of the impact of xenobiotics on the semen quality of different fish species have focused mainly on the unlimited occurrence in waters of compounds such as heavy metals, nitrates, and nitrites (Kime et al. 1996, Epler et al. 2000, Chyb et al. 2000, 2001, Dietrich et al. 2004) and a few organic compounds including bisphenol A, 4-nonylphenol, and tributyltin (Rurangwa et al. 2002, Demska-Zakęś et al. 2005, Lahnsteiner et al. 2005a, 2005b).

The aim of the current study was to evaluate the *in vitro* impact of dibutyl phthalate and benzyl butyl phthalate on selected parameters of sperm motility in European pikeperch, *Sander lucioperca* (L.), at concentrations occurring in aquatic environments and at much higher ones bordering on their solubility in water.

Materials and methods

The biological material comprised semen collected from five European pikeperch spawners (aged 3+, body weight from 1.78 to 3.43 kg). The males had been reared from larvae at the Department of Sturgeon Fish Breeding, Inland Fisheries Institute in Olsztyn. The milt was obtained through out-of-season spawning in winter (Zakęś 2007). The fish were stimulated hormonally (single intraperitoneal injection) with human chorionic gonadotropin (200 IU hCG kg $m.c.^{-1}$). Twenty-four hours following injection, the milt was collected with a syringe in quantities of 2.1 to 3.0 cm^3, transferred to glass containers with a volume of 25 cm^3, and covered with polyethylene film. The containers were placed on a layer of ice (at a temperature of 4°C) and were transported to the Department of Gamete and Embryo Biology, Polish Academy of Sciences in Olsztyn (transport time was approximately two hours).

The concentration of sperm was determined in the initial semen samples using the method described by Ciereszko and Dąbrowski (1993). To do this, the milt samples were diluted with a 0.7% NaCl solution (Sigma-Aldrich) at a ratio of 1:1000. The absorbance of the samples was measured using a Beckman DU-640 spectrophotometer (Analytical Instruments, LLS, USA) at a wavelength of α = 530 nm. The results of the absorption measurements were fit to a standard curve that had been formulated previously for pikeperch based on a cytometric model (Bielański 1979), and then the concentration values were read. The initial concentration of sperm in five pikeperch semen samples ranged from 7.01 to 8.34×10^9 cm^{-3}.

The sperm motility parameters of MOT – percentage of motile sperm (%), VSL – straight line velocity (μm s^{-1}), VAP – average path velocity (μm s^{-1}), VCL – curvilinear velocity (μm s^{-1}), and ALH – amplitude of lateral sperm head displacement (μm), were analyzed with computer assisted sperm analysis (CASA). The analysis was conducted with two methods that simulate two different phenomena that occur in nature.

The direct impact of on sperm of xenobiotics present in the water was simulated by activating their motility in the presence of phthalates (experiment 1). To elicit the sub-lethal effects of pollution on the reproductive system (toxin bio-accumulation), semen was incubated with phthalates for 48 h (experiment 2). All of the tests were conducted in the semen plasma since it is the natural environment of the sperm.

Experiment 1: exposing fresh semen to phthalates

Five samples of fresh semen were subjected to short-term exposure to chosen phthalates. Two solutions were prepared containing mixtures of DBP or BBP and solvent (99.8% ethanol; the initial concentration of DBP was 1000 mg dm^{-3}, while that of BBP was 200 mg dm^{-3}). These were then diluted with the appropriate quantity of activating fluid (40 mM NaCl, 20 mM Tris, 0.5% bovine albumin BSA fraction V, pH 8.5), to obtain the final target concentrations of 100, 1000, 10000 μg DBP dm^{-3} and 20, 200, 2000 μg BBP dm^{-3}. The control group comprised semen mixed with ethanol and activating fluid. The final concentration of ethanol was 1%. Measurements of sperm motility immediately following activation (0 h) were recorded in two replicates.

Experiment 2: incubating semen with phthalates (bio-accumulation effect)

The solutions containing the phthalates were mixed with sperm motility immobilizing fluid (200 mM NaCl, 2 mM $NaHCO_3$, 10 mM KCl, 2 mM $CaCl_2$, 20 mM Hepes, pH 8.0). The solution obtained was mixed with semen at a ratio of 1:1. The final concentrations of phthalates were: 100, 1000, 10000 μg DBP dm^{-3} and 20, 200, 2000 μg BBP dm^{-3} respectively. The control group contained ethanol (1%). Sample incubation was conducted in an oxygen atmosphere at a temperature of 4°C. Sperm motility was activated three times, at 4, 24, and 48 h following incubation in the respective doses of xenobiotic. All of the measurements were taken in two replicates.

Evaluating the motility parameters of sperm using CASA

Just prior to recording, 1 mm^3 of semen was placed in the well of a microscope slide and sperm motility was induced with activation fluid. The activity was recorded with a Basler a202K digital camera (Basler, Germany) integrated with an Olympus BX51 microscope (Olympus, Japan) (objective with 20x magnification). The film speed was 47 frames s^{-1}. Sperm movement was recorded for 8 s following activation. The first 200 frames from each recording were analyzed using Crismas (Image House, Germany).

Statistical analysis

The mean values of fresh semen quality immediately following the activation of the sperm (experiment 1) were compared with Levene's test, single factor analysis of variance ANOVA, and Tukey's post hoc test. The mean values of the quality parameters of semen after incubation (experiment 2) were analyzed with multi-factor analysis of variance MANOVA, taking into consideration the impact of exposure time (T), dose (D), and dose and time (D x T). All of the tests were performed at a level of significance of $\alpha < 0.05$ using STATISTICA 7.0.

Results

Experiment 1: exposing fresh semen to phthalates

In the control group, MOT was 27.24%, VCL – 66.29 μm s^{-1}, VSL – 30.80 μm s^{-1}, VAP – 50.36 μm s^{-1}, while ALH was a mean of 0.7 μm. The tested concentrations of DBP were not noted to significantly impact the parameters analyzed. The percentage of motile sperm moving with an average velocity of 45.3-53.6 μm s^{-1} oscillated within the range of 24.6-31.4% (Table 1). BBP also did not have a negative impact on the motility of the sperm that were tested immediately after the phthalates were mixed with the semen (Table 2). All of

Table 1
Impact of dibutyl phthalate (DBP) on selected parameters of European pikeperch sperm motility (means (SD)). Parameters analyzed immediately after DBP was mixed with fresh sperm 8 s following the activation of sperm motility. No significant differences were noted among groups (P > 0.05) (N = 5). MOT – percentage of motile sperm. VCL – curvilinear velocity, VSL – straight line velocity, VAP – average path velocity, ALH – amplitude of lateral sperm head displacement

	Concentration of dibutyl phthalate (μg DBP dm^{-3})			
Parameter	0	100	1000	10000
MOT (%)	27.24 (14.59)	24.62 (12.10)	31.39 (14.49)	27.40 (8.23)
VCL (μm s^{-1})	66.29 (8.64)	52.69 (25.60)	66.12 (18.00)	54.68 (7.99)
VSL (μm s^{-1})	30.80 (3.87)	27.90 (11.77)	29.20 (7.79)	24.29 (8.65)
VAP (μm s^{-1})	50.36 (7.32)	45.32 (16.64)	53.60 (9.37)	43.56 (3.84)
ALH (μm)	0.70 (0.14)	0.56 (0.31)	0.72 (0.14)	0.58 (0.17)

Table 2
Impact of benzyl butyl phthalate (BBP) on selected parameters of European pikeperch sperm motility (means (SD)). The parameters were analyzed immediately after mixing BBP with fresh sperm 8 s following the activation of sperm motility. No significant differences were noted among groups (P > 0.05) (N = 5). See Table 1 for definitions of abbreviations

	Concentration of benzyl butyl phthalate (μg BBP dm-3)			
Parameter	0	20	200	2000
MOT (%)	27.24 (14.59)	28.38 (11.62)	23.08 (10.15)	22.32 (10.66)
VCL (μm s^{-1})	66.29 (8.64)	68.97 (21.66)	66.25 (15.98)	61.13 (24.58)
VSL (μm s^{-1})	30.80 (3.87)	37.56 (19.89)	38.42 (12.98)	33.06 (14.22)
VAP (μm s^{-1})	50.36 (7.32)	56.70 (21.49)	53.93 (13.46)	49.57 (17.22)
ALH (μm)	0.70 (0.14)	0.71 (0.23)	0.67 (0.18)	0.66 (0.39)

the parameters tested were close to those of the control group (P > 0.05).

Experiment 2: incubating semen with phthalates (bio-accumulation effect)

None of the concentrations of DBP or BBP tested had an impact on the pikeperch following 4 and 24 h of exposure (Tables 3 and 4). The highest values of the parameters tested were noted in the control group. Along with increasing concentrations of xenobiotics, a decreasing trend was noted in all of the parameters analyzed, and this was particularly pronounced following 24 h of incubation. However, because of the large differences among individuals in the various sperm motility parameters, the differences between groups were not significant (P > 0.05). No significant interactions were noted between exposure times and the phthalate concentrations applied and the values of the parameters tested. Only the incubation time was identified as a factor that influenced semen quality. After the pikeperch milt had been incubated for 24 h with DBP or BBP, the number of motile sperm decreased significantly (MOT; P < 0.01) with a simultaneous increase in average path velocity and straight line velocity (P < 0.05) in comparison to the values of these parameters obtained after 4 h of exposure (Tables 3 and 4). The analysis of the various motility parameters following 48 h of incubation was impossible because of the low percentage of motile sperm in the control and experimental groups (from 0 to 2%).

Table 3
MANOVA of overall differences and sperm motility parameters (means (SD)) after 4- and 24-hour exposure of pikeperch sperm to dibutyl phthalate (DBP) (N = 5). See Table 1 for definitions of abbreviations

Concentration DBP ($\mu g\ dm^{-3}$)	MOT (%)		VCL ($\mu m\ s^{-1}$)		VSL ($\mu m\ s^{-1}$)		VAP ($\mu m\ s^{-1}$)		ALH (μm)	
	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h
0	53.52 (12.58)	29.36 (17.08)	107.21 (7.75)	122.8 (37.22)	49.27 (10.99)	68.34 (34.63)	74.49 (10.65)	101.51 (41.33)	1.19 (0.08)	1.32 (0.30)
100	49.15 (6.76)	23.81 (5.99)	91.03 (12.14)	104.3 (36.29)	39.17 (13.28)	52.21 (26.17)	62.14 (14.69)	81.78 (37.24)	1.10 (0.12)	1.08 (0.26)
1000	48.51 (20.64)	19.35 (3.96)	93.10 (10.78)	108.9 (23.26)	36.69 (7.76)	58.76 (9.99)	61.67 (11.12)	81.65 (15.33)	1.11 (0.11)	1.12 (0.22)
10000	51.76 (14.27)	19.68 (5.75)	99.99 (21.62)	92.7 (28.58)	39.96 (9.39)	41.53 (19.41)	67.86 (19.69)	69.02 (22.58)	1.17 (0.19)	1.02 (0.31)
MANOVA	F	P	F	P	F	P	F	P	F	P
Dose (D)	0.68	0.5688	1.21	0.3237	1.66	0.1952	1.32	0.2836	1.29	0.2954
Time (T)	50.68	0.0000	1.44	0.2393	5.54	0.0248	4.90	0.0339	0.01	0.9064
D x T	0.22	0.8837	0.51	0.6778	0.58	0.6290	0.52	0.6695	0.70	0.5592

Table 4
MANOVA of overall differences and sperm motility parameters (means (SD)) after 4- and 24-hour exposure of pikeperch sperm to benzyl butyl phthalate (BBP) (N = 5). See Table 1 for definitions of abbreviations

Concentration BBP ($\mu g\ dm^{-3}$)	MOT (%)		VCL ($\mu m\ s^{-1}$)		VSL ($\mu m\ s^{-1}$)		VAP ($\mu m\ s^{-1}$)		ALH (μm)	
	4h	24h	4h	24h	4h	24h	4h	24h	4h	24h
0	53.52 (12.58)	29.36 (17.08)	107.21 (7.75)	122.76 (37.22)	49.27 (10.99)	68.34 (34.63)	74.49 (10.65)	101.51 (41.33)	1.19 (0.08)	1.32 (0.30)
20	55.84 (10.90)	24.19 (16.98)	108.40 (14.12)	111.42 (30.41)	47.34 (14.52)	51.43 (14.13)	73.95 (15.87)	89.49 (24.52)	1.21 (0.06)	1.20 (0.25)
200	48.47 (15.47)	27.05 (9.41)	92.20 (19.91)	105.95 (25.78)	36.21 (11.11)	54.63 (19.43)	61.91 (14.69)	87.55 (22.72)	1.07 (0.22)	1.09 (0.30)
2000	54.62 (9.01)	17.46 (10.85)	87.87 (9.03)	89.80 (34.63)	32.69 (6.02)	39.09 (17.34)	58.41 (8.38)	70.29 (28.77)	1.07 (0.11)	0.99 (0.42)
MANOVA	F	P	F	P	F	P	F	P	F	P
Dose (D)	0.33	0.8035	2.21	0.1058	2.81	0.052	1.91	0.1478	1.87	0.1539
Time (T)	47.28	0.0000	1.19	0.2826	4.48	0.042	7.45	0.0102	0.04	0.8434
D x T	0.74	0.5357	0.2	0.8926	0.48	0.698	0.26	0.8548	0.30	0.8245

Discussion

The *in vitro* tests performed for this study indicate that pikeperch sperm are highly tolerant of dibutyl and benzyl butyl phthalates even at concentrations that are nearly twenty-fold higher than their contents in surface waters. Since sperm motility lasts from 40 to 120 s (Sarosiek et al. 2004), the results of the current study permit concluding that phthalates potentially occurring in aquatic basins cannot disrupt the movement of the sperm to the egg during this short period of motility.

It should also be emphasized that pikeperch sperm cannot be used as a sensitive bio-marker for assessments of the risk of pollutants occurring in waters since the highest analyzed concentration of both

phthalates exceeded known lethal values for fish (Adams et al. 1995). Similar phenomena were observed by Lahnsteiner et al. (2004), who conducted wide-ranging studies on the impact of non-organic compounds (cadmium, copper, mercury, lead, zinc, and nitrites), and organic compounds such as cyclohexane and 2.4-dichlorophenol on the sperm motility parameters in four species of freshwater fish. It was noted that in the natural environment, only the preceding organic compounds occurred in concentrations that could significantly lower both sperm motility and velocity. The concentrations of the other compounds, which efficiently lower the quality of semen, significantly exceeded the values set for surface waters by the World Health Organization (Scholz 2000), and were usually lethal to fish. Simultaneously, it was also observed that the sperm of fish that are susceptible to pollution, such as brown trout, *Salmo trutta* m. *fario* L., and burbot, *Lota lota* (L.), are characterized by substantial resistance to environmental toxic compunds. It has been suggested that the high tolerance of the sperm of these fish also applies to other species of fish that require high quality water (Lahnsteiner et al. 2004). The current study of the semen of pikeperch, which is a species sensitive to pollution, confirms this dependence (Siwicki et al. 2003).

It has been demonstrated repeatedly that the detrimental impact of toxic chemicals becomes apparent only after longer exposure time. Significant sperm motility reduction was confirmed in African catfish, *Clarias gariepinus* (Burchell), after 24 h of incubation in mercury chloride at a concentration of just 0.001 mg dm^{-3} (Rurangwa et al. 1998), while the motility of goldfish, *Carassius auratus* L., sperm was affected at concentrations of 0.1 mg dm^{-3} (Van Look and Kime 2003). Decreases in the values of this parameter were also observed in African catfish sperm following 24 h of exposure to 0.27 μg dm^{-3} of tributyltin (Rurangwa et al. 2002). Exposing pikeperch sperm to dibutyl phthalate or benzyl butyl phthalate for 24 h did not cause a significant decrease in the parameters studied. However, it cannot be confirmed unequivocally that these compounds do not have a detrimental impact on fish gametes until additional *in vivo* studies are conducted. It was demonstrated that when these phthalates are delivered *per os* to pikeperch during the sex differentiation stage, there is a delay in testis development and the feminization of the male gonads is induced (Jarmołowicz et al., unpublished data). Additionally, there is a link between the presence of some phthalates (including DBP) in mammals and oxidative stress caused by excess reactive oxygen species (ROS) (Barlow et al. 2003, Lehmann et al. 2004, Liu et al. 2005). In humans, ROS can cause lipid peroxidation in sperm cell membranes, changes in the structure of membrane receptors, and in enzyme and transport proteins, and it can also increase anomalies in chromatin structure and breaks in sperm DNA strands (Hughes et al. 1996).

In the current study, it was noted that as time passed there was a statistically significant decrease in the percentage of motile sperm, while there were increases in average path velocity and straight line velocity. Other researchers observed similar effects after short-term semen storage in an oxygen atmosphere (Lahnsteiner et al. 1996, 1998, Kowalski et al. 2004). The physiology of this phenomenon is not yet fully understood. Presumably, the heightened velocity of the sperm might be linked with hyperactivity in metabolism, which, in teleost fish, is oxidizing (Lahnsteiner et al. 1995, Lahnsteiner and Patzner 1997). This might have also resulted from the application of the immobilizing fluid.

The results obtained in the current study indicate that pikeperch semen is not a good bio-indicator of phthalate pollution in aquatic ecosystems. The high tolerance of pikeperch sperm to these compounds can likely be explained by the protective function of the seminal plasma, which plays a crucial role in fish that fertilize externally. Recently, Wojtczak and Ciereszko (2005) succeeded in isolating an enzyme belonging to the carboxylesterase group, which is linked to the bio-transformation and detoxification of organic xenobiotics. Seminal plasma improves the survival of sperm (metabolism and cell membrane integrity), motility ability, fertilizing ability, and it also protects the genetic material (Glogowski and Ciereszko 2007).

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Streszczenie

Wpływ ftalanu dibutylowego i benzylowobutylowego na parametry ruchu plemników sandacza europejskiego *Sander lucioperca* (L.)

W badaniach analizowano dodatek do nasienia *in vitro* ftalanów dibutylowego (DBP) i benzylowobutylowego (BBP) w stężeniach występujących w środowisku oraz w koncentracjach blisko granicy ich rozpuszczalności w wodzie na wybrane parametry ruchliwości plemników sandacza europejskiego, *Sander lucioperca* (za pomocą systemu CASA – *Computer-aided-sperm analysis*). Analizę przeprowadzono dwiema metodami. Zastosowano natychmiastową aktywację ruchu plemników w obecności ftalanów (0 h), przez co uzyskano efekt bezpośredniego wpływu ksenobiotyków na plemniki oraz inkubowano nasienie z ftalanami przez 4, 24 i 48 h w celu wywołania subletalnego efektu działania zanieczyszczeń na układ rozrodczy (efektu bioakumulacji toksykanta). Nie wykazano istotnego wpływu badanych stężeń DBP i BBP na parametry ruchu plemników sandacza. Odsetek ruchliwych plemników, poszczególne prędkości ruchu: krzywoliniowa (VCL), prostoliniowa (VSL) i całkowita (VAP) oraz amplituda odchyleń bocznych główki plemnika nie różniły się istotnie statystycznie od grupy kontrolnej, zarówno po zastosowaniu natychmiastowej aktywacji ruchu, jak i po 4 oraz 24 h inkubacji ($P > 0,05$). Na podstawie uzyskanych wyników można przypuszczać, że oba ftalany w naturalnych ekosystemach wodnych nie wpłyną na jakość nasienia oddawanego podczas aktu tarła, pośrednio na zdolność plemników do zapłodnienia komórki jajowej. Istnieje potrzeba prześledzenia wpływu ftalanów *in vivo* na jakość nasienia ryb w stężeniach występujących w środowisku wodnym.