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2013 Pavla Linhartová

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DIPLOMA THESIS

The effect of xenobiotics on DNA integrity and physiology of fish spermatozoa

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Zásady pro vypracování:

Nowdays more and more pollutants and toxic substances occur in the nature due to human activity and development of industry. The majority of these hazardous pollutants is soluble in water and accumulates in aquatic environment. Xenobiotics are a class of pollutants foreign to an entire biological system, which had not exist in nature before they were synthesized by human. Xenobiotics are known to enhance production of reactive oxygen species (ROS) that can attack a variety of biological macromolecules such as DNA, proteins and lipids, leading to oxidative damages. Thus water pollution could impair reproductive success of aquatic organisms through decreasing the quality of gametes, which in turn may affect fertilization success. The gametes of many fish species are released into water environment where they can be directly exposed to various pollutants that are capable to interrupt cellular metabolism on different levels. Therefore the goal of the present study will be to investigate effects of short-term (2 h) in vitro exposure to different xenobiotical compounds on quality parameters and oxidative stress in spermatozoa of two fish species, namely sterlet (*Acipenser ruthenus*) and brook trout (*Salvelinus fontinalis*).
During the master study student will prepare literature review considering the problematic of water pollution and its effects on reproduction of aqutic animals. Experiments will be performed on the basis of Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology in Vodnany. The student will learn methods of sperm motility assessment, analysis of oxidative stress parameters and antioxidant activity, techniques for evaluation of DNA damage, ATP content and protein analysis. Student will collect sperm from 6 adult males of sterlet and brook trout. Sperm samples will be exposed for 2 h to different concentrations of four xenobiotical compounds (duroquinone, bisphenol A, tetrabromobisphenol A and vinclozolin) and changes in sperm motility and velocity, oxidative stress indices, DNA fragmentation, ATP content and protein profiles will be analyzed. The results of diploma work will reveal the effect of environmentally related concentrations of xenobiotics on quality of fish spermatozoa.

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děkan

doc. Ing. Pavel Kozák, Ph.D.
ředitel

V Českých Budějovicích dne 3. února 2012

1 Introduction

Pollution of the aquatic environment with xenobiotics or pollutants has become a serious health concern in recent years. Nowdays more pollutants and toxic substances occur in the nature due to human activity and development of industry. The majority of these hazardous pollutants is soluble in water and accumulates in aquatic environment. Some of the most common xenobiotics occurring in surface waters are bisphenol A (BPA), tetrabrombisphenol A (TBBPA), vinclozolin (VIN) and duroquinone (DQ). As the majority of pollutants are discharged into free waters (rivers, lakes, coastal waters) fishes inhabiting these areas are often chronically exposed to excessive concentrations of toxic compounds. Apart from possible effects on the endocrine system leading to disruptions in reproductive physiology (Kime et al., 1999, Arukwe, 2011) gametes of fish with external fertilization can be impacted by the ambient environment. Disruption in gamete development or physiology may have a deleterious effect on reproduction, a prerequisite for survival of any aquatic species. During the past decade, toxicity tests involving fish gametes have received wide attention (Rurangwa et al., 2002, Li et al., 2010). Many aquatic organisms are nowadays threatened, because water pollution disrupts the balance and life cycle it the aquatic environment. That´s why many water animals, especially fishes, are involved in IUCN red list. Several studies have shown that ova are not ideal pollution bio-indicators since, at present, their quality can only be measured through time-consuming fertilization experiments. Moreover, the results of these experiments are subject to variability in sperm and uncontrolled factors in ova, such as over-ripening (Brooks et al., 1997). Toxicity screening using fish sperm has been considered as a simple and valid model, since it does not require expensive sterile cell culture conditions (Rurangwa et al., 2002).

The aim of this thesis was to observe the effect of xenobiotics (BPA, TBBPA, DQ and VIN) on DNA integrity, sperm quality parameters, and oxidative stress indices in sterlet and brook trout sperm. The level of oxidative stress indices (LPO, CP), superoxide dismutase activity (SOD) and ATP content were observed. It should be mentioned that all these compounds can induce ROS stress in fish spermatozoa, which could impair the sperm DNA integrity, quality and antioxidant defense system. In this

study quality of sperm was assessed by measurement of spermatozoa curvilinear velocity, percentage of motile sperm and the level of DNA damage expressed as a % DNA in Tail and Olive Tail Moment. Based on obtained results, the destroyable effect of BPA, TBBPA, DQ and VINwas confirmed on fish sperm. My results also suggeste that the use of fish spermatozoa for *in vitro* assays may provide a novel and efficiently means for monitoring residual pollution in aquatic environment.

2 Xenobiotics

It has been clearly established over the past 20 years that fish possess the ability to perform a wide variety of biotransformation reactions (Li et al., 2012, Thomas et al., 1998). Nowdays more pollutants and toxic substances, like xenobiotics, occur in the nature due to human activity and development of industry. Majority of these hazardous pollutants are soluble in water and accumulates in aquatic environment. Recent studies on the biotransformation of xenobiotic chemicals in fish have been focused on the specific metabolic products, since these metabolic reactions affect distribution, accumulation, and toxicity of chemicals (Hulak et al., 2008; Mikula et al., 2009). On the other hand, xenobiotics are also known to enhance production of reactive oxygen species (ROS) (Livingstone, 2001) that can attack a variety of biological macromolecules such as DNA, proteins and lipids, leading to oxidative damages (Kelly et al., 1998). Consequently, increased levels of ROS are believed to be involved in male infertility due to lipid peroxidation (LPO) and oxidative stress that affects sperm physiology and integrity (Rashmi et al., 2008).

All these used xenobiotics are soluble in water, what creates a huge problem for fishes and other water animals. These xenobiotics appeared in waters in not only low or normal concentrations, but in concentrations, which can endanger the health and the reproductive system of fishes. In recent studies next concentrations were used:

- For sterlet (*Acipenser ruthenus*):

- Duroquinone (DQ): 10, 50, 100 and 150 μM
- Tetrabrombisphenol A (TBBPA): 0.5, 1.75, 2.5, 5 and 10 μg/l
- Bisphenol A (BPA): 0.5, 1.75, 2.5, 5 and 10 μg/l
- Vinclozolin (VIN): 0.5, 2, 10, 15, 20 and 50 μg/l

➢ For brook trout (*Salvenilus fontinalis*):

- Duroquinone (DQ): 12.5, 25, 50, 100 and 150 µM
- Tetrabrombisphenol A (TBBPA): 1.75, 2.5, 5 and 7.5 µg/l
- Bisphenol A (BPA): 1.75, 2.5, 5 and 7.5 µg/l
- Vinclozolin (VIN): 0.1, 0.5, 2, 10 and 50 µg/l

2.1.1 Duroquinone (DQ)

Duroquinone (DQ, Fig. 1) is a derivate of 1, 4-benzoquinone which is well soluble in water and in low concentrations (50 - 150 µM) may have a deleterious effect on fish spermatozoa (Zhou et al., 2006, Linhartova P. et al., 2013).

Quinones are widespread in our environment, occurring both naturally and as pollutants. Quinones also form an important class of toxic metabolites generated as a result of the metabolism of phenols and related compounds, including phenol itself, 1-naphthol, and diethyl stilbesterol. Thus it can be said, that duroquinone can enter freshwater and marine ecosystems from man-made sources (Smith et al., 1985).

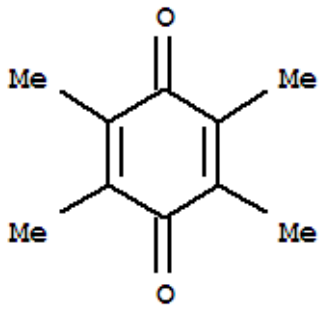


Fig.1: Duroquinone

2.1.2 Tetrabrombisphenol A (TBBPA)

Tetrabromobisphenol A (TBBPA, Fig. 2) is a brominated flame retardant and is a derivative of bisphenol A (BPA). Most commercial TBBPA products are of a relatively low purity, in fact containing a mixture of products brominated to varying extents. TBBPA can be used as reactive and additive flame retardant. In the reactive application, TBBPA is bound chemically to the polymers. The main use of TBBPA is epoxy resins

of printed circuit boards. As an additive flame retardant it is used in acrylonitrile butadiene styrene, which are used e.g. in TVs. The annual consumption worldwide has been estimated as 119,600 tons in 2001, of which 11,600 tons were used by the European industry. TBBPA emits from different processes to the environment and can be found in trace concentration in the atmosphere, hydrosphere, soil, and sediments. It also occurs in sewage sludge and house dust (Sellström and Jansson, 1995). TBBPA has been the subject of an eight year evaluation under the EU Risk Assessment procedure which reviewed over 460 studies. The Risk Assessment was published in the EU Official Journal in June 2008. TBBPA will now go through REACH registration. TBBPA is in the process of being classified in the EU as an R50-53 substance, which means that it is toxic to aquatic organisms, but it loses this classification when it is reacted into the printed circuit board resin, which represents more than 80% of its uses In fish, TBBPA is acutely toxic at low concentrations as shown in a number of studies. LC50 concentrations reported include 0.51 mg/l (0.9 μM) in bluegill sunfish, 0.40 mg/l (0.7 μM) in rainbow trout , and a relatively high 3 mg/l (5.5 μM) in zebrafish (Lee et al., 1993).

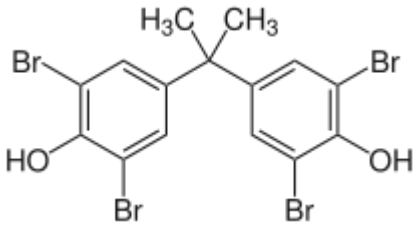


Fig.2: Tetrabrombisphenol A

2.1.3 Bisphenol A (BPA)

Bisphenol A (BPA, Fig. 3), a synthetic estrogen used to harden polycarbonate plastics and epoxy resin. Actually there is increased focus on this substance and growing number of research studies and legislative actions. Traceable BPA concentration exposure has been shown to disrupt the endocrine system and trigger a wide variety of disorders, including chromosomal and reproductive system abnormalities, impaired brain and neurological functions (Belfroid et al., 2002). The results of some recent studies using novel low-dose approaches and examining different endpoints describe subtle effects in laboratory animals at very low concentrations (Hatef et al., 2012; Lahnsteiner et al., 2005). Some of these low-dose studies are potentially of concern for the environment because the concentration levels identified with effects are similar to some current environmental levels to which sensitive aquatic organisms may be exposed (Martí et al., 2011; Mikula et al., 2009).

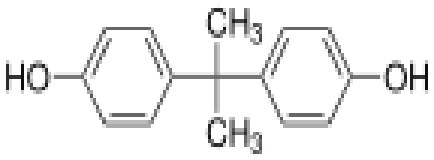


Fig.3: Bisphenol A

2.1.4 Vinclozolin

Last xenobiotic, that was used in my study is a well-characterized anti-androgenic compound vinclozolin (VIN [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine 2,4-dione], Fig. 4). It is a fungicide widely used in the United States and Europe to control disease in fruits and vegetables (Lambropoulou and Albanis, 2004, Gazo et al., 2013). It is rapidly metabolized into two active metabolites, M1 and M2, which have high affinity for the androgen receptor and can block gene expression, causing anti-androgenic effects (Wong et al., 1995). Previous studies have shown that VIN inhibits the development and maintenance of a variety of male traits in the guppy (*Poecilia reticulata*), including male coloration patterns, sperm count, and sexual behavior (Baatrup and Junge, 2001, Bayley et al., 2002).

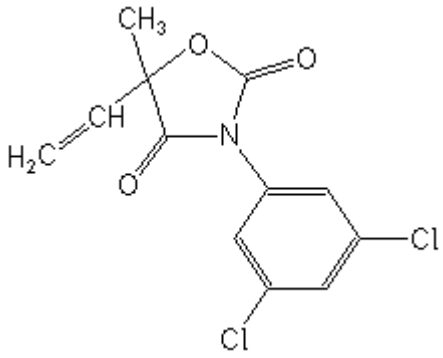


Fig.4: Vinclozolin

2.2 Experimental animals

For my study two model organisms were used:

1. Sterlet (*Acipenser ruthenus*), which is the representative of cartilaginous fish.
2. Brook trout (*Salvenilus fontinalis*), which is the representative of bony fish.

2.2.1 Sterlet (*Acipenser ruthenus*)

The sterlet (*Acipenser ruthenus*) (Fig. 5) is one of the smallest Eurasian species of sturgeon. It is a common domestic species in many European (Russia, Belarus,

Bulgaria, Hungary, Romania) and world countries (China Main, Turkey). Though species has undergone significant decline, the local populations still survive in most parts of its distribution range (rivers draining to the Black, Azov and Caspian Seas; Siberia from Ob eastward to Yenisei drainages and Danubian estuaries). Sterlet is also known for its production of caviar , which is a very expensive and prized delicacy. It is distinguishable between other European species of sturgeons by the presence of a great number of whitish lateral scutes, fringed barbels, and an elongated and narrow snout, highly variable in length. The sterlet may reach usually 3-4 kg in weight and 40 to 60 cm in length (maximum published lenght is 125 cm and weight is 16 kg). It is quite variable in coloration, but usually has a yellowish ventral side. Sterlet is usually sexually mature in age of 3–4 years (males), 4–5 years old (females).

Sturgeons have been classified as endangered fish by many international organizations and populations of these species have drastically decreased mostly due to over-fishing, destruction of their natural habitat, as well as water pollution (Birstein and DeSalle, 1998). However, for large species such as sturgeon, it is logistically difficult and expensive to conduct toxicity evaluations on broodfish (Tashjian et al., 2006). The sterlet constitutes an attractive model for biological studies of sturgeon because of its freshwater status, its small size and therefore low cost of maintenance and its early sexual maturation.

Fig.5: Sterlet (*Acipenser ruthenus*), representative of chondrostean fish

The morphology of sterlet sperm differs from primitive sperm of teleost fish (Billard, 1986). There is a presence of acrosome (Psenicka et al., 2007) with acrosin- and trypsin-like activities (Ciereszko et al., 1994, 1996), sperm behavior (Cosson et al., 2000) and size is different from brook trout sperm. The length of the head is 6 μm, the central part is 1μm long with 2-9 mitochondria, flagellum is 50-100 μm long with the

visual completion of central tubules (Fig. 6).

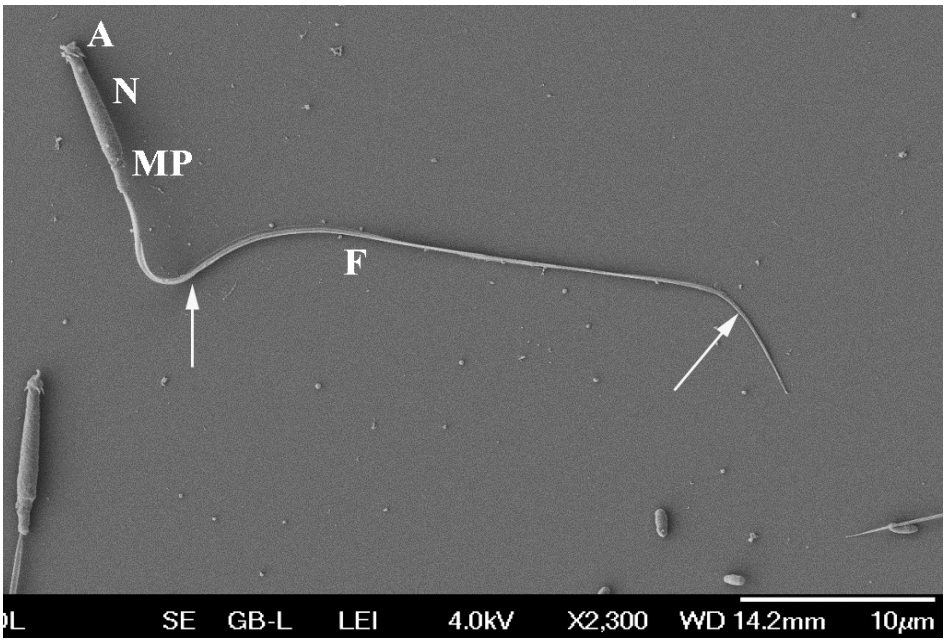


Fig. 6: Structure of sperm of sterlet (A- acrosom, N- nukleus, MP- midpiece, F- flagellum), (Linhartova et al., 2013)

2.2.2 Brook trout (*Salvenilus fontinalis*)

The brook trout, *Salvelinus fontinalis* (sometimes called brook charr or eastern brook trout), is a species of fish in the salmon family of order Salmoniformes. The specific epithet "*fontinalis*" comes from the Latin "of or from a spring or fountain."

Coloration of this species is green to brown in basic coloration, with a distinctive marble pattern (called vermiculations) of lighter shades across the flanks and back and extending at least to the dorsal fin, and often to the tail (Fig. 7). Fish could be distinguished by the combination of dark green marbling on its back and dorsal fin and by the red spots with blue halos on its sides. Usually, the belly, of the males, becomes very red or orange when the fish are spawning. Typical lengths vary from 25 to 65 cm and weights vary from 0.3 to 3.0 kg. In comparison with sterlet, brook trout has faster sexual maturation. Brook trout is sexually maturated in 2 years old (male), 3 years old (female).

The brook trout is a popular game fish for anglers, particularly for fly fishermen. This fish is very sensitive to basic pH in comparison with acidic Ph, to which is brook trout resistant. The typical pH range of brook trout waters is 5.0 to 7.5, with pH extreme of 3.5.

Fig.7: Brook trout (*Salvenilus fontinalis*), representative of teleost fish

For the sperm called aqua sperm is typical a round head shape of spermatozoa head (2-3 μm) and is reduced the central portion of the distal and proximal centriole 1-2 of the modified mitochondria. The parameters of flagellum are 40-60 μm in lenght, 0.1 μm in diameter (Fig. 8).

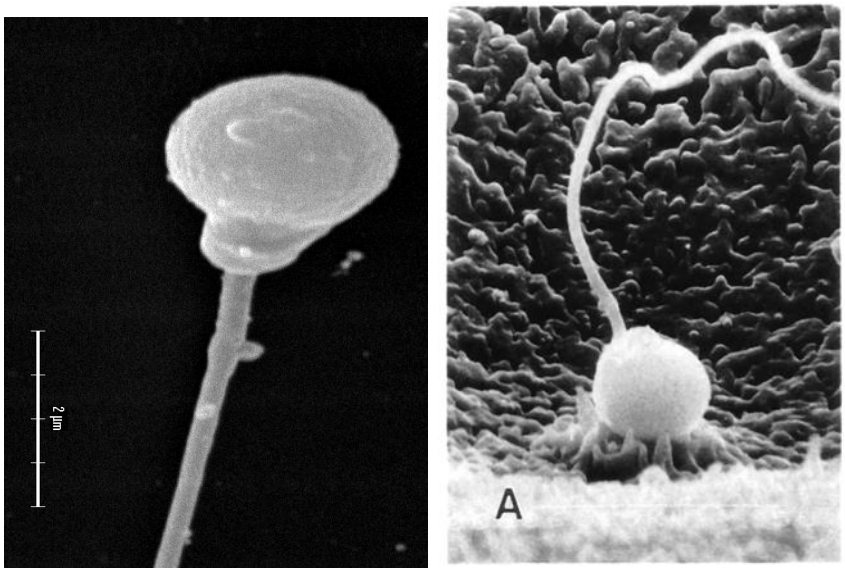


Fig.8: Structure of sperm "aqua sperm" of tench (Methodological Publication, Linhart et al., 2011)

3 Materials and methods

3.1 Broodstock handling and collection of gametes of sterlet (*Acipenser ruthenus)* and brook trout (*Salvenilus fontinalis)*

Sperm of sterlet (*Acipenser ruthenus*) was obtained from six different males (age: 6–7 years old; body weight: 0.5-2 kg; body length: 55 ± 9.3 cm) raised in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Prior to hormonal stimulation, fish were kept in tanks with water temperature varying between 14 and 15°C. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl solution at doses of 5 mg/kg of body weight, 48 h before sperm collection. Semen was collected from urogenital papilla by aspiration through a plastic catheter (5-7 mm diameter) connected to a 20 ml syringe. Sperm from brook trout (*Salvelinus fontinalis*) was obtained during the natural reproduction season without hormonal stimulation. Sperm was obtained from 6 different males (2–3 years old; body weight: 0.5-1 kg; body length: 30 ± 10 cm) raised in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Special care was taken to avoid contamination with mucus, feces or water. Sperm samples were stored on ice (0–4 °C) until processing. Sperm concentrations of each of six males were estimated microscopically (Olympus BX 41) at 200x using a Burker cell hemocytometer.

3.2 Preparation of xenobiotics

3.2.1.1 Duroquinone

All chemicals used were of analytical grade and purchased from Sigma (St. Louis, MO, USA). Duroquinone (duroquinone, subsequently referred to as DQ) [2,3,5,6-Tetramethyl-1,4-benzoquinone or Tetramethyl-p-benzoquinone; empirical formula: $C_{10}H_{12}O_2$; MW: 164.20] was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 10 $mg.ml^{-1}$.

3.2.1.2 Tetrabrombisphenol A

Tetrabrombisphenol A (TBBPA) [2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol or 2,2-bis(3,5-dibrom-4 hydroxyfenyl)propane; empirical formula: $C_{15}H_{12}Br_4O_2$; MW: 543.85), (≥99%; Sigma-Aldrich, USA)] was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 0.1 g/l.

3.2.1.3 Bisphenol A

Bisphenol A (BPA) [2,2-Bis (4-hydroxyphenyl) propane; empirical formula: $C_{15}H_{16}O_2$; MW: 228,29), (≥99%; Sigma-Aldrich, USA)] was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 0.1 g/l.

3.2.1.4 Vinclozolin

Vinclozolin (VIN) [3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4 oxazolidinedione; empirical formula: $C_{12}H_9Cl_2NO^3$; MW: 286.1, (PESTANAL®, analytical standard; Sigma-Aldrich, USA)] was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 0.1 g/l.

3.2.2 Sample preparation

Sperm from six individual males was centrifuged at 300×g, 4°C for 30 min to remove seminal plasma. Obtained pellet was resuspended in an immobilizing medium (IM) (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) to obtain spermatozoa density of 5 x 10^8 cells/ml. The sperm sub-samples were then exposed for 2 h to final concentrations of BPA (sterlet: 0.5, 1.75, 2.5, 5 and 10 µg/l, brook trout: 1.75, 2.5, 5 and 7.5 µg/l), TBBPA (sterlet: 0.5, 1.75, 2.5, 5 and 10 µg/l, brook trout: 1.75, 2.5, 5 and 7.5 µg/l), DQ (sterlet: 25, 50, 100 and 150 µM, brook trout: 12.5, 25, 50, 100 and 150 µM) and VIN (sterlet: 0.5, 2, 10, 15, 20 and 50 µg/l, brook trout: 0.1, 0.5, 2, 10 and 50 µg/l). A group exposed to immobilizing medium was used as a control. Each experimental condition was duplicated.

3.3 Sperm motility and velocity recording

Spermatozoa velocity (µm/s, measured only motile sperm) and percentage of motile sperm cells (%) were determined after triggering sperm motility under dark-field microscopy (Olympus BX 50, Japan) (20 x objective magnification). For triggering, sperm motility activating medium (10 mM NaCl, 1 mM CaCl, 10 mM Tris pH 8.5) at

a dilution ratio of 1:5000 was used. To avoid sperm sticking to the microscopic slide, 0.1% BSA was added to the activating solution. Sperm motility was recorded with a CCD video camera (SONY DXC-970MD, Japan) mounted on the microscope, illuminated with the stroboscopic flash (ExposureScope®, Czech Republic). Record of sperm motility was done in triplicate for each sample using video-recorder (SONY DVD Recorder-1000MD, Japan).

3.4 Sperm motility and velocity evaluation

Velocity and motility were assessed 10, 30, 60, 90 and 120 s after activation for sterlet and 10, 15, 20, 25 and 30 s after activation for brook trout. The successive positions of the video recorded sperm heads were analyzed from video frames by means of Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S). Velocity and percentage of sperm motility were calculated from sperm head positions on five successive frames with three different colors (frame 1 red, frames 2–4 green and frame 5 blue),described by Rodina et al., 2007. Twenty to 40 sperm were counted for each frame. Sperm that moved were visible in three colors, while non-moving sperm were white. The percentage of motile sperm was calculated from the number of white and red cells. Sperm velocity was calculated as μms^{-1} based on length traces of sperm from blue to green and red heads, calibrated for magnification.

3.5 Assessment of DNA Damage

In present study DNA integrity was assessed using the Comet assay or single cell gel electrophoresis assay following the method described by Li P. et al. (2008). Unless otherwise stated, molecular grade, DNAse-free reagents (Sigma Aldrich, USA) were used throughout. Microscope slides (OxiSelectST; Cell Biloabs, INC. USA) were used for the assay and each slide was prepared in the following manner. The 100 µl of sperm sample was diluted in 5 ml of PBS (phosphate buffer solution; Ca^{2+} and Mg^{2+} free) to obtain a sperm density of $1x10^7$ cells ml^{-1}. Diluted samples (200 µl) were mixed with 700 µl of 0.8% NuSieve GTG low melting temperature agarose (OxiSelectST; Cell Biloabs, INC. USA). Finally, a 55 µl of this mixture was added to the slide, and allowed the agarose to solidify for 1 hour at 4°C. The slides were then immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10% Lysis Solution [OxiSelect™ Comet Assay Kit],

10% DMSO, pH 10) at 4°C. Thereafter the slides were subjected to enzymatic treatment with proteinase K (1mg/ml in 2.5M NaCl, 100mM EDTA, 10% DMSO, pH 7.4) and incubated over night at 32 °C. After the proteinase K solution was drained, the slides were immersed in a horizontal gel tank filled with freshly made electrophoresis buffer (90 mM Tris Base, 90 mM Boric acid, 2.5 mM EDTA). Electrophoresis was carried out for 20 min at 35 V and 170 mA. Slides were then carefully transferred from the electrophoresis chamber to a clean container with pre-chilled DI H_2O for 2 min. The washing procedure was repeated three times. The slides were drained well, dehydrated by dipping into absolute ethanol for 5 min and air-dried for storage. Prior to the analysis, 50 µl of Vista Green DNA Staining Solution (OxiSelectST; Cell Biloabs, INC. USA) was added to each agarose spot of the slide and analyzed with an Olympus BX50 fluorescence microscope at 20x magnification. A sum of 100 cells was scored for each sample and the captured images were analyzed using CometScore image analysis software (TriTek Corporation, USA). Tail length (measured from the middle of the head to the end of the tail) and tail DNA content (tail % DNA) were measured. Percentage of DNA in tail and Olive Tail Moment were calculated using the following formulas:

Tail DNA% = 100 x Tail DNA Intensity/Cell DNA Intensity

Olive Tail Moment = Tail DNA% x Tail Moment Length

3.6 Oxidative stress indices

Sperm samples were centrifuged at 5000×g at 4°C for 10 min. The supernatant was carefully collected and discarded. The spermatozoa pellet was diluted with 50mM potassium phosphate (KPi) buffer, pH 7.0, containing 0.5 mM EDTA and 0.1 mM PMSF to obtain a spermatozoa concentration of 5 x 10^8 cells/ml, then homogenized in an ice bath using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany). The homogenate was divided into two: one part for measuring thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP), and a second part that was centrifuged at 12 000 x g for 30 min at 4°C to obtain the post mitochondrial supernatant for the antioxidant enzyme activity assay.

The TBARS method described by Li et al. (2010) was used to evaluate sperm lipid peroxidation (LPO). Its concentration was calculated by absorption at 535 nm and a molar extinction coefficient of 156 mM/cm. The content of TBARS was expressed as nanomoles per 10^8 cells. Carbonyl derivatives of proteins (CP) were detected by reaction with 2, 4-dinitrophenylhydrazine (DNPH) according to the method described by Lenz et al. (1989). The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM/cm and expressed as nmol per 10^8 cells.

3.7 Superoxide dismutase activity (SOD)

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Marklund and Marklund (1974). This assay depends on the autoxidation of pyrogallol. The activity of SOD in supernatant was assessed spectrophotometrically at 420 nm. One unit of SOD activity is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical per minute. Activity was expressed in international units (or milliunits) mU per 10^8 cells.

3.8 ATP bioluminiscence assay

ATP content in spermatozoa was determined after 2 h of exposure to BPA, TBBPA, DQ and VIN using bioluminescence method described by Boryshpolets et al. (2009). Sperm samples were added to a boiling extraction medium consisting of 100mM Tris–HCl, pH 7.75 and 4mM EDTA. After boiling for 2min at 100°C samples of the sperm suspension were centrifuged at 12 000×*g* for 20 min using a Heraeus Model 400-R centrifuge (Hanau, Germany). ATP content in the supernatants was evaluated by bioluminescence method, using a Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany). Luminescence was read with a multifunctional microplate reader Infinite M200 (Tecan, Austria). ATP content was expressed as nmol ATP/10^9 spermatozoa.

3.9 2D electrophoresis (SDS-Page)

Rehydration buffer (RB), DL-Dithiothreitol (DDT), ampholyte mixture for IpG (Bio-Lyte pH 3-10 Buffer) and thawed pellet of sperm (5 x 10^8 cells/ml) with added concentration of xenobiotic were mixed together to obtain a volume of 130 µl. Then the sample was centrifuged for 20 min at 16 000 g and 25°C. By this way the protein denaturation was achieved. Obtained mixture was applied to 11 cm IpG strips (pH range

3-10, linear). Isoelectric focusing was performed using the IPGphor system (Amersham Bioscience). Electrical current conditions for the separation were set as follows: passive rehydration for 14 h; isolelectric focusing: 250 V for 1 h; 500 V for 1 h; 1000 V for 1 h (gradient); 5000 V for 3 h (gradient). After isoelectric focusing, the IPG strips were equilibrated with a solution containing 6 M urea, 29.3% glycerol, 2% SDS, 75 mM Tris-HCl pH 8.8 and 2% (w/v) dithiothreitol for 15 min, and in the second step with a solution containing 2.5% (w/v) iodacetamide instead of dithiothreitol for another 15 min. For second dimension electrophoresis each IpG strip was laid onto a 12% SDS-PAGE gel:

12% basic gel:

- 4ml 30% acrylamide, 0.8% bis-acrylamide + 3,5 ml ddH_20+ 2,5 ml Tris-HCl (pH 8,8)+ 100 µl 10% SDS+ 150 µl Ammonium persulfate (APS) + 30 µl N,N,N′,N′-Tetramethylethylenediamine (TEMED)

4% stacking gel:

- 0,65 ml 30% acrylamide, 0.8% bis-acrylamide + 3,05 ml ddH_20+ 1,25 ml Tris-HCl (pH 6,8)+ 50 µl 10% SDS+ 75 µl APS+ 30 µl TEMED (the comb was immediately placed onto the gel with one hole for marker).

The SDS-PAGE was carried out at 15 mA per gel for 1 hour. Gels were stained with a silver staining method and scanned to allow analysis and future processing.

3.10 Statistical analysis

All measurements were conducted in triplicate. Normality and homogeneity of dispersions in studied values were tested using Shapiro–Wilk's and Levene test. Statistical comparison was made by analysis of variance (ANOVA) with subsequent post hoc Tukey's honest significant difference (HSD) test. The values were expressed as mean ± SEM (n = 6). The observed relationships among the parameters were confirmed and quantified according to the Spearman's test. Values for motility and velocity only at 10 sec post-activation were used. The *t*-test was used to establish if the correlation coefficients are significantly different from zero. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 software for Windows.

4 Results

4.1 Xenobiotics

Results described below elucidate the effect of DQ, VIN, TBBPA and BPA on spermatozoa of brook trout. In annexes several papers from Pavla Linhartova, Ievgenia Gazo, Anna Shaliutina, Martin Hulak and Vojtěch Kašpar are added. These papers consider the same topic, using the sterlet as a model organism.

4.1.1 Evaluation of DNA damage

An illustration of the outcome of this particular assay for DNA damage is presented in Figure 9. The level of DNA fragmentation in spermatozoa of brook trout exposed to all used xenobiotics increased significantly in a dose-dependent manner after 2 h of *in vitro* exposure (Fig. 9).

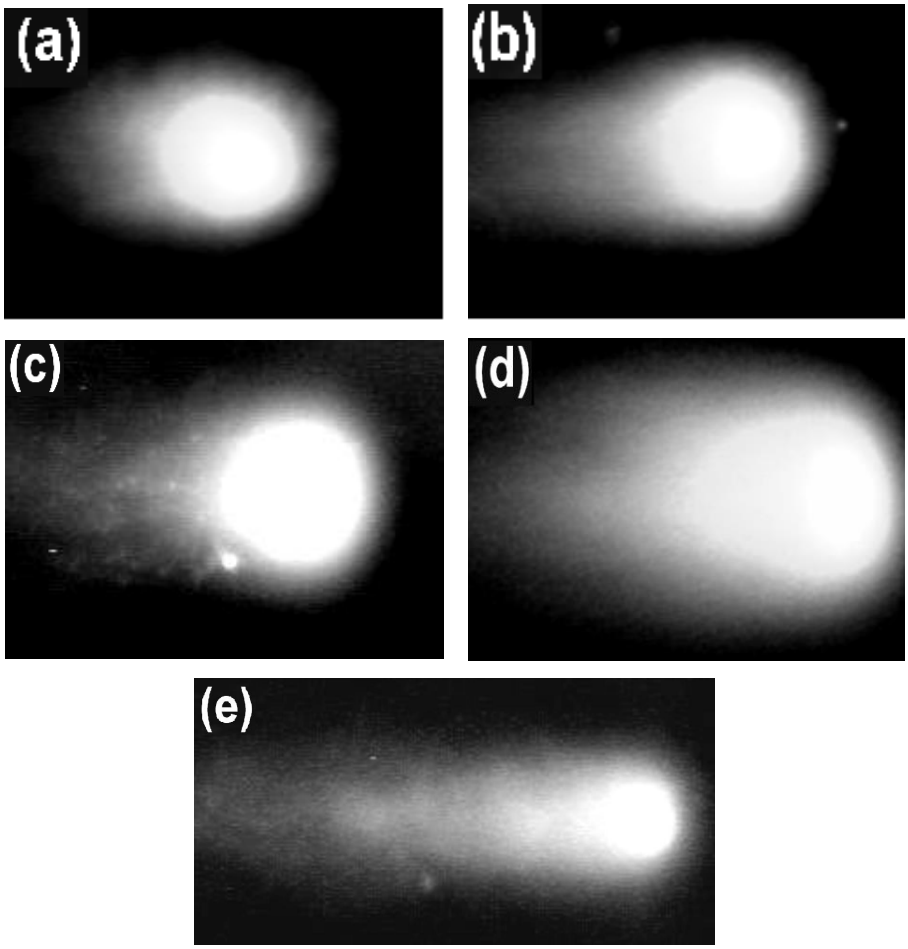
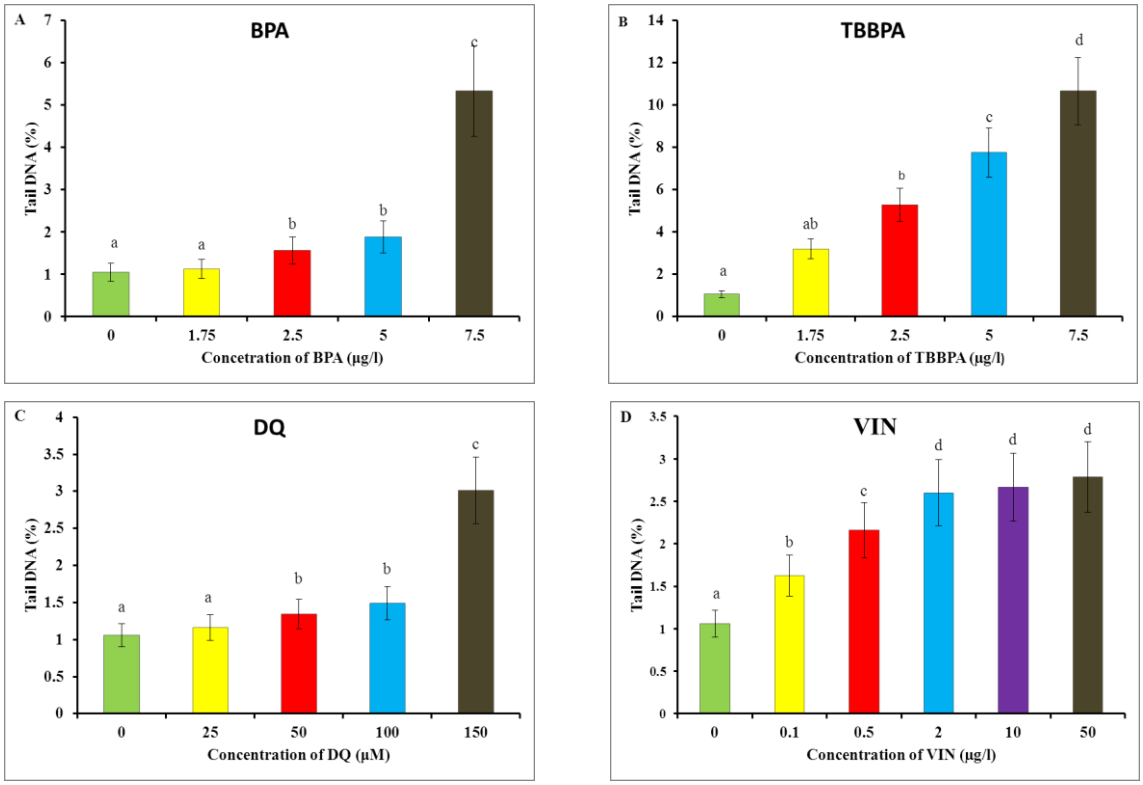


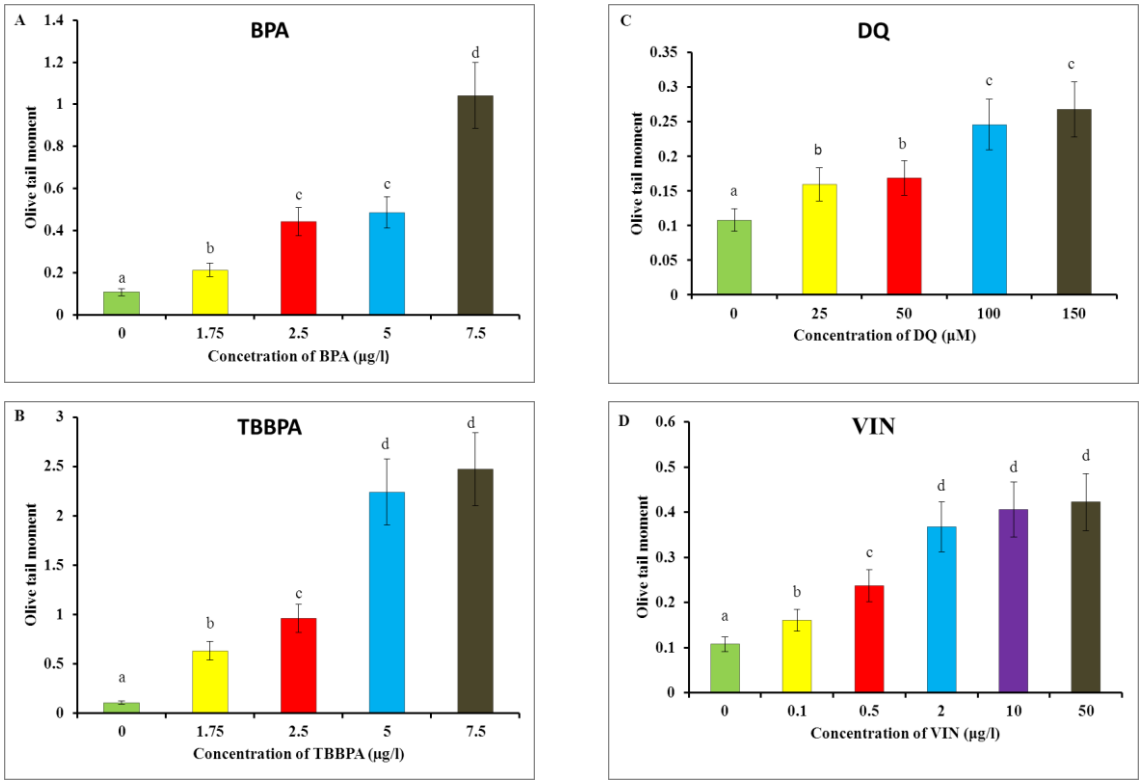
Fig. 9: Example of figures from Comet assay of *Salvelinus fontinalis* spermatozoa exposed to different concentrations of DQ. The different degrees of DNA damage in spermatozoa are arranged in five classes (a- no damage-control, % of DNA in Tail < 5%; b- small damage- %DNA in Tail < 15% (25 μM of DQ); c, -medium damage- % of DNA in Tail < 50% (50 μM of DQ); d-higher damage-% of DNA in Tail < 60% (100 μM of DQ); e- the highest damage - % DNA in Tail >60% (150 μM DQ).

Graphs 1A-1D show the effects of *in vitro* exposure to BPA, TBBPA, VIN and DQ on DNA damage. The level of DNA fragmentation, expressed as the mean percent of DNA in Tail, significantly increased ($df = 4$, $P < 0.05$) after *in vitro* exposure of sperm to concentrations above 2.5 μg/l of BPA and TBBPA, then 50 μM of DQ and 0.1 μg/l of VIN as compared with the control (graphs 1A-1D). The sperm exposed to the highest concentration of TBBPA (7.5 μg/l) had a DNA fragmentation degree almost 11%, as compared with the control samples (1-2%). Otherwise lower DNA fragmentation degree (2.9%) appeared in sperm treated with 50 μg/l of VIN.



Graphs1 (A,B,C,D): Percentage of DNA in Tail (the % of DNA in Tail measured in Comet assay) after *in vitro* treatment of brook trout (*S. fontinalis*) sperm with nominal concentrations of all used xenobiotics (BPA, TBBPA, DQ and VIN). Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.

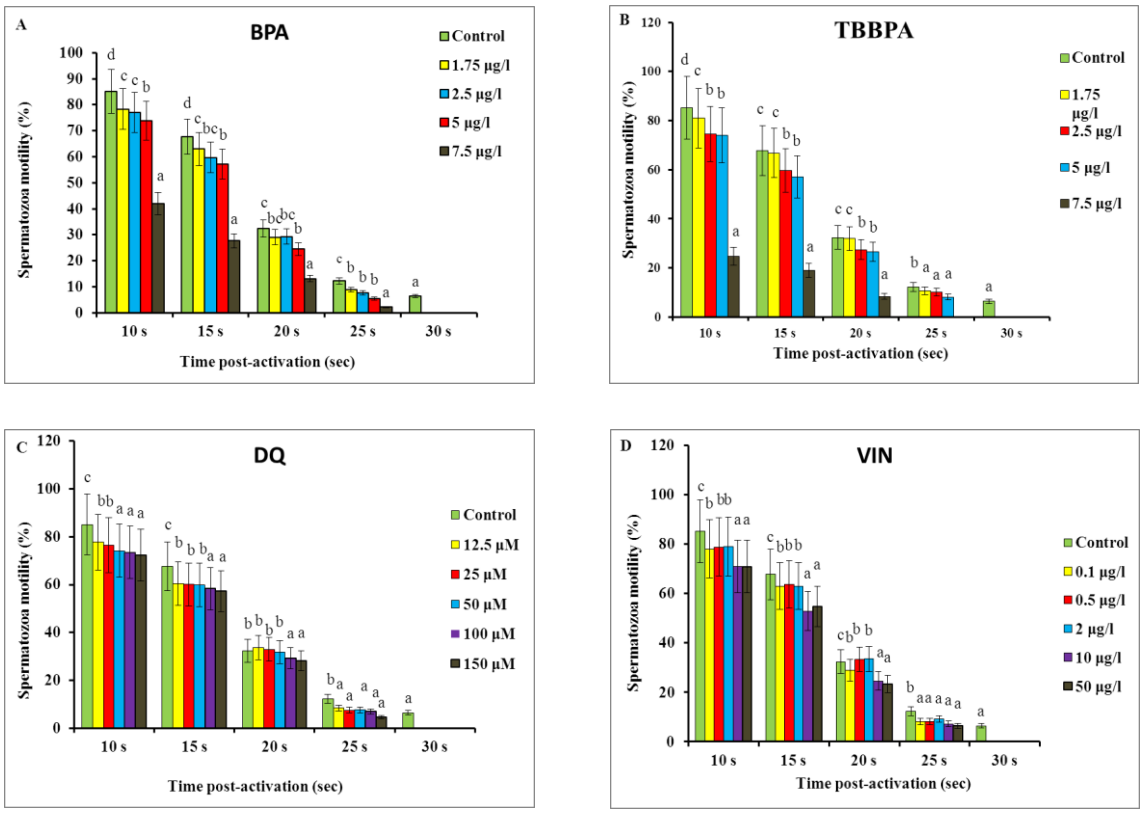
The level of DNA fragmentation, expressed as Olive Tail Moment significantly increased ($df = 4$, $P < 0.05$) after *in vitro* exposure of sperm to concentrations above 1.75 µg/l of BPA and TBBPA as compared with the control (graphs 2A, 2B). It is worth to mention that treatment with DQ and VIN led to the same dose-dependent increasing in Olive Tail Moment as the one observed for % of DNA in Tail.



Graphs2 (A,B,C,D): Olive Tail Moment of the spermatozoa after *in vitro* treatment of brook trout (*S. fontinalis*) sperm in nominal concentrations of all used xenobiotics.. Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.

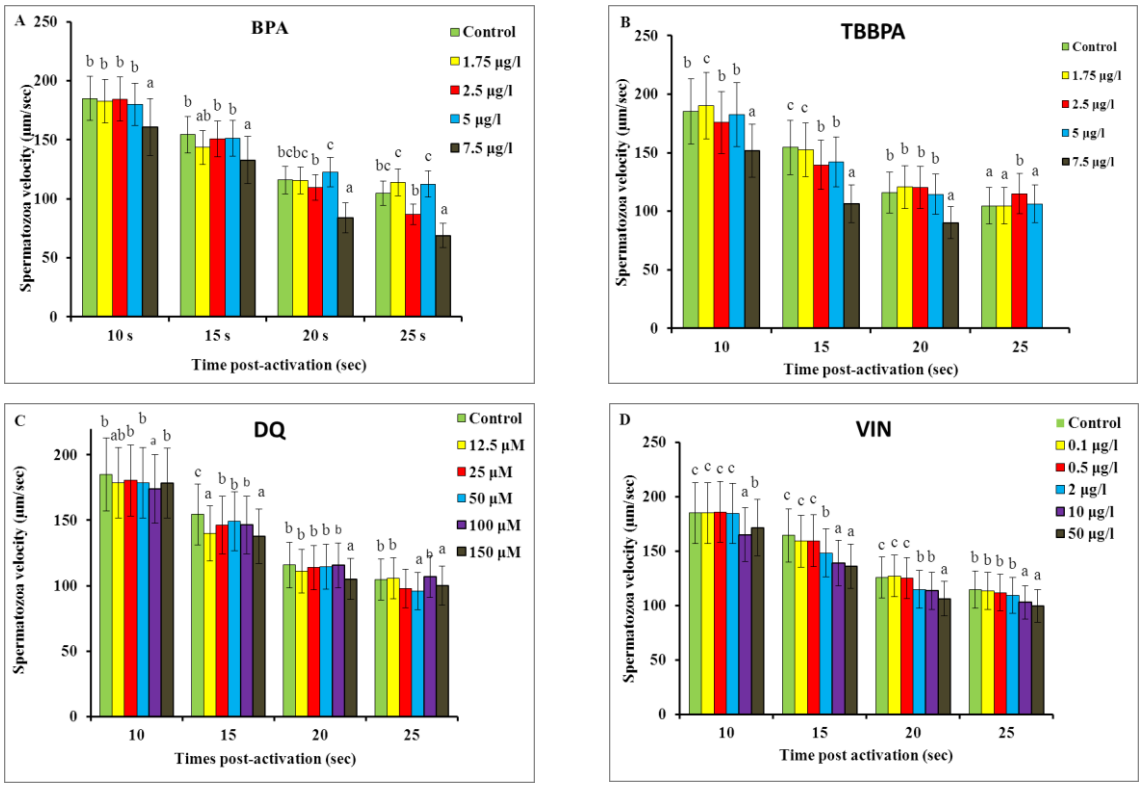
4.1.2 Spermatozoa motility and velocity

Significant effect of BPA, TBBPA, VIN and DQ on sperm motility was observed at different times post activation (df = 4, P < 0.05, graphs 3A-3D). The duration of spermatozoa motility decreased with increasing concentrations of used xenobiotics. In the graph 3A it is shown that in control group, 15.3% of spermatozoa were motile at 25 s post activation, while only 2.3% were motile in group exposed to the highest concentration of BPA (7.5 µg/l). The similar trend was observed for VIN and DQ in graphs 3C and 3D. But it is worth to mention, that no spermatozoa motility was observed in 25 s post-activation at the highest concentration of TBBPA (7.5 µg/l). Actually, large difference between control and 7.5 µg/l of BPA and TBBPA (graphs 3A, 3B) was observed in 10 s post-activation, compared to VIN and DQ (graphs 3C, 3D) where the difference between control and the highest used concentration of xenobiotic was not so perceptible.



Graphs 3 (A,B,C,D): Effect of BPA, TBBPA, DQ and VIN on spermatozoa motility after *in vitro* exposure of brook trout (*S. fontinalis*) sperm to all used concentrations of xenobiotics. Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.

The similar trend was observed for spermatozoa velocity. Typically, at 10 s post-activation the spermatozoa velocity in control group (graphs 4A and 4B) reached 187 µm.s^{-1} compare to 172 µm. s^{-1} in 7.5 µg/l of BPA and TBBPA. Otherwise the results of DQ and VIN point out, that there was not a big difference between spermatozoa velocities in control group and other samples treated with mentioned xenobiotics (graphs 4C and 4D).



Graphs 4 (A,B,C,D): Effect of BPA, TBBPA, DQ and VIN on spermatozoa velocity (µm.s^{-1}) after *in vitro* exposure of brook brook trout (*S. fontinalis*) sperm in nominal concentrations of all four used xenobiotics. Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.

I observed the changes in flagella movement and C shaped flagella (Fig. 10), which are typical for dead sperm, or sperm with very slow motility mostly at 90 and 120 s post-activation.

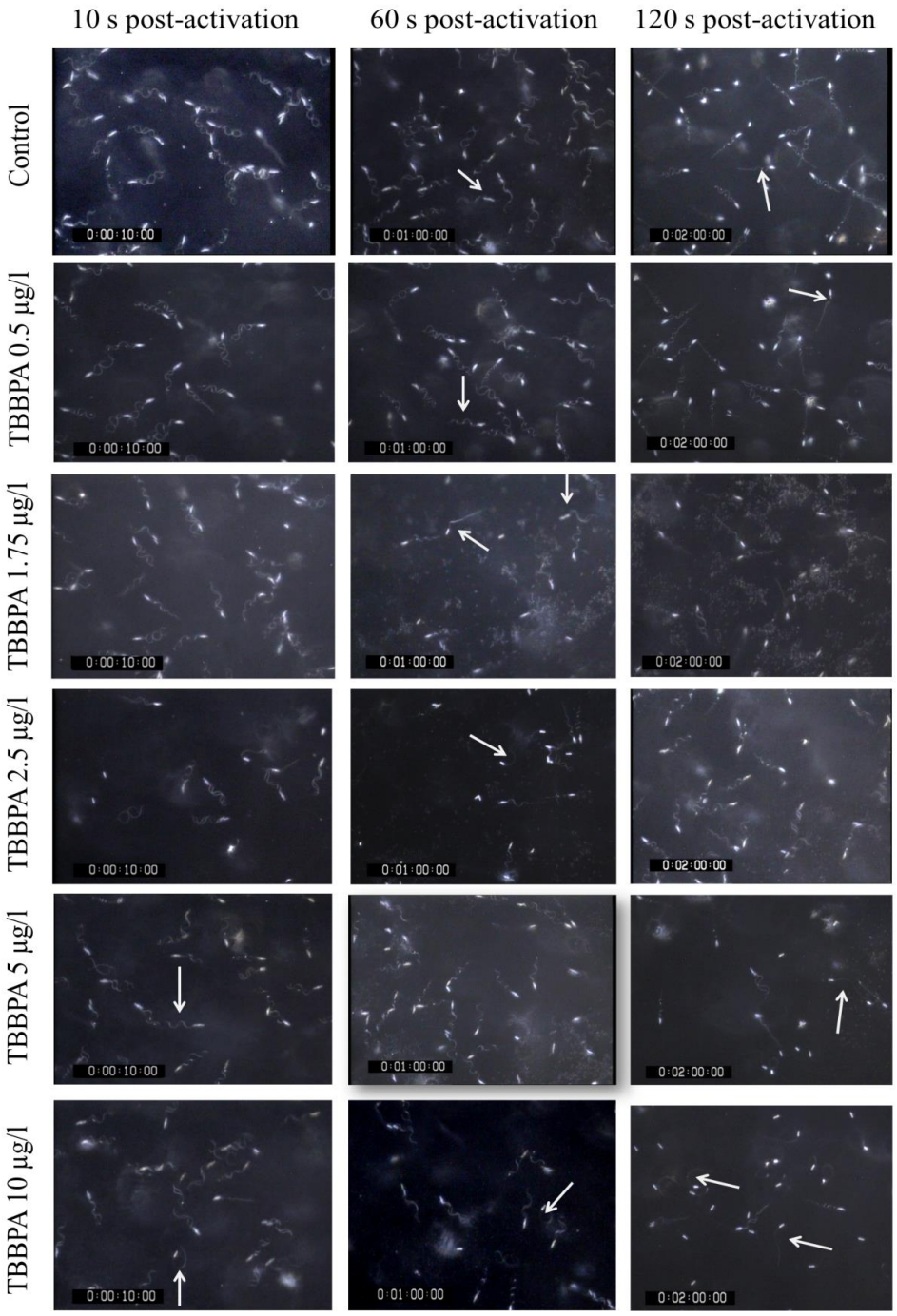
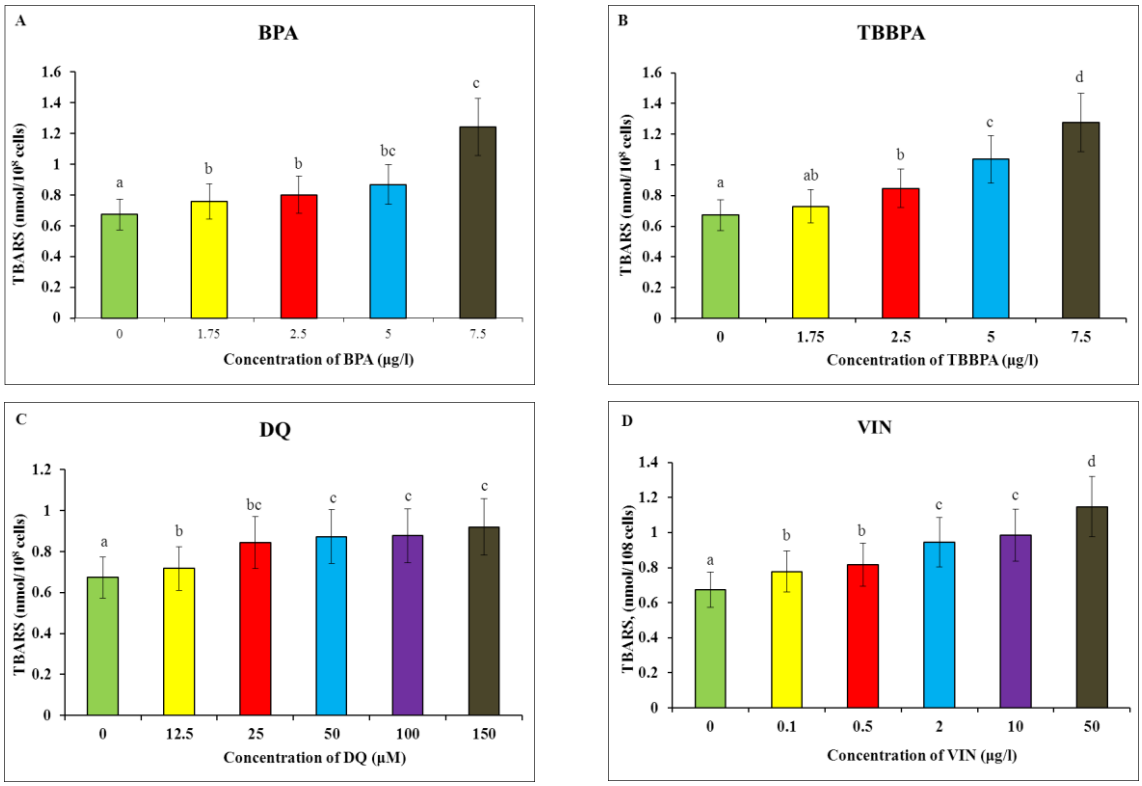


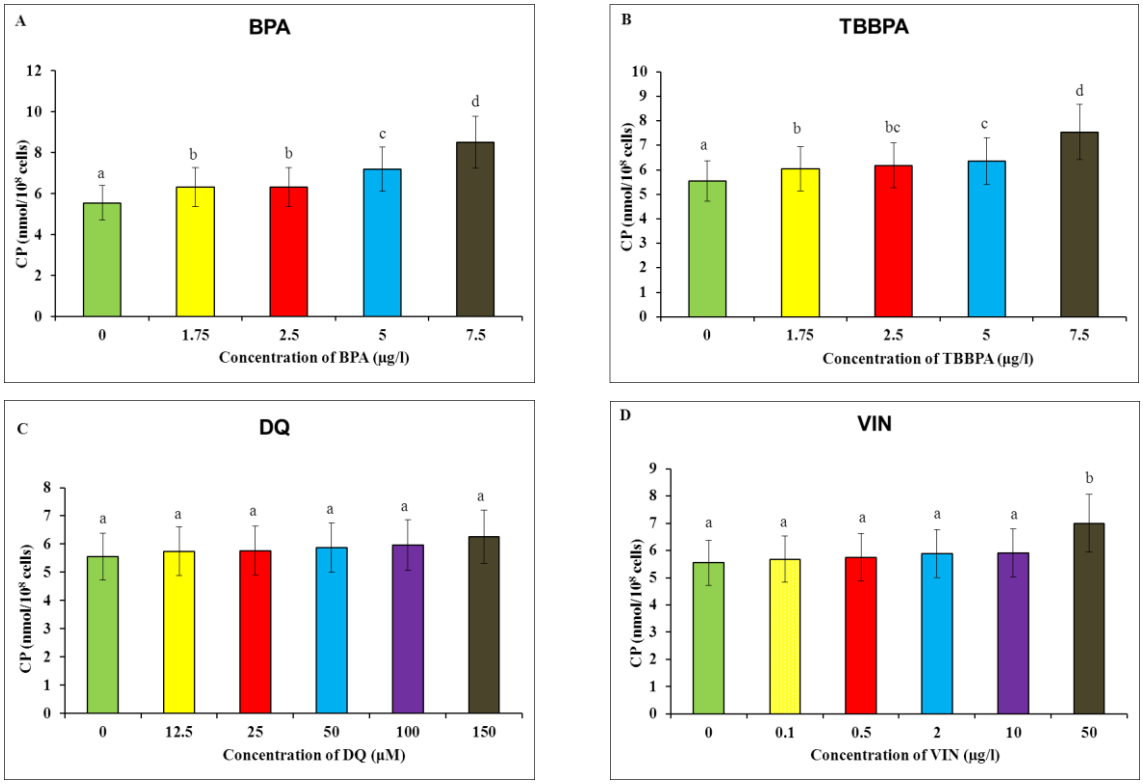
Figure 10: Swimming behavior of sterlet (*Acipenser ruthenus*) spermatozoa. Flagella movement was recorded under dark field microscopy with stroboscopic lamp at 10, 60 and 120 s post-activation of spermatozoa treated with TBBPA (annex 9.3, p. 127, fig. 2). Different forms of flagella were observed during spermatozoa movement as well as once motility period of sperm cell is finished. The white arrows indicate changes in flagella shape, such as decrease in beat frequency and occurring of C shape, in various concentrations of TBBPA compared to control.

4.1.3 Oxidative stress indices and antioxidant response

In order to verify the presence of oxidative imbalance induced by BPA,TBBPA, DQ and VIN, LPO level (indicated by spermatozoa TBARS level) and CP level in all groups were measured (graphs 5 and 6). LPO level significantly ($df= 4$, $P < 0.05$) increased upon exposure to 5 µg/l of BPA and TBBPA and above (graphs 5A-5B). The same trend was observed for 50 µM of DQ and 2 µg/l and above of VIN (graphs 5C-5D). LPO production was markedly higher than the control value when exposed to the highest concentrations of BPA, TBBPA, DQ and VIN. The level of CP activity was used as an indicator of protein oxidation in fish spermatozoa during *in vitro* exposure to xenobiotics (graphs 6A-6D). The result shows that the CP level increased significantly ($df=4$, $P < 0.05$, graphs 6) as compared with the control in groups treated with BPA, TBBPA and VIN, but not in group treated with DQ, and reached the maximum value when exposed to the highest concentrations of BPA and TBBPA. The smallest effect on oxidative stress activity was observed at VIN (graphs 6C and 6D), where the difference between control group and all used concentrations was not so destroyable.

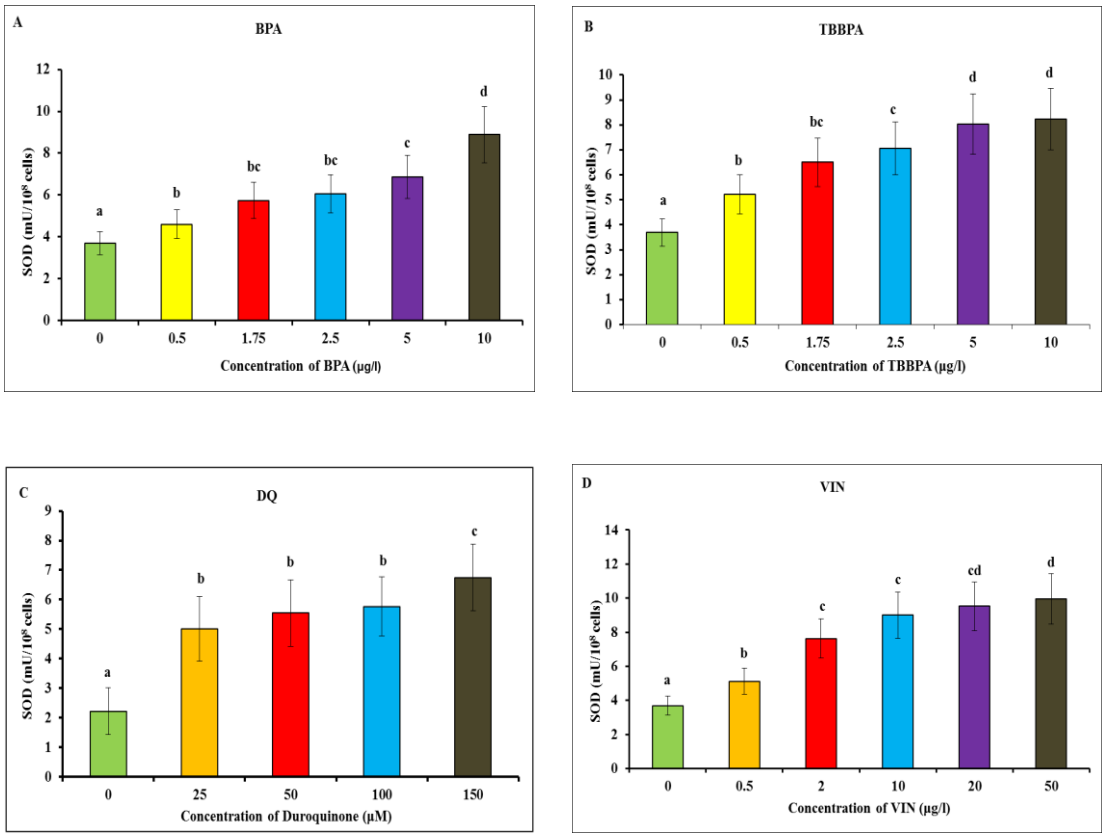


Graphs 5 (A,B,C,D): Effect of BPA, TBBPA, DQ and VIN on TBARS level during *in vitro* treatment of brook trout (*S. fontinalis*) sperm with all used concentrations of xenobiotics. Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.



Graphs 6 (A,B,C,D): Effect of BPA, TBBPA, DQ and VIN on CP level during *in vitro* treatment of brook trout (*S. fontinalis*) sperm with nominal concentrations of all used xenobiotics. Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.

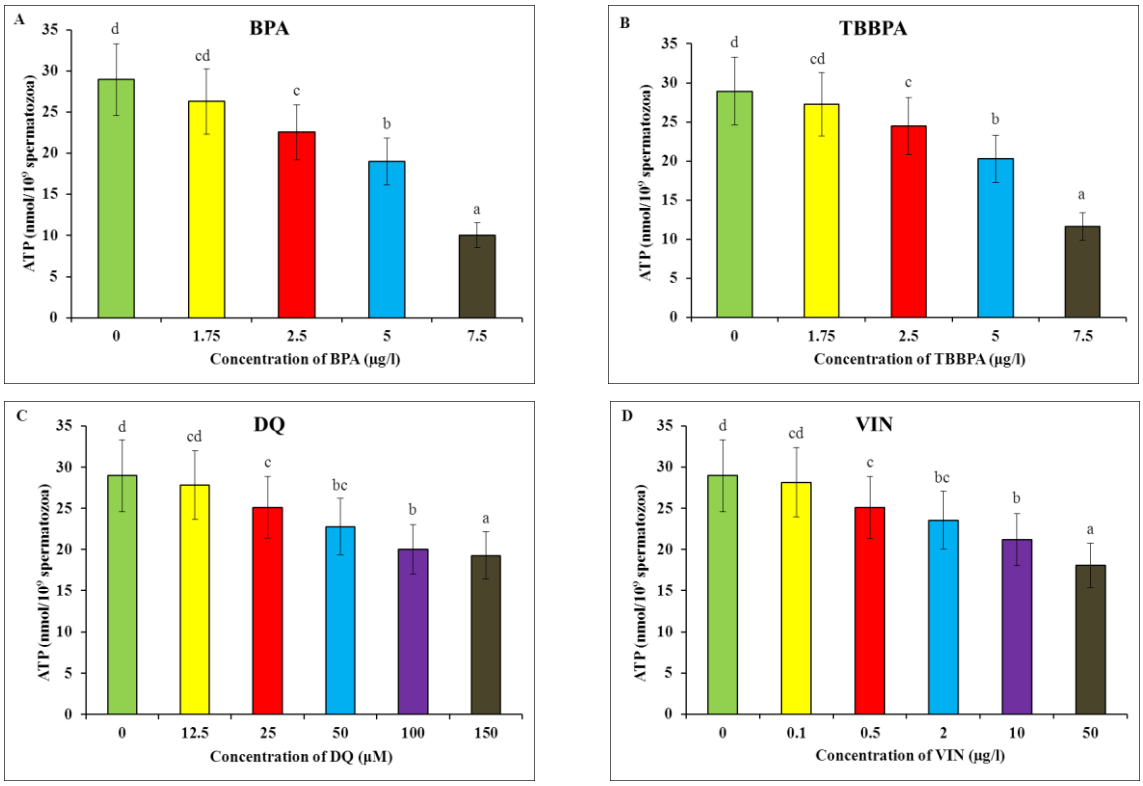
The superoxide dismutase (SOD) activity was used as an indicator of antioxidant activity of sterlet spermatozoa during *in vitro* exposure to different xenobiotics (annex 9.1, p. 74, fig. 7, annex 9.2, p. 103, fig. 8 and annex 9.3, p. 131, fig. 7). The results show that the SOD activity increased significantly in all treatments as compared to the control ($df=4$, $P < 0.05$, graphs 7). It is worth to mention that SOD activity in sterlet spermatozoa increased gradually and in dose-dependent manner. On the other hand no results for SOD activity were obtained in studies on brook trout.



Graphs 7 (A,B,C,D): Effect of BPA, TBBPA, DQ and VIN on activity on superoxide dismutase (SOD) in sterlet (*A. ruthenus*) sperm after 2 h exposure to all used xenobiotics. Data are presented as means ± SEM, n=6. Different letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

4.1.4 Evaluation of ATP content

To investigate further the potential mechanism underlying the decrease in sperm quality, the intracellular ATP content in spermatozoa was analyzed. The total level of ATP in spermatozoa from control was found as 27-28 nmol ATP/10^9 spermatozoa. Significant differences (df=4, $P < 0.05$) in intracellular ATP level were found upon exposure to 5 and 7.5 µg/l of BPA, where the lowest ATP content was 12 nmol ATP/10^9 spermatozoa (graph 8A). The intracellular level of ATP for the highest concentrations of DQ (150 µM) and VIN (50 µg/l) was twice higher, almost 23 nmol ATP/10^9 spermatozoa.



Graphs 8 (A,B,C,D): Effect of BPA, TBBPA, DQ and VIN on intracellular ATP content in brook trout (*S. fontinalis*) sperm after 2 h exposure to all used xenobiotics. Data are presented as means ± SEM, n=6. Different letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

4.1.5 2D electrophoresis

The results from 2D electrophoresis are shown on figures 10A-10E. The control group (fig. 10 A) was not influenced by any xenobiotic, meaning any changes in the protein structure. The use of all four xenobiotics (BPA, TBBPA, VIN and DQ) at highest concentrations led to changes in protein profiles of brook trout spermatozoa. All changes are highlighted by black rounds (fig. 10B-10E). In response to the presence of xenobiotics protein spots changed their position or concentration. After incubation with BPA – 8 spots changed, in TBBPA – 10 spots, in DQ – 8 spots, in VIN – 9 spots. Some proteins presented in control group disappeared after treatment with xenobiotics: in BPA – 5, in TBBPA – 5, in DQ – 4 and in VIN – 2 proteins.

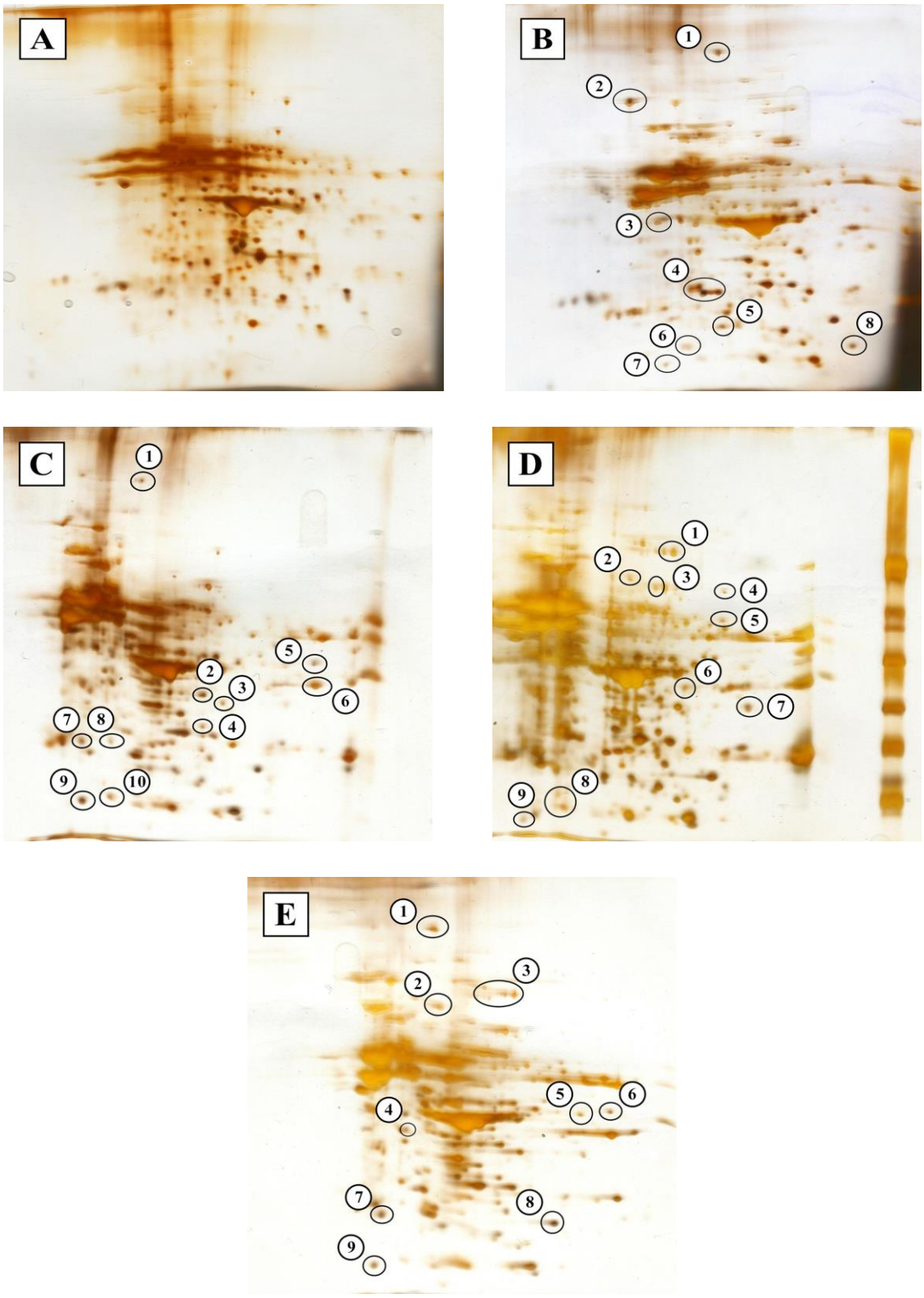


Fig. 10: The 2DE protein maps of fresh (A) and treated with xenobiotics [B- BPA (5 µg/l), C- TBBPA (5 µg/l), D- DQ (150 µM) and E- VIN (50 µg/l)] brook trout´s permatozoa. Proteins were stained with silver. Labeled spots represent proteins with significantly altered amounts after treatment.

4.2 Duroquionone

The results on the effect of DQ on spermatozoa of sterlet (*Acipenser ruthenus*) are in annex 9.2 in a paper: Pavla Linhartova, Ievgenia Gazo, Anna Shaliutina and Martin Hulak, The effect of duroquinone on physiology and DNA integrity of fish spermatozoa (*Acipenser ruthenus*). Paper was accepted 4. 4. 2013 in journal Toxicology in Vitro.

4.3 Tetrabisphenol A

The results on the effect of TBBPA on spermatozoa of sterlet (*Acipenser ruthenus*) are in annex 9.3 in a paper: Pavla Linhartova, Ievgenia Gazo, Anna Shaliutina and Martin Hulak, Tetrabrombisphenol A (TBBPA) and its effects on DNA integrity, oxidative stress indices and quality of spermatozoa of sterlet (*Acipenser ruthenus*). Paper is in preparation to journal Cell Biology and Toxicology.

4.4 Bisphenol A

The results on the effect of BPA on spermatozoa of sterlet (*Acipenser ruthenus*) are written in a paper: Martin Hulak, Ievgenia Gazo, Anna Shaliutina, Pavla Linhartova, In vitro effects of bisphenol A on the quality parameters, oxidative stress, DNA integrity and Adenosine triphosphate content in sterlet (*Acipenser ruthenus*) spermatozoa. Paper is under minor revision in journal Comparative Biochemistry and Physiology.

4.5 Vinclozolin

The results on the effect of VIN on spermatozoa of sterlet (*Acipenser ruthenus*) are in annex 9.1 in a paper: Ievgenia Gazo, Pavla Linhartova, Anna Shaliutina and Martin Hulak, Influence of environmentally relevant concentrations of vinclozolin on quality, DNA integrity, and antioxidant responses of sterlet *Acipenser ruthenus* spermatozoa. Paper is in online version in journal Chemico-Biological interaction from 25. 4. 2013.

5 Discussion

Aim of the the present study was to observe and quantify effect of xenobiotic substances on DNA damage and spermatozoa motility or velocity. Parameters of spermatozoa motility (% of motile cells, velocity of movement) were significantly reduced after *in vitro* treatment with all four xenobiotics compared to control. I could observe decreasing trend of both parameters (% of motile cells, velocity of movement) with increasing concentration of xenobiotics. It is worth to mention, that the reduction of both studied parameters was significant in case of both studied model species - brook trout (*Salvenilus fontinalis*) and sterlet (*Acipenser ruthenus*). However, brook trout sperm was found to be more susceptible for treatments with xenobiotics at highest concentrations and reported motility of brook trout spermatozoa was up to 50% lower than in control. Same treatment (VIN) of sterlet spermatozoa resulted in reduction of motility up to 30% compared to control (annex 9.1, p. 72, fig. 4A), (Gazo et al., 2013). On the other hand, brook trout spermatozoa did not shown any significant dose-dependent decrease of motility or velocity at lower concentrations of xenobiotics in contrast to sterlet spermatozoa. In previous studies it was also shown that *in vitro* exposure to DQ has the harmful effect on motility of common carp (*Cyprinus carpio)* spermatozoa but dose-dependent reduction was not clearly visible at applied concentrations (Zhou et al., 2006). My results are in agreement with several other studies performed with brown trout (*Salmo trutta*) and goldfish (*Carassius auratus auratus*) that showed lower spermatozoa motility and velocity of sperm cells exposed to environmentally related concentrations of BPA either *in vivo* or *in vitro* (Hatef et al., 2012; Lahnsteiner et al., 2005).

It is well known that plasma membrane plays an important role in the initiation of sperm motility due to hypoosmotic signals (Li P. et al., 2009, 2012). Therefore any modification of plasma membrane can significantly modify spermatozoa movement. Lipid peroxidation (LPO) is particularly important for aquatic animals since they normally contain greater amounts of highly unsaturated fatty acids (HUFA) than the other species. This has been reported to be a major contributor to the loss of cell function under oxidative stress (Storey, 1996). The most common indicator of LPO in fish is the level of Thiobarbituric Acid Reactive Substances (TBARS), (Oakes and Van der Kraak, 2003). My results showed that applied xenobiotics significantly increased

level of TBARS after 2 h of sperm exposure. If I compare the sensitivity of sperm of two model organisms, it can be suggested that brook trout spermatozoa were more sensitive to oxidative stress than sterlet spermatozoa. The basic level of TBARS in control group of brook trout spermatozoa was 0.67 nmol/10^8 cells (graphs 5, p. 32) , whereas in sterlet spermatozoa level of TBARS in control group reached 0.17 nmol/10^8 cells (annex 9.1, p. 73, fig. 5, annex 9.3, p. 130, fig. 5) or 0.24 nmol/10^8 cells (annex 9.2, p. 100). The level of TBARS in brook trout spermatozoa treated with the highest concentration of DQ (150 μM) reached 0.91 nmol/10^8 cells (graph 5C, p. 32), compared to sterlet spermatozoa, where the level of TBARS after treatment with 150 μM of DQ reached 0.35 nmol/10^8 cells (annex 9.2, p. 102, fig. 7A), (Linhartova P. et al., 2013).

The CP is a result of protein oxidation. The formation of CP is nonreversible, causing conformational changes, decreased catalytic activity in enzymes and ultimately resulting in breakdown of proteins by proteases, due to increased susceptibility to protease action (Zhang et al., 2008). Though the level of CP significantly increased with increasing concentrations of DQ, TBBPA, BPA and VIN in both tested species, the sensitivity to protein oxidation induced by xenobiotics in spermatozoa of sterlet and brook trout was different. In control group the level of CP in brook trout spermatozoa (5.5 nmol/10^8 cells) significantly exceeded the level of CP in sterlet sperm (2.7 nmol/10^8 cells), (graphs 6, p. 33 and annexes 9.1, p. 73, fig. 6, 9.2, p. 103. fig. 7B, 9.3, p. 130, fig. 7). However, the level of CP in sperm treated by 50 μg/l of VIN reached 13 nmol/10^8 cells for sterlet (annex 9.1, p. 73, fig. 6), whereas for brook trout sperm it was twice lower (6 nmol/10^8 cells). Based on obtained results can be hypothesized, that the loss of spermatozoa motility and velocity in brook trout and sterlet sperm was caused by adding increasing doses of xenobiotics. This had the effect on oxidative stress that significantly interrupt cellular metabolism of spermatozoa.

In the present study the antioxidant activity expressed by total superoxide dismutase (SOD) activity in spermatozoa was measured. Significantly higher values of SOD activity were observed in spermatozoa of sterlet exposed to the highest concentrations of xenobiotics. From my results can be hypothesized that the level of antioxidant enzymatic activity in sterlet spermatozoa is not sufficient to prevent cellular damages and DNA fragmentation caused by oxidative stress resulted from short-term exposure to xenobiotic compounds. Unfortunately I did not get good results for the

antioxidant activity in brook trout sperm. It could be caused by worse quality of spermatozoa or by the dysfunction of some compound used for sample processing.

It is worth to mention, that sperm DNA damage assessment gained a special attention as a sperm quality marker (Peknicova et al, 2002). Comet assay have been used for the evaluation of DNA damage associated with *in vitro* exposure of sterlet and brook trout spermatozoa to all used xenobiotics. Thanks to this method I am able to demonstrate that the short-term exposure of sterlet and brook trout spermatozoa to xenobiotics caused a dramatic increase in DNA damage expressed by two parameters: Olive Tail Moment and percentage of DNA in Tail. Environmental pollution can be a substantial problem, which is responsible for dramatic increase of DNA fragmentation in gametes of animals with external fertilization. Except from DNA damage, which can affect sperm, DNA modifications could take place in this study, because they are generated by many chemical reactions (Box et al, 2001). If I compare the spermatozoa of two model organisms, it can be concluded that basic level of DNA fragmentation in control group was approximately the same for both species (1.5% of DNA in Tail for sterlet spermatozoa and 1.2% of DNA in Tail for brook trout spermatozoa). The % of DNA in Tail for sterlet spermatozoa treated with 10 μg/l of TBBPA was 4% (annex 9.3, p.129, fig. 4A), but in brook trout spermatozoa (where the highest used concentration of TBBPA was just 7.5 μg/l) it reached the level of DNA damage 11%, (graph 1B, p. 26). By contrast, when sperm was treated with xenobiotic duroquinone (150 μM), the % of DNA in Tail was higher in sterlet spermatozoa (7%) than in brook trout spermatozoa (just 3.5% of DNA in Tail), (annex 9.2, p. 101, fig. 5, graph 1C, p. 26).

It is already known that non-estrogenic as well as estrogenic organic compounds such as TBBPA, BPA, VIN, DQ or estradiol (Thomas et al, 1998) can bind to the sperm membrane receptors and unregulated spermatozoa motility. ATP metabolism is another potential target of my used xenobiotics. The effect of BPA, TBBPA, VIN and DQ on sperm energetic is another option that could be considered. It is well known, that ATP is the most important energy source required for axonemal beating in fish sperm (Perchec-Poupard et al, 1998; Rurangwa et al, 2002). ATP is essential for spermatozoa motility and decreased ATP stores results in decreased spermatozoa movement. My results point to a decreasing trend in intracellular ATP concentration during in vitro treatment. A positive significant correlation between intracellular ATP content and spermatozoa motility was reported.

However, protein degradation, showed in electrophoregrams of 2D seems to be responsible for changes in protein amount and structure. The effect of BPA, TBBPA, VIN and DQ has some devasting effect on protein composition. Similar results have been reported in human (Cao et al., 2003), boar (Huang et al., 1999) and bull spermatozoa (Lessard et al., 2000). Furthermore, nine or ten protein spots were changed. Unfortunately these proteins were not identified.

Thus it can be concluded that environmentally relevant concentrations of DQ, TBBPA, BPA and VIN are able to induce oxidative stress in spermatozoa leading to reduced motility, DNA integrity, and ATP content. Though the antioxidant system apparently responds to increased lipid peroxidation and carbonylation of proteins, it seems that the antioxidant capacity of spermatozoa is not sufficient to prevent cell damage.

6 Conclusion

In short, the results of my present study demonstrated that environmentally relevant concentrations of all used xenobiotics (DQ, TBBPA, BPA and VIN) can induce oxidative stress in fish spermatozoa in vitro, which results in accumulation of LPO and CP, together with the significant inhibition of ATP metabolism, leading to significant reduction of sperm quality. Based on obtained results it seems that the use of sperm of brook trout and sterlet for *in vitro* assay may provide a novel and efficient means for evaluation of the effect of xenobiotics in aquatic environment.

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8 List of symbols and abbreviations

- ATP- adenosinetriphosphate
- BPA- bisphenol A
- CP- carbonyl derivatives of proteins
- DI H_2O- distilled water
- DNA- deoxyribonucleic acid
- DQ- duroquinone
- LPO- lipid peroxidation
- ROS- reactive oxygen species
- SOD- superoxide dismutase
- TBARS- thiobarbituric acid reactive substances assay
- TBBPA- tetrabrombisphenol A
- VIN- vinclozolin

9 Annexes

Revised manuscript

The journal Chemico-Biological interaction allowed to state this article in present thesis.

9.1 Influence of environmentally relevant concentrations of vinclozolin on quality, DNA integrity, and antioxidant responses of sterlet *Acipenser ruthenus* spermatozoa

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ABSTRACT

The effects of vinclozolin (VIN), an anti-androgenic fungicide, on quality, oxidative stress, DNA integrity, and ATP level of sterlet (*Acipenser ruthenus*) spermatozoa were investigated *in vitro*. Fish spermatozoa were incubated with different concentrations of vinclozolin (0.5, 2, 10, 15, 20 and 50 μg/l) for 2 h. A dose-dependent reduction in spermatozoa motility and velocity was observed at concentrations of 2-50 μg/l. A dramatic increase in DNA fragmentation was recorded at concentrations 10 μg/l and above. After 2 h exposure at higher test concentrations (10-50 μg/l), oxidative stress was apparent, as reflected by significantly higher levels of protein and lipid oxidation and significantly greater superoxide dismutase activity. Intracellular ATP content of spermatozoa decreased with increasing concentrations of VIN. The results demonstrated that VIN can induce reactive oxygen species stress in fish spermatozoa, which could impair the sperm quality, DNA integrity, ATP content, and the antioxidant defense system.

Keywords: Spermatozoa motility, DNA damage, oxidative stress, ATP, sturgeon

1. Introduction

As the majority of pollutants are discharged into coastal waters [1], fishes inhabiting these areas are often chronically exposed to excessive concentrations of toxic compounds. Apart from possible effects on the endocrine system leading to disruptions in reproductive physiology [2,3], gametes of teleosts with external fertilization can be impacted by the ambient environment. Disruption in gamete development or physiology may have a deleterious effect on reproduction, a prerequisite for survival of any aquatic species. During the past decade, toxicity tests involving fish gametes have received wide attention [4,5]. Several studies have shown that ova are not ideal pollution bio-indicators since, at present, their quality can only be measured through time-consuming fertilization experiments. Moreover, the results of these experiments are subject to variability in sperm and uncontrolled factors in ova, such as over-ripening [6]. Toxicity screening using fish sperm has been considered a simple and valid model, since it does not require expensive sterile cell culture conditions [4].

Sturgeon have been classified as endangered by many international organizations. The population of sturgeon species has drastically declined, primarily due to over-fishing, habitat destruction, and water pollution [7]. In large species such as sturgeon, it is logistically difficult and costly to conduct toxicity evaluations on broodstock-size animals, and the need for sensitive, rapid toxicity testing has led to the increasing use of sturgeon sperm [5]. Fish spermatozoa are generally characterized by short-term motility, most less than 60 s. Considering the limited time available for fertilization after release of sperm into water, any negative effect on sperm quality may dramatically decrease fertilization success [8-10]. Spermatozoa are highly susceptible to oxidative injury, due not only to a lack of protective cytoplasmic enzymes, but also to the high content of poly-unsaturated fatty acids (PUFA) in their plasma membranes [11]. This leads to a decrease in the potential for successful fertilization and an increase in the probability of intracellular structure damage upon exposure to oxidizing agents [12].

In the present study, we investigated potential adverse effects of the well-characterized anti-androgenic compound vinclozolin (VIN) on sturgeon spermatozoa. Vinclozolin [3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-oxazolidine 2,4-dione] is a fungicide widely used in the United States and Europe to control disease in fruits and

vegetables [13]. According to Steeger and Garber [14] the peak estimated environmental concentration of vinclozolin is 52.0 μg/l. It degrades via hydrolysis quickly in neutral water (half-life = 1.3 d). In buffered aqueous solutions half-life of vinclozolin was shown to be temperature and pH dependent, so that in phosphate buffer with pH 7 at 13°C measured half-life was 140 h [15]. In aerobic and anaerobic environments, vinclozolin breaks down via microbial degradation, with half-lives ranging 17.6-134 days [14]. It is rapidly metabolized into two active metabolites, M1 and M2, which have high affinity for the androgen receptor and can block gene expression, causing anti-androgenic effects [16]. Previous studies have shown that VIN inhibits the development and maintenance of a variety of male traits in the guppy (*Poecilia reticulata*), including male coloration patterns, sperm count, and sexual behavior [17, 18].

This study was undertaken to investigate effects of short-term (2 h) *in vitro* exposure to VIN on quality parameters and oxidative stress in spermatozoa of the endangered sterlet *Acipenser ruthenus* by analyzing oxidative stress indices, such as lipid oxidation and protein carbonylation (CP) and superoxide dismutase (SOD) activity along with their motility and velocity, ATP content, and DNA integrity.

2. Materials and methods

2.1. Broodstock handling and collection of gametes

Sterlet sperm was obtained from six males (6–7 years old; body weight: 0.5-2 kg; body length: 55 ± 9.3 cm) reared in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Prior to hormone stimulation, fish were kept in tanks with water temperature varying between 14 and 15°C. Spermiation was stimulated by intramuscular injection of carp pituitary powder dissolved in 0.9% (w/v) NaCl at a dose of 5 mg/kg of body weight, 48 h before sperm collection. Semen was collected from the urogenital papilla by aspiration through a plastic catheter (5-7 mm diameter) connected to a 20 ml syringe. Special care was taken to avoid contamination with mucus, feces, or water. Samples were stored on ice (0–4°C) until processing. Spermatozoa concentration of each of the six males was estimated microscopically (Olympus BX 41) at 200x using a Burker cell hemocytometer.

2.2. Sample preparation

Vinclozolin (3-(3,5-dichlorophenyl)-5-ethenyl-5-methyl-2,4 oxazolidinedione; empirical formula: $C_{12}H_9Cl_2NO^3$; MW: 286.1), (PESTANAL®, analytical standard; Sigma-Aldrich, USA) was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 0.1 g/l. Stock solutions were prepared daily. Fish sperm from six individual males was centrifuged at 300×g, 4°C for 30 min to remove seminal plasma and diluted with an immobilization medium (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) to obtain spermatozoa densities of 5 x 10^8 cells/ml. The sperm sub-samples were then exposed for 2 h to final concentrations of 0.5, 2, 10, 15, 20, and 50 µg/l of VIN at 4°C. A group exposed to immobilization medium only was used as a control. Each experimental condition was duplicated.

2.3. Spermatozoa motility and velocity recording

Spermatozoa velocity (µm/s, measuring only motile spermatozoa) and percent motile spermatozoa (%) were determined after triggering motility under dark-field microscopy (Olympus BX 50, Japan) (20x objective magnification). For triggering, sperm was diluted 1:5000 in activation medium (10 mM Tris, 10 mM NaCl, 1 mM $CaCl_2$, pH 8.5). To avoid spermatozoa sticking to the microscope slide, 0.2% (w/v) Pluronic (Sigma-Aldrich, USA) was added to the swimming solution. Spermatozoa motility was recorded with a CCD video camera (SONY DXC-970MD, Japan) mounted on the microscope. Analysis of spermatozoa motility was made in triplicate for each sample.

2.4. Spermatozoa motility and velocity evaluation

Velocity and motility were assessed 10, 30, 60, 90, and 120 s post-activation. The successive positions of the video recorded spermatozoa heads were analyzed from five video frames using Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S). Velocity and percent motility were calculated from spermatozoa head positions on five successive frames with three colors (frame 1 red, frames 2–4 green, and frame 5 blue). Twenty to 40 spermatozoa were counted for each frame. Those that moved were visible in three colors, while non-moving spermatozoa were white. The percent motile was calculated from the number of white and red cells. Spermatozoa velocity was calculated as µm/s based on length traces of spermatozoa

from blue to green and red heads, calibrated for magnification.

2.5. Assessment of DNA damage

DNA integrity was assessed using the Comet assay, or single cell gel electrophoresis assay, following the method described by Li et al. [19]. Unless otherwise stated, molecular grade, DNAse-free reagents (Sigma Aldrich, USA) were used throughout. Microscope slides (OxiSelectST; Cell Biloabs, INC. USA) used for the assay were prepared in the following manner: The 100 µl sperm sample was diluted in 5 ml of PBS (phosphate buffer solution; Ca^{2+} and Mg^{2+} free) to obtain a spermatozoa density of $1x10^7$ cells/ml. Diluted samples (200 µl) were mixed with 700 µl 0.8% NuSieve GTG low melting point agarose (OxiSelectST; Cell Biloabs, INC. USA). Finally, 55 µl of this mixture was added to the slide, and the agarose was allowed to solidify for 1 h at 4°C. The slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10% Lysis Solution [OxiSelect™ Comet Assay Kit], 10% DMSO, pH 10) at 4°C. Slides were then subjected to enzyme treatment with proteinase K (1mg/ml in 2.5 M NaCl, 100 mM EDTA, 10% DMSO, pH 7.4) and incubated overnight at 32°C. After the proteinase K solution was drained, slides were immersed in a horizontal gel tank filled with freshly mixed electrophoresis buffer (90 mM Tris Base, 90 mM Boric acid, 2.5 mM EDTA). Electrophoresis was carried out for 20 min at 35 V and 170 mA. Slides were carefully transferred from the electrophoresis chamber to a clean container with pre-chilled DI H_2O for 2 min. The washing procedure was repeated three times. Slides were drained well, dehydrated by dipping into absolute ethanol for 5 min, and air-dried for storage. Prior to the analysis, 50 µl of Vista Green DNA Staining Solution (OxiSelectST; Cell Biloabs, INC. USA) was added to each agarose spot on the slide and analyzed using an Olympus BX50 fluorescence microscope at 20x magnification. One hundred spermatozoa were scored for each sample, and the captured images were analyzed using CometScore image analysis software (TriTek Corporation, USA). Tail length (measured from the middle of the head to the end of the comet tail) and % Tail DNA (content of DNA in comet tail) were measured. The percent DNA in tail and Olive tail moment were calculated using the following formulas:

% Tail DNA = 100 x Tail DNA Intensity/Cell DNA Intensity

Olive Tail Moment = % Tail DNA x Tail Moment Length.

2.6. Oxidative stress indices

Sperm samples were centrifuged at 5000 × g at 4°C for 10 min. The supernatant was carefully collected and discarded. The spermatozoa pellet was diluted with 50 mM potassium phosphate (KPi) buffer, pH 7.0, containing 0.5 mM EDTA to obtain a spermatozoa concentration of 5 x 10^8 cells/ml, then homogenized in an ice bath using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany). The homogenate was divided into two portions, one for measuring thiobarbituric acid reactive substances (TBARS) and carbonyl derivatives of proteins (CP) and a second that was centrifuged at 12 000 x g for 30 min at 4°C to obtain the post mitochondrial supernatant for the antioxidant enzyme activity assay.

The TBARS method described by Lushchak et al. [20] was used to evaluate sperm lipid oxidation (LO). Its concentration was calculated by absorption at 535 nm and a molar extinction coefficient of 156 mM/cm. The content of TBARS was expressed as nanomoles per 10^8 cells. Carbonyl derivatives of proteins were detected by reaction with 2,4-dinitrophenylhydrazine according to the method described by Lenz et al. [21]. The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM/cm, expressed as nmol per 10^8 cells.

2.7. Superoxide dismutase activity

Total SOD (EC 1.15.1.1) activity was determined by the method of Marklund and Marklund [22]. This assay depends on the autoxidation of pyrogallol. The activity of SOD in supernatant was assessed spectrophotometrically at 420 nm. One unit of SOD activity is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical per min. Activity was expressed in international units (or milliunits) mU per 10^8 cells.

2.8. Evaluation of ATP content

ATP content in spermatozoa was determined after 2 h exposure to VIN using the bioluminescence method described by Boryshpolets et al. [23]. Sperm samples were diluted in 5 ml boiling medium containing 100 mM Tris-HCl, pH 7.5 and 4 mM EDTA. After boiling for 2 min at 98°C samples were centrifuged at 12 000 x g for 20 min. Supernatant was collected and stored at -80°C before analysis. A Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany) was used for determination of

ATP content. Luminescence was recorded with a multifunctional microplate reader Infinite M200 (Tecan, Austria). ATP content was expressed as nmol ATP/10^8 spermatozoa.

2.9. Data analysis

All measurements were conducted in triplicate. Normality and the homogeneity of variance of all data were first tested with the Kolmogorov test and the Bartlett test, respectively. Data obtained from the Comet assay were log-transformed if necessary. Values are expressed as means ± SD (n = 6) and analyzed by factorial ANOVA. Statistical comparison was made by analysis of variance (ANOVA) followed by Tukey's HSD test for each analyzed parameter. The relationships among the parameters were quantified according to the Spearman's correlation tests. Values for motility and velocity only at 10 sec post-activation were used. The t-test was used to establish if the correlation coefficients are significantly different from zero. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 software for Windows.

3. Results

3.1. Spermatozoa motility and velocity

The effect of VIN on percent motile spermatozoa and their curvilinear velocity was assessed 10, 30, 60, 90, and 120 s post-activation (Fig. 1, A and B). Motility and velocity significantly lower than in the control were observed after exposure to concentrations of VIN from 2 to 50 μg/l ($P < 0.05$) at all post-activation times. Motility parameters of spermatozoa exposed to the lowest concentration of VIN (0.5 μg/l) were significantly different from the control only at 10 and 30 sec post-activation. In general, motility and velocity of spermatozoa exposed to VIN concentrations of 15-50 μg/l were 16-20% lower that in control. A significant positive correlation ($r = 0.713$, $P < 0.05$) was found between percent motile spermatozoa and ATP content (Table 1). A significant negative correlation ($r = - 0.628$, $P < 0.05$) between spermatozoa motility and the level of protein carbonylation was observed. Fig. 2 illustrates changes in flagella movement after exposure of spermatozoa to VIN. The beat frequency and number of waves along the flagellum were significantly lower at the highest concentration of VIN compared to control. Atypical C shaped flagella were observed.

3.2. DNA fragmentation

An illustration of the outcome of this assay for DNA damage is presented in Fig. 3. Obtained pictures of comets were used to evaluate following parameters: % of DNA in Tail (calculated by formula: % Tail DNA = 100 x Tail DNA Intensity/Cell DNA Intensity) and Olive Tail Moment (Olive Tail Moment = % Tail DNA x Tail Moment Length). Comet assay analysis indicated a dramatic increase in DNA fragmentation in spermatozoa nuclei after 2 h exposure to VIN at concentrations of 10-50 μg/l compared to controls ($P < 0.05$; Fig. 4, A and B). No significant differences ($P > 0.05$) in DNA integrity were found between the control group and spermatozoa exposed to nominal VIN concentrations of 0.5 to 2 μg/l. The highest VIN concentration was associated with 20% DNA fragmentation (Fig. 4A). There was significant correlation between % DNA in tail and TBARS level (Table 1) ($r = 0.354$, $P < 0.05$).

3.3. Oxidative stress indices and antioxidant response

The level of TBARS and CP were used as indicators of the extent of LO and protein oxidation, respectively. In the control group, TBARS level was typically around 0.16 nmol/10^8 spermatozoa (Fig. 5). A significantly higher level of LO was observed in spermatozoa exposed to VIN at 10 μg/l and above. The highest concentration of VIN enhanced the TBARS level to 0.33 nmol/10^8 spermatozoa. There was no significant difference in CP level between spermatozoa in the control group and group exposed to 0.5 μg/l VIN (Fig. 6). A gradual increase in CP level was observed in groups exposed to concentrations greater than 2 μg/l. The maximum detected CP was 11.6 nmol/10^8 in spermatozoa exposed to 50 μg/l VIN. Spearman's correlation revealed significant negative correlation between ATP content and TBARS ($r = - 0.66$, $P < 0.05$) and CP ($r = - 0.61$, $P < 0.05$) levels (Table 1).

The antioxidant activity was assessed by total SOD activity. The antioxidant response was significantly enhanced in all treatment groups (Fig. 7). Total SOD was 6.5 mU/10^8 in spermatozoa at 50 μg/l of VIN compared to 2 mU/10^8 in controls. An increase in this parameter was not observed with increasing concentrations of VIN. A positive correlation was found between SOD activity and CP level ($r = 0.41$, $P < 0.05$).

3.4. ATP content

The ATP levels can indicate decline in quality of fish spermatozoa. Intracellular ATP content in the control group was 6.8 nmol ATP/10^8 spermatozoa (Fig. 8). After 2 h exposure to VIN at concentrations higher than 2 μg/l, the ATP level declined significantly in a dose-dependent manner. The lowest ATP content was approximately 5.0 nmol ATP/10^8 spermatozoa at VIN concentrations of 15-20 μg/l. ATP content in spermatozoa exhibited a significant positive correlation with motility (r = 0.71, P < 0.05) and velocity (r = 0.29, P < 0.05) and showed a significant negative correlation with TBARS level (r = - 0.66, P < 0.05) and CP (r = - 0.61, P < 0.05) (Table 1).

4. Discussion

Environmental contaminants have been shown to induce reproductive dysfunction in both wildlife and humans [12,24]. Despite the volume of research into the effects of chemicals having endocrine-disrupting properties in fish, there is limited evidence that endocrine disruption adversely affects individual fertility and, hence, poses problems for the wider population. In this study an attempt was made to determine *in vitro* the effects of anti-androgen VIN on spermatozoa performance, using sterlet as a model organism. Results of the present study suggest that sturgeon spermatozoa quality, DNA integrity, ATP metabolism, and the antioxidant defense system can be impaired by VIN-mediated oxidative stress.

Because variations in spermatozoa motility might reflect quantitative and qualitative toxic effects of ectogenous contaminants, monitoring of spermatozoa motility parameters is an important approach in toxicity experiments [25]. In the current study, spermatozoa motility and velocity were significantly reduced after *in vitro* exposure to environmentally relevant concentrations of VIN. There are several possible ways in which anti-androgens may affect spermatozoa motility activation and movement. In mammals, it has been shown that anti-androgens repress androgen receptor (AR) mediated transcriptional activation, by competitive inhibition of endogenous androgens binding to their receptor [26-30]. Binding of anti-androgens may result in a conformational change of the ligand binding domain of AR to bring about interaction with co-repressors instead of co-activators [31,32]. Besides classical intracellular steroid hormone receptors, several membrane steroid receptors, capable of mediating non-genomic steroid actions, have been described [33,34]. Despite the

limited amount of information about membrane receptors in fish spermatozoa, it is noteworthy that Thomas and Doughty [35] observed that non-estrogenic as well as estrogenic organic compounds could interfere with a rapid non-genomic progestin action to up-regulate spermatozoa motility in Atlantic croaker (*Micropogonias undulatus*).

ATP metabolism is another potential target of VIN. ATP is essential for spermatozoa motility, and decreased ATP equates with decreased spermatozoa movement [36]. Several studies have shown that aquatic pollution might result in intracellular ATP depletion within cells [4,36]. Pathways to inhibition of ATP production may differ with pollutant. In general, anti-androgen agents inhibit the mitochondrial respiratory chain and ATP formation [37]. In the present study, the percent of motile spermatozoa was strongly correlated with intracellular ATP content. Both parameters decreased after 2 h incubation with VIN. ATP concentration within the cell determines motility duration and flagella beat frequency [36,38]. Therefore decrease of ATP content could explain changes in flagella behavior observed in the present study.

Studies have demonstrated that VIN can induce production of ROS in cell cultures [39,40]. Oxidative stress results in direct or indirect ROS-mediated damage of nucleic acids, proteins, and lipids. The toxic effect of ROS on fish spermatozoa has been reported for several species [41,42]. In mammals it has been shown that an accumulation of peroxidation products in mitochondria leads to a decrease in ATP production and compromises the maintenance of cell homeostasis [43]. In our study a significant correlation was found between ATP content and the level of TBARS and CP. Therefore we hypothesize that the accumulation of free radicals (LO, CP) in spermatozoa could indirectly modify energy metabolism.

Increased levels of LO and CP observed in the present study also suggest spermatozoa susceptibility to VIN-induced free radical production. TBARS levels were greater in spermatozoa incubated with VIN at concentrations > 10 μg/l. This is not surprising, since a major mechanism of action of VIN is lipid peroxidation and membrane disruption [44]. Peroxidation of PUFAs in spermatozoa cell membranes is an autocatalytic, self-propagating reaction, which can give rise to cell dysfunction [11].

Lipid oxidation can trigger the loss of membrane integrity, causing increased cell permeability, enzyme inactivation, and structural damages of DNA [45,46].

Increase in CP level also indicates oxidative stress in the cell. Protein carbonylation is the most frequently used marker of protein oxidation. There is evidence that oxidative modification leads to proteolytic degradation, which may affect the structure, function, and integrity of proteins [41,47,48]. Previous studies have shown that protein oxidation and accumulation of lipid hydroperoxides in the plasma membrane can profoundly affect fertilization ability of spermatozoa [48]. In this study we observed increased carbonylation of proteins after 2 h exposure to concentrations of VIN higher than 2 μg/l. This demonstrates that environmentally realistic concentrations of anti-androgens such us VIN are capable of inducing protein oxidation as well as lipid oxidation in sterlet spermatozoa.

To counteract the damaging effects of ROS, a variety of antioxidant enzymes are present in fish spermatozoa [41,49]. Superoxide dismutase is considered the first line of defense against the effects of oxyradicals in the cell through catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen [5]. In the present study, significantly increased SOD activity was observed in all treatment groups. This is likely to be an adaptive response to toxicant stress and serves to neutralize the impact of increased ROS generation.

Studies of human spermatozoa have revealed that oxidative stress may induce significant DNA damage in both the mitochondrial and nuclear genomes [50]. Lack of repair mechanisms renders spermatozoan DNA vulnerable to damage, and this damage is linked to male infertility [51]. Vinclozolin is known to cause DNA methylation in germ cells of rats and mice and to promote transgenerational disease [52]. However, no data have been reported on the effect of VIN on DNA integrity of spermatozoa of aquatic organisms. In the present study, the Comet assay was used to evaluate the effect of xenobiotic VIN on DNA integrity. Exposure to VIN at concentrations higher than 10 μg/l enhanced DNA fragmentation, expressed as percent of DNA in comet tail and Olive tail moment. Positive correlation was found between the level of DNA fragmentation and oxidative stress indices, indicating that the observed DNA damage was a result of a free radical production. Correlation between loss of DNA integrity and increased TBARS level is in accordance with other reports [53,54], indicating that lipid

oxidation results in the production of reactive products which are a major source of endogenous DNA damage.

Results of the present study demonstrated that sterlet spermatozoa are highly sensitive to the presence of small concentrations of anti-androgenic compounds such as VIN. Environmentally relevant concentrations of this fungicide are able to induce oxidative stress in spermatozoa leading to reduced motility, DNA integrity, and ATP content. Though the antioxidant system apparently responds to increased lipid oxidation and carbonylation of proteins, it seems that the antioxidant capacity of spermatozoa is not sufficient to prevent cell damage.

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Figure legends:

Fig. 1. *In vitro* effects of vinclozolin on spermatozoa motility (A) and velocity (B) in *Acipenser ruthenus*. Data are presented as means ± SD. Superscript letters indicate significant differences among samples at the same time post-activation (ANOVA, $P < 0.05$).

Fig. 2. Swimming behavior of *Acipenser ruthenus* spermatozoa incubated with three concentrations of vinclozolin (VIN) (0.5, 15, 50 µg/l). Flagella movement was recorded under dark field microscopy with stroboscopic illumination at 10, 60, and 120 s post-activation of spermatozoa.

Fig. 3. Examples from Comet assay of *Acipenser ruthenus* spermatozoa exposed to differing concentrations of vinclozolin (a– control, b– 0.5 µg/l, c– 2 µg/l, d– 10 µg/l, e– 15 µg/l, f– 20 µg/l, g– 50 µg/l). Relative changes in DNA fragmentation are represented by an increasing amount of DNA in the comet tail.

Fig. 4. Influence of vinclozolin on DNA fragmentation in *Acipenser ruthenus* spermatozoa measured by Comet assay as a percentage of DNA in the tail (A) and Olive

tail moment (B). Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Fig. 5. Effect of vinclozolin on TBARS level in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Fig. 6. Effect of vinclozolin on CP level in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Fig. 7. Effect of vinclozolin on SOD activity in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Fig. 8. Influence of vinclozolin on ATP content in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SD. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Table 1. Correlation coefficients among the parameters measured in *Acipenser ruthenus* spermatozoa after 2 h exposure to vinclozolin. The t-test is used to establish significance of the correlation between pairs of parameters.

Figure 1A

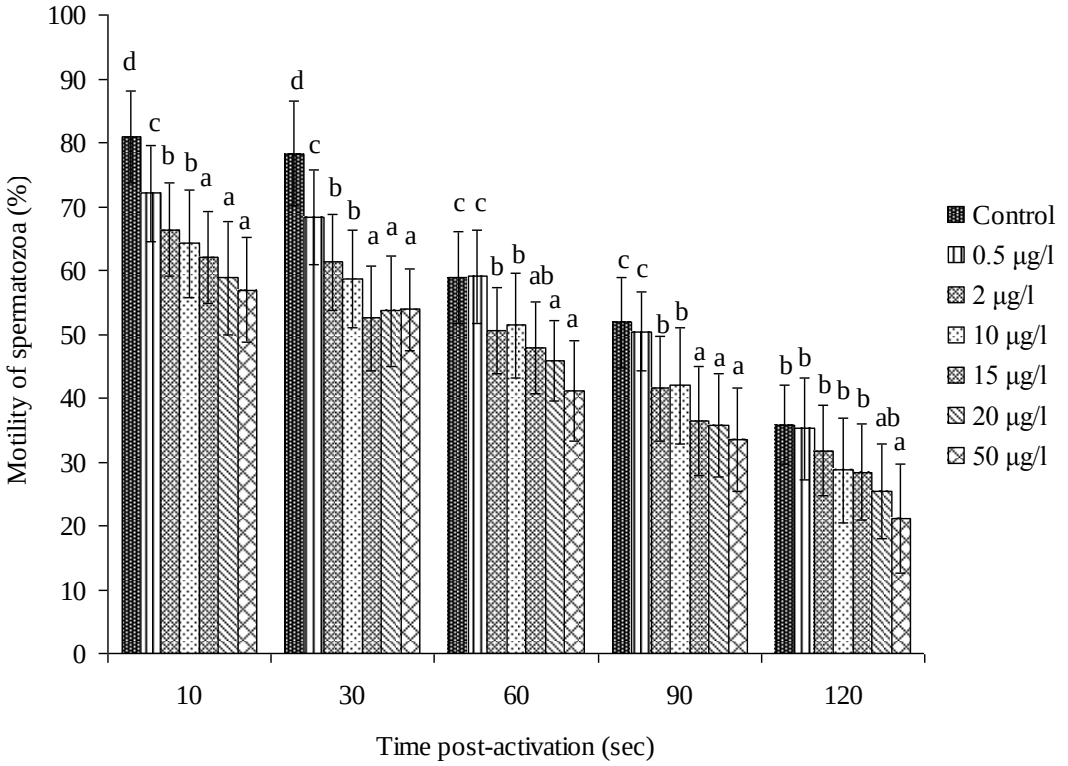


Figure 1B

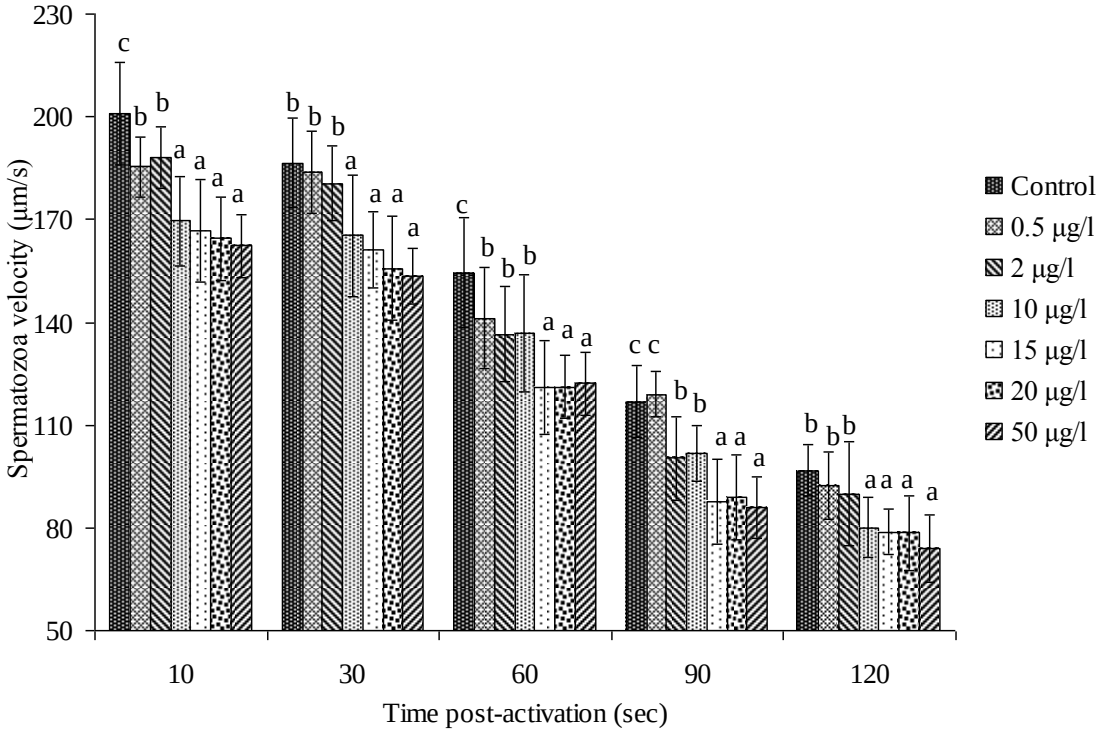


Figure 2.

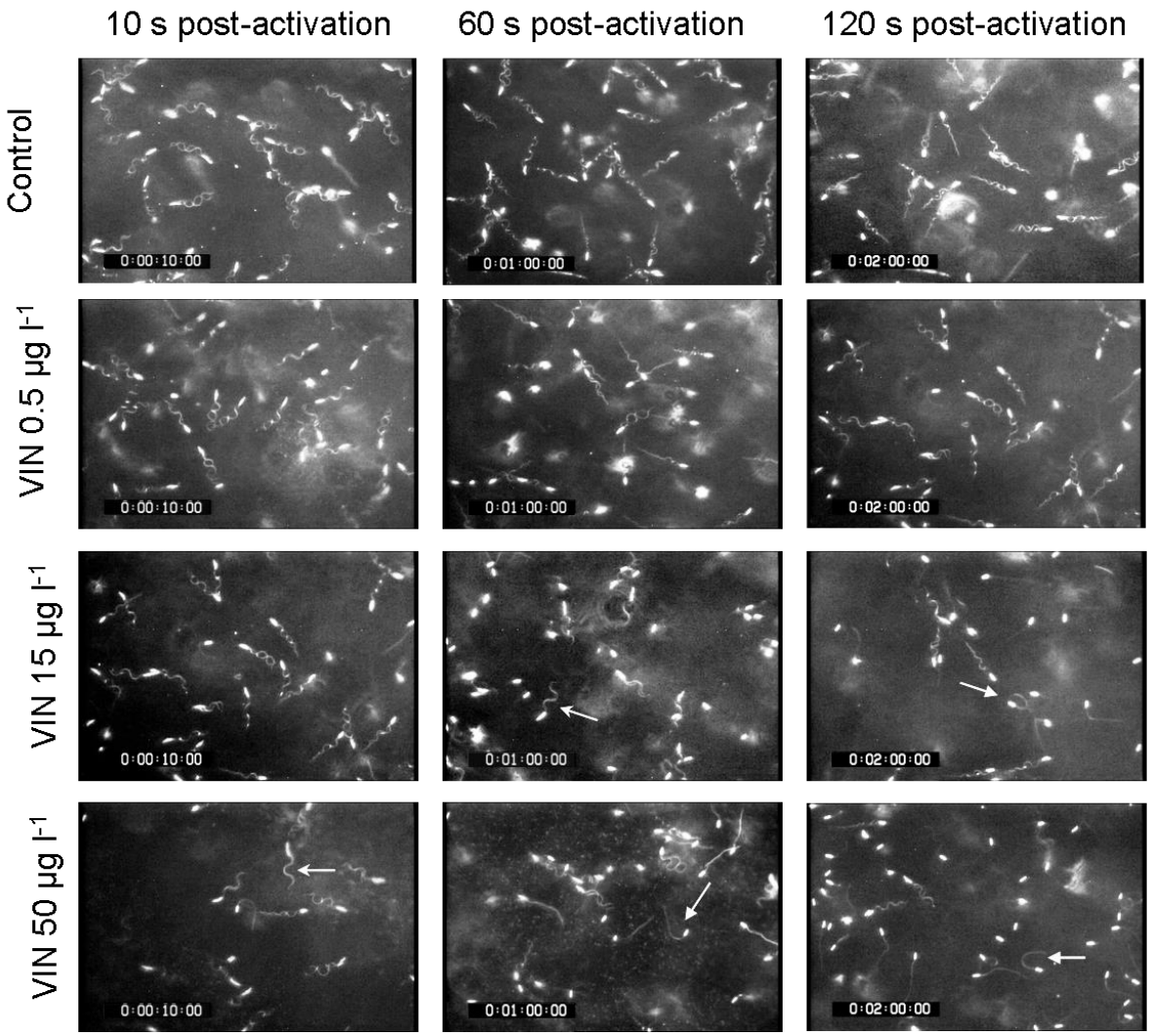

10 s post-activation
60 s post-activation
120 s post-activation
Control
VIN 0.5 µg l-1
VIN 15 µg l-1
VIN 50 µg l-1
0:00:10:00
0:01:00:00
0:02:00:00

Figure 3

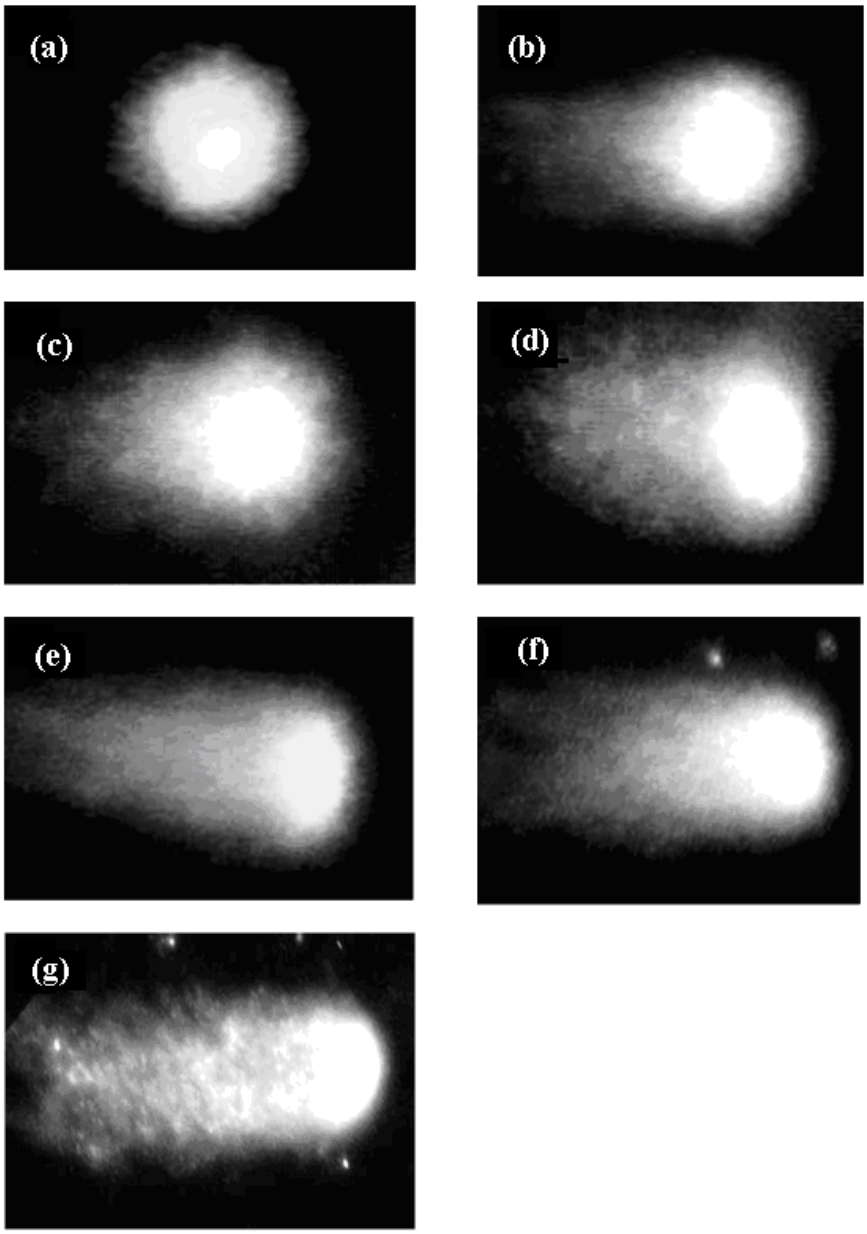


Figure 4A.

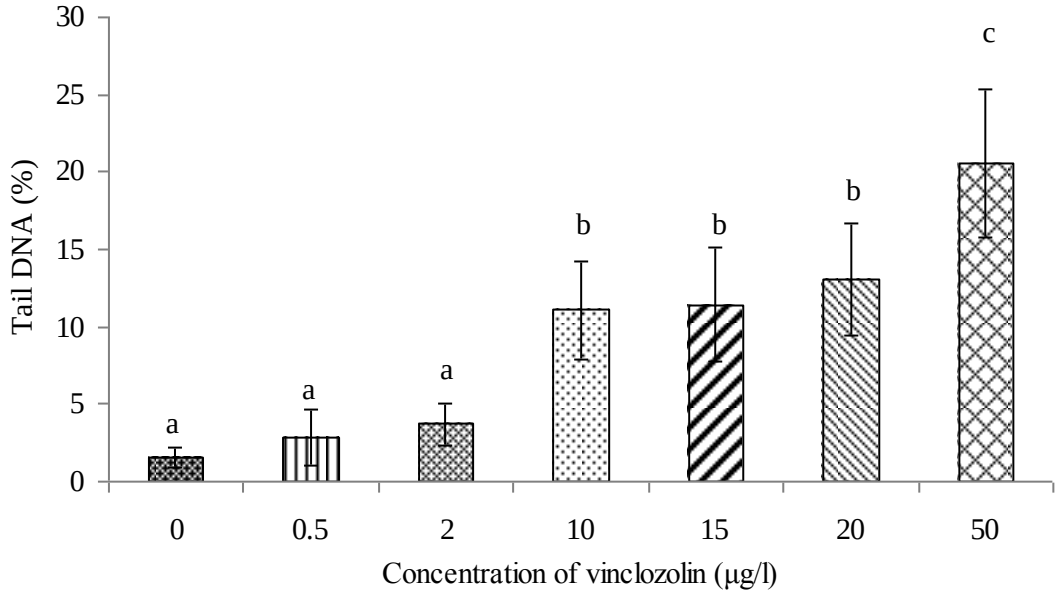


Figure 4B.

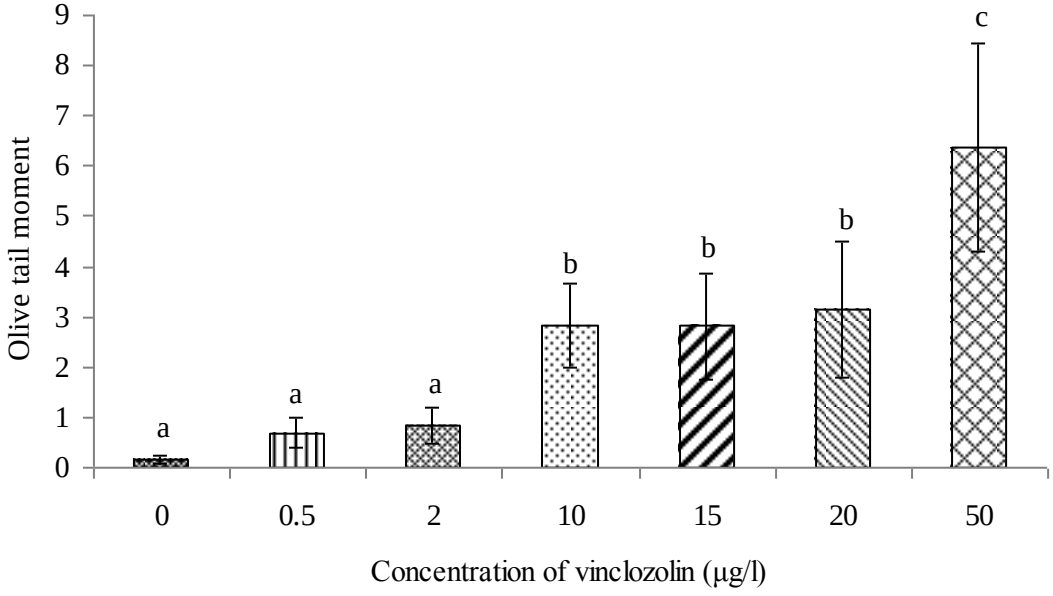


Figure 5

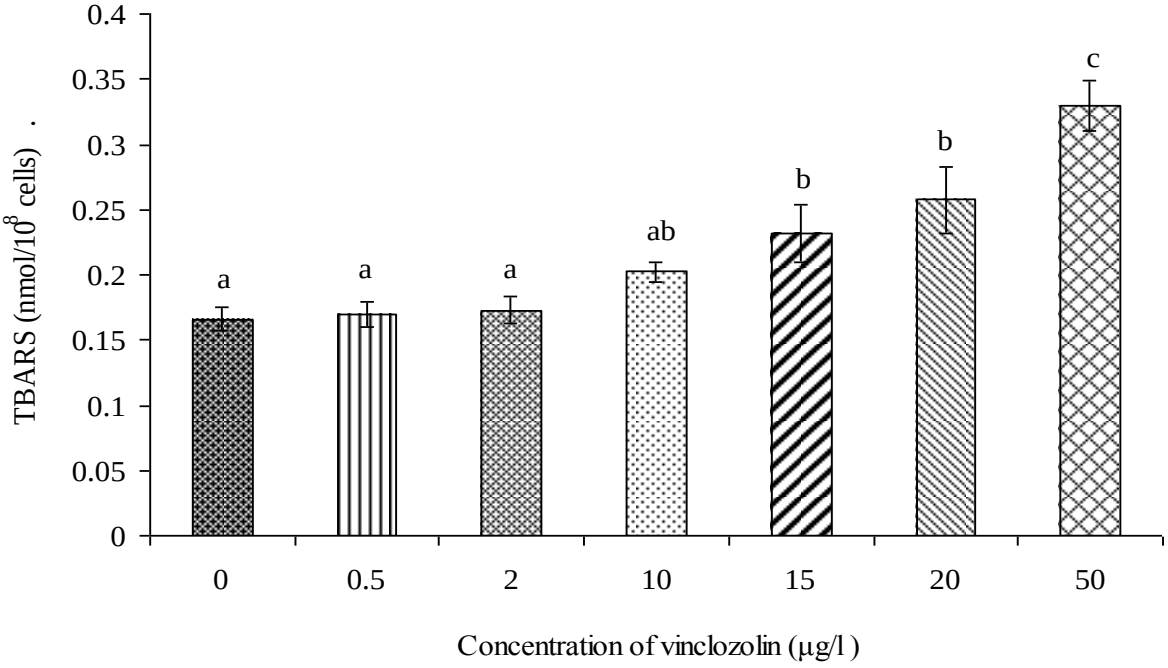
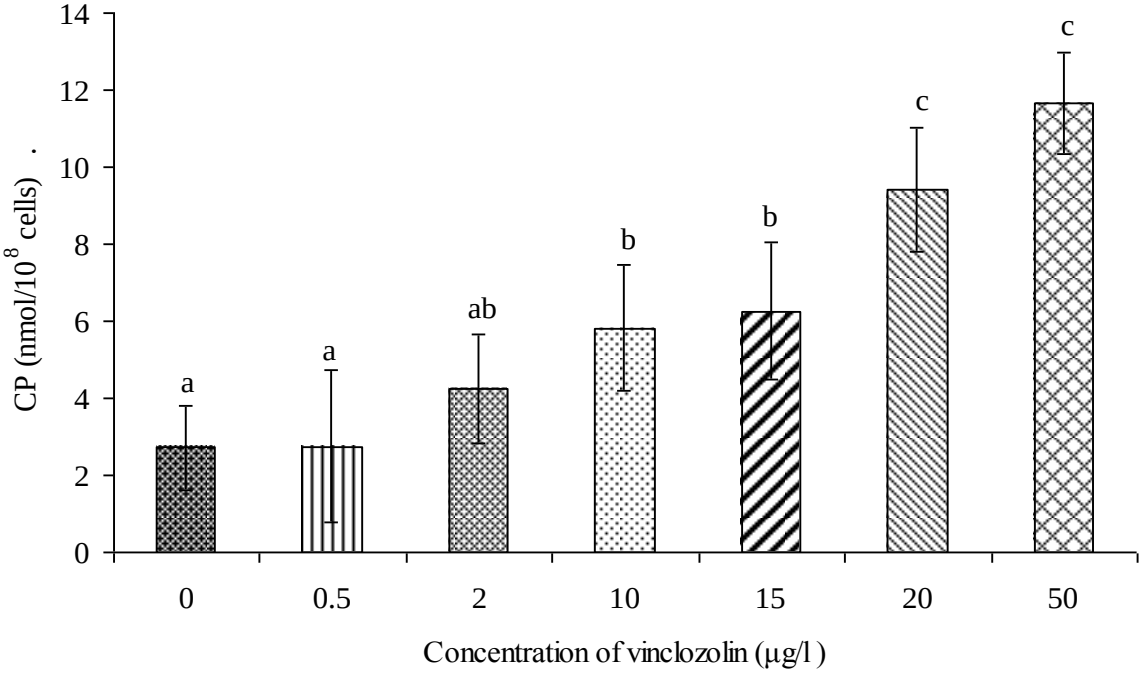


Figure 6



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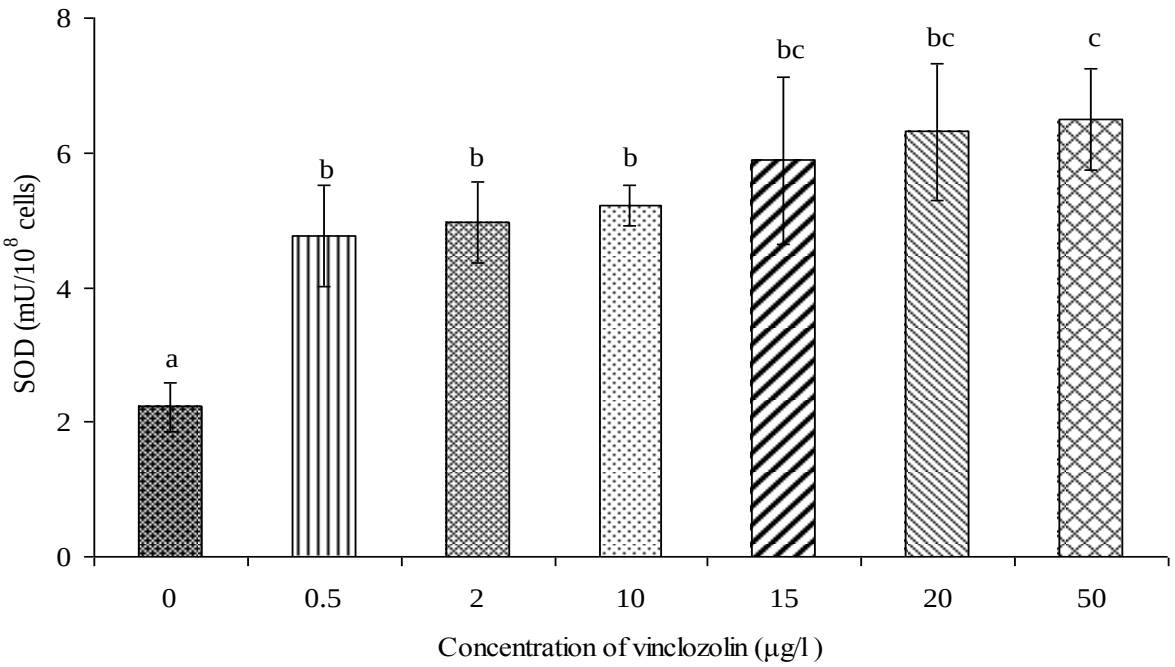

Figure 8.

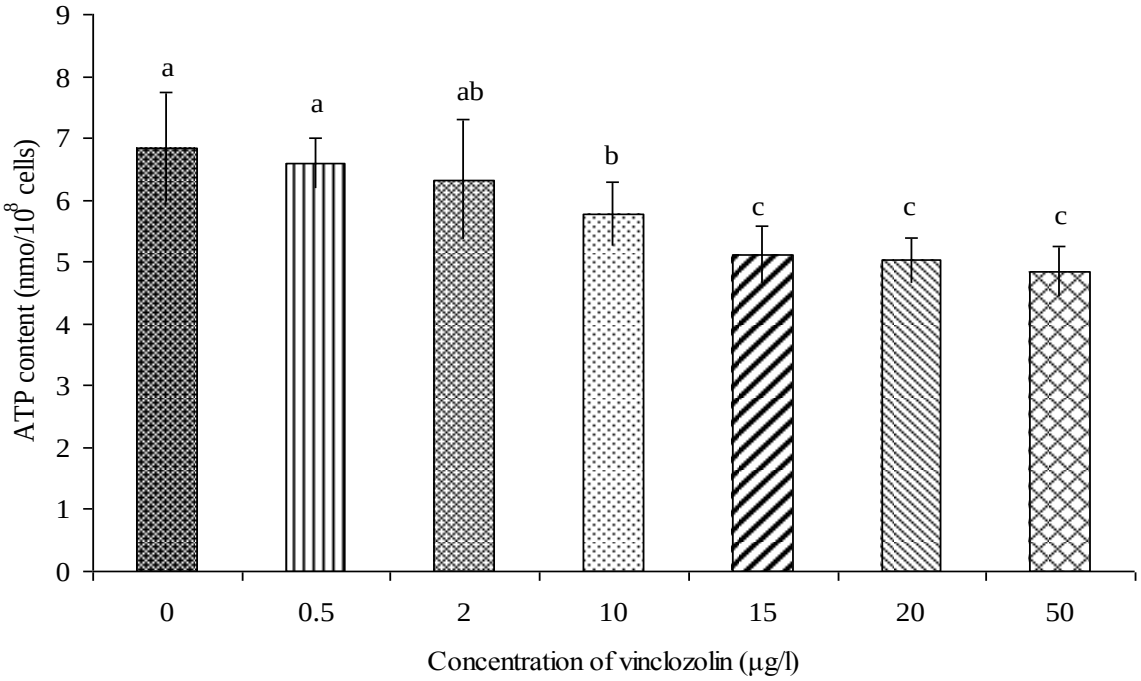

Table 1.

	Motility	Velocity	%DNA in tail	Olive tail moment	TBARS	CP	SOD	ATP
Motility	1.00							
Velocity	0.28[1]	1.00						
%DNA in tail	-0.36*	-0.19*	1.00					
Olive tail moment	-0.33*	-0.23*	0.92*	1.00				
TBARS	-0.43*	-0.202	0.35*	0.29*	1.00			
CP	-0.62*	-0.30*	0.15	0.14	0.24	1.00		
SOD	-0.45*	0.02	0.11	0.18	0.21	0.41*	1.00	
ATP	0.71*	0.29*	-0.27*	-0.2	-0.66*	-0.61*	-0.28*	1.00

$P < 0.05$ according to the Spearman's test.

9.2 The *in vitro* effect of duroquinone on functional competence, genomic integrity, and oxidative stress indices of sterlet (*Acipenser ruthenus*) spermatozoa

The journal Toxicology in Vitro allowed to state this article in present thesis.

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ABSTRACT

The sturgeon is a highly endangered fish species mostly due to over-fishing, habitat destruction, and water pollution. Duroquinone (derivative of 1,4-benzoquinone) is a xenobiotic compound widespread in the environment. The effect of duroquinone on motility, DNA integrity, and oxidative stress indices in sterlet, *Acipenser ruthenus*, spermatozoa was investigated. Sterlet sperm was exposed for 2 h to duroquinone at concentrations of 25, 50, 100, and 150 μM. Spermatozoa motility, velocity, and ATP content were significantly decreased with exposure to duroquinone. The level of DNA damage significantly increased at concentrations of 50 μM and above. Oxidative stress indices (lipid peroxidation and content of carbonyl proteins) and superoxide dismutase (SOD) activity increased significantly with increasing concentrations of duroquinone. Oxidative stress in sterlet spermatozoa induced by duroquinone was shown to impair spermatozoa DNA integrity, motility parameters, and the antioxidant defense system. Spermatozoa motility, content of carbonyl proteins, and SOD activity were shown to be sensitive biomarkers, exhibiting strong responses to low concentrations of the xenobiotic. Results also suggested that fish spermatozoa *in vitro* assays may provide a simple and efficient means of monitoring residual pollutants in the aquatic environment.

Keywords: reactive oxygen species, DNA damage, Comet assay, spermatozoa motility, sturgeon

1. Introduction

Over the past 20 years it has been established that fish are capable of a wide variety of biotransformation reactions (Li et al., 2012, Thomas et al., 1998). Recent studies on the biotransformation of xenobiotic chemicals in fish have focused on the specific metabolites produced, since these metabolic reactions affect distribution, accumulation, and toxicity of chemicals (Hulak et al., 2008; Mikula et al., 2009). Xenobiotics are also known to enhance production of reactive oxygen species (ROS) (Livingstone, 2001) that can attack a variety of biological macromolecules such as DNA, proteins, and lipids, leading to oxidative damage (Kelly et al., 1998). Increased levels of ROS are believed to be involved in male infertility associated with lipid peroxidation and oxidative stress, which affect sperm physiology and integrity (Sikka et al., 1995).

Duroquinone is a potentially hazardous xenobiotic compound receiving recent attention. Duroquinone is a derivate of 1,4-benzoquinone which is well soluble in water, and, at low concentrations (50 - 150 μM), was shown to induce cytotoxic effects through ROS production (Lemaire et al., 1994; Zhou et al., 2006).

A wide variety of quinones (Smith et al., 1985), including duroquinone, enter freshwater and marine ecosystems from anthropogenic sources. Quinones are widespread in the environment and form an important class of toxic metabolites generated by the metabolism of phenols and related compounds, including phenol, 1-naphthol, and diethyl stilbesterol. They occur both naturally and as water pollutants at concentrations to 480 μg/l (Usenko, 2012). One of the more common types of quinones is benzoquinone and its derivatives. Among them, duroquinone is of prime interest due to its ability to induce mitochondrial Ca^{2+} release through H_2O_2 generation by redox cycling followed by NADPH oxidation and gluthatione oxidation (Moore et al., 1987). Hence if the antioxidant system is robust, duroquinone has only a minimal cytotoxic effect, as shown in rat hepatocytes (Moore et al., 1987), and may be more damaging to cell types lacking protective cytoplasmic enzymes.

Reproductive success is a key factor in determining species survival. Water pollution can decrease the quality of gametes of aquatic organisms, which in turn may affect fertilization success, hatching of embryos, and subsequent survival of offspring. The gametes of most fish species are usually released into water, where they can be

directly exposed to manmade compounds capable of interrupting cellular metabolism at various levels. Since spermatozoa movement is a major prerequisite for successful fertilization, assessment of motility and velocity may be a sensitive and accurate bio-indicator of aquatic pollution (Li Z. H. et al., 2010a).

The sturgeon (Acipenseridae) is among the oldest living vertebrates and is classified as endangered fishes by many international organizations. Their population has drastically decreased, mostly due to over-fishing and destruction of natural habitat, as well as to water pollution (Birstein and DeSalle, 1998). For a large species such as sturgeon, it is logistically difficult and costly to conduct toxicity evaluations on broodstock-sized animals (Tashjian et al., 2006). In the present study we used sterlet *Acipenser ruthenus* as a model to investigate potential adverse effects of duroquinone on spermatozoa in sturgeon. *Acipenser ruthenus* is a common Eurasian sturgeon, that has undergone significant decline, but local populations survive in most parts of its distribution range (rivers draining to the Black, Azov, and Caspian Seas; Siberia from Ob eastward to the Yenisei drainages; and Danubian estuaries). The sterlet is an attractive model for biological studies of sturgeon because of its freshwater status, its small size and therefore low cost of maintenance, and its early sexual maturation. Sterlet spermatozoa differ from those of teleost fish (Billard, 1986) in the presence of an acrosome (Psenicka et al., 2007) with acrosin- and trypsin-like activities (Ciereszko et al., 1994, 1996), as well as in size and behavior (Cosson et al., 2000). The presence of acrosomal reactions in sturgeon spermatozoa means differing enzyme activity from teleosts (Ciereszko et al, 1996). The differences in sperm morphology and biochemistry between chondrostean and teleost fish species may translate to different reactions to environmental stimuli.

The aim of this study was to investigate how spermatozoa physiology is affected by short-term (2 h) *in vitro* exposure of sterlet sperm to environmentally relevant concentrations of duroquinone, by analyzing DNA integrity, oxidative stress indices (lipid peroxidation - LPO and protein carbonylation - CP), and antioxidant enzyme (superoxide dismutase - SOD) activity of sturgeon sperm, as well as spermatozoa motility and velocity. The effect of duroquinone on intracellular ATP level was also investigated.

2. Materials and methods

2.1. Broodstock handling and collection of gametes

Sperm of sterlet was obtained from six males (age: 6–7 years; body weight: 1.5 ± 0.2 kg; body length: 55 ± 9.3 cm) reared in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia. Spermiation was stimulated by intraperitoneal injection of carp pituitary powder dissolved in 0.9% NaCl solution at doses of 4–5 mg/kg of body weight, 48 h before sperm collection. Sperm was collected in 250 ml cell culture containers and kept on ice (0–4 ° C) until processing. Sperm concentration of each male was estimated microscopically (Olympus BX 41) at 20× using a Burker cell hemocytometer.

2.2. Chemicals

All chemicals used were analytical grade and purchased from Sigma (St. Louis, MO, USA). The stock solution of duroquinone (DQ) was dissolved in 96% ethanol and diluted with distilled water to obtain a stock solution of 10 mg/ml.

2.3. Sperm dilution and exposure

Sturgeon sperm was diluted with an immobilization medium (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) to obtain a spermatozoon concentration of 5×10^7 cells/ml. We use the standard concentration of spermatozoa per egg in sterlet to ensure fertilization success. Sperm sub-samples (n = 6) were then exposed for 2 h to environmentally realistic DQ concentrations of 25, 50, 100, and 150 μM. A control group was exposed to immobilization medium with 1% ethanol equal to the amount of ethanol in the experimental samples. Each experimental condition was conducted in duplicate.

2.4. Sperm motility and velocity

Spermatozoa velocity and percent of motile spermatozoa were determined after triggering motility under dark-field microscopy (Olympus BX 50, Japan) (20 x objective magnification). For triggering, sperm was diluted in activation medium (10

mM NaCl, 1 mM CaCl, 10 mM Tris pH 8.5) at a dilution ratio of 1:5000. To avoid sperm sticking to the microscope slide, Pluronic 0.2% (w/v) (Sigma-Aldrich, USA) was added to the swimming solution. Spermatozoa motility was recorded with a CCD video camera (SONY DXC-970MD, Japan) mounted on the microscope, illuminated with a stroboscopic flash (ExposureScope®, Czech Republic). Analysis of sperm motility was done in triplicate for each sample.

Velocity and motility were assessed at 10, 30, 60, 90, and 120 s post-activation. The successive positions of the video recorded spermatozoa heads were analyzed from video frames using Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S). Velocity and percent motility were calculated from spermatozoa head positions on five successive frames with three colors (frame 1 red, frames 2–4 green, and frame 5 blue). Twenty to 40 spermatozoa were counted for each frame. Spermatozoa that moved were visible in three colors, while non-moving spermatozoa were white. The percent of motile spermatozoa was calculated from the number of white and red cells. Spermatozoa velocity was calculated at μm/s based on length traces of spermatozoa from blue to green and red heads, calibrated for magnification.

2.5. Assessment of DNA Damage

The alkaline single-cell gel electrophoresis (Comet) assay followed the methods of Li et al. (2010). Unless otherwise stated, molecular grade DNAse-free reagents (Sigma Aldrich, USA) were used throughout. Microscope slides (OxiSelectST; Cell Biolabs, INC. USA) were used for the assay, with each slide prepared in the following manner: 100 μl of sperm ($5x10^7$ cells/ml) was diluted in 5 ml of PBS. Diluted samples (200 μl) were mixed with 700 μl of 0.8% NuSieve GTG low melting temperature agarose (OxiSelectST; Cell Biolabs, INC. USA). Finally, 55 μl of this mixture was added to the slide, and the agarose was allowed to solidify for 1 hour. Slides were immersed in lysis buffer (2.5M NaCl, 100mM EDTA, 10% of 10x Lysis Solution [OxiSelect™ Comet Assay Kit], 10% DMSO pH 10). The slide was then subjected to enzyme treatment with proteinase K (20 mM Tris-HCl, 1 mM $CaCl_2$, and 50% glycerol, pH 7.4) and incubated for 12 h at 32 ° C. After the proteinase K solution was drained, the slides were immersed in a horizontal gel tank filled with running buffer, and electrophoresis

was carried out for 20 min at 35 V and 170 mA. The slides were drained well, dehydrated by dipping into absolute ethanol for 5 min, and air-dried for storage. Fifty µl of Vista Green DNA Staining Solution (OxiSelectST; Cell Biolabs, INC. USA) was added to each agarose spot, and the slide was examined using an Olympus BX50 fluorescence microscope at 20x magnification. One hundred cells were scored for each sample, and the captured images were analyzed using CometScore image analysis software (TriTek Corporation, USA). Tail length (measured from the middle of the head to the end of the tail) and tail DNA content (% tail DNA) were measured. Percent of DNA in tail and Olive tail moment were measured using the following methods:

Tail DNA% = 100 x Tail DNA Intensity/Cell DNA Intensity

Olive Tail Moment = Tail DNA% x Tail Moment Length.

2.6. Oxidative stress indices and antioxidant activity

The sperm samples were centrifuged at 13000 x g at 4 ° C for 10 min, and the pellet, in an ice bath, was homogenized using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany) with potassium phosphate buffer (50 mM KPi-buffer pH 7.0, 0.5 mM EDTA, 0.1 mM PMSF) to obtain density of 5×10^8 cells/ml. The homogenate was divided into two portions, one for measuring TBARS and CP, and a second centrifuged at 12000 x g for 30 min at 4 ° C to obtain the post-mitochondrial supernatant for other antioxidant enzymes activity studies.

The TBARS method described by Zhou et al. (2006) and by Lushchak et al. (2005) was used to evaluate sperm LPO. The TBARS concentration was calculated by absorption at 535 nm and a molar extinction coefficient of 156 $mMcm^{-1}$. The content of TBARS was expressed as nanomoles per 10^8 cells. Carbonyl derivatives of proteins (CP) were detected by reaction with 2, 4-dinitrophenylhydrazine (DNPH) according to the method described by Lenz et al. (1989). The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 $mMcm^{-1}$ and was expressed as nanomoles per 10^8 cells.

Total superoxide dismutase (SOD) activity was determined by the method of Marklund and Marklund (1974). The homogenized samples were centrifuged at 12000 ×g at 4 ◦ C for 30 min. The SOD activity was assessed spectrophotometrically at 420

nm. One unit of SOD activity is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical per min. Activity was expressed in international units (or milliunits) mU per 10^8 cells.

2.7. ATP bioluminescence assay

ATP content in spermatozoa was determined after 2 h of exposure to DQ using the bioluminescence method described by Boryshpolets et al. (2009). Sperm samples were added to a boiling extraction medium consisting of 100 mM Tris–HCl, pH 7.75 and 4mM EDTA. After boiling for 2 min at 100 ° C, samples of the sperm suspension were centrifuged at 12000 x g for 20 min. ATP content in the supernatants was evaluated by bioluminescence, using a Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany). Luminescence was read with a multifunctional microplate reader Infinite M200 (Tecan, Austria). ATP content was expressed as nmol ATP/10^8 spermatozoa.

2.10. Statistical analysis

Normality and homogeneity of dispersions in studied values were tested using Shapiro–Wilkox and Levene tests. Statistical comparison was made by analysis of variance (ANOVA) with subsequent post hoc Tukey's honest significant difference (HSD) test. The values were expressed as mean ± SEM (n = 6). The observed relationships among the parameters were confirmed and quantified according to Spearman's test. Values for motility and velocity at 10 sec post-activation were used. The t-test was used to establish whether the correlation coefficients were significantly different from zero. All analyses were performed at a significance level of $p < 0.05$ using STATISTICA 9.0 software for Windows.

3. Results

3.1. Spermatozoa motility and velocity

A significant effect of DQ on sperm motility was observed at each tested post activation time (df = 4, $p < 0.05$, Fig. 1). The duration of spermatozoa motility decreased with increasing DQ concentrations. In the control, 38.3% of spermatozoa were motile at 120 s post-activation, while 25.3% were motile in the group exposed to

150 μM of DQ. A significant positive correlation (r = 0.67, $p < 0.05$) was found between percent of motile spermatozoa and ATP content (Table 1). A similar trend was observed for spermatozoa velocity. Typically, at 10 s post-activation the spermatozoa velocity in the control group reached 200 μm/s compares to 168 μm/s for spermatozoa exposed to 150 μM DQ (Fig. 2). Fig. 3 shows swimming behavior of sterlet spermatozoa after activation at tested times post-activation for DQ concentrations and in controls. A C-shaped flagellum was observed at 25 and 50 μM DQ at 60 s post-activation and for 150 μM at 10 s post-activation, and when motility ceased. In the control, the flagellum remained straight when motility ceased at 120 s post-activation.

3.2 Evaluation of DNA damage

An illustration of the outcome of a typical assay for DNA damage is presented in Fig. 4. The level of DNA fragmentation in spermatozoa exhibited a significant dose related response to DQ after 2 h of *in vitro* exposure (Figs. 5 and 6). The level of DNA fragmentation, expressed as the mean Olive tail moment, after *in vitro* exposure of spermatozoa to concentrations over 50 μM of DQ was significantly greater than in the control (df = 4, $p < 0.05$) (Fig. 6). Fig. 5 shows the effects of *in vitro* exposure to DQ on the percent of tail DNA. The spermatozoa exposed to the highest concentration of duroquinone (150 μM) showed DNA fragmentation of almost 8%, as compared with 1-2% for the controls. A significant positive correlation was found between percent of DNA in tail and CP (r = 0.66, $p < 0.05$), as well as between Olive tail moment and CP (r = 0.72, $p < 0.05$; Table 1). A significant negative correlation (r = -0.69, $p < 0.05$) was found between percent of DNA in tail and ATP (Table 1).

3.3. Oxidative stress indices and antioxidant response

To verify the presence of oxidative imbalance induced by duroquinone, LPO levels (as indicated by spermatozoa TBARS level) and CP levels were measured in all groups (Figs. 7 A, B). LPO level significantly (df = 4, $p < 0.05$) increased upon exposure to DQ at 50 μM and greater, and LPO production was 1.96-fold that of the control value when exposed to 150 μM. Compared to the control, the level of CP significantly increased upon exposure to 25 μM DQ (df = 4, $p < 0.05$). The SOD activity, an indicator of antioxidant activity in fish spermatozoa, increased significantly in all treatment groups compared to the control (df = 4, $p < 0.05$, Fig. 8) and reached the maximum value (three-fold the control value) when exposed to the highest (150 μM) concentration of DQ.

3.4. Evaluation of ATP content

To further investigate the potential mechanism underlying the decrease in sperm quality, the intracellular ATP content in spermatozoa was analyzed. The level of ATP in control spermatozoa was 68 nmol ATP/10^8 spermatozoa. Significant differences (df = 4, $p < 0.05$) in intracellular ATP levels were observed with exposures at 100 and 150 μM DQ, with the lowest ATP content at 45 nmol ATP/10^8 spermatozoa (Fig. 9).

4. Discussion

As a first step toward understanding the harmful effects of duroquinone on sterlet sperm, an *in vitro* spermatozoa motility assay was analyzed using a computer-aided motion-analysis system. Spermatozoa motility and velocity decreased significantly in all treatment groups compared to the control. The percent motile spermatozoa was reduced in a dose-dependent manner, while reduction of spermatozoa velocity was dose-dependent only at 120 s post activation. This is in agreement with a previous study in common carp *Cyprinus carpio* (Zhou et al., 2006). Duroquinone was associated with damage to the spermatozoa flagella, and atypical flagella positions were observed during spermatozoa movement as well as when motility ceased. It is not known whether the observed flagella positions were due to damage of the plasma membrane or of the axoneme. The plasma membrane plays an important role in the initiation of sperm

motility via hypo-osmotic signals (Li et al., 2009, 2012), and modification of the plasma membrane can significantly alter spermatozoa movement.

It is known that non-estrogenic as well as estrogenic organic compounds, such us zearalenone, BPA, and estradiol (Thomas et al., 1998) can bind to the spermatozoa membrane receptors and upregulate spermatozoa motility. However, the opposite, a reduction of motility, can also be observed when xenobiotic compounds, such as duroquinone, affect spermatozoa energy (intracellular ATP content). We observed a decreasing trend in intracellular ATP during *in vitro* treatment as well as a significant positive correlation between intracellular ATP content and spermatozoa motility. ATP is the main source of energy required for axonemal beating in fish spermatozoa (Perchec-Poupard et al., 1998; Rurangwa et al., 2002). In mammalian spermatozoa there is evidence that intracellular ATP concentrations decrease upon exposure to oxidative stress (Cummins et al., 1994). ATP depletion by ROS has been explained by inactivation of mitochondrial ATP synthase or by activation of the nuclear enzyme poly (ADP-ribose) polymerase-1 (PARP). Activation of PARP as a consequence of ROS-induced DNA damage, which causes excessive consumption of substrates such as nicotinamide adenine dinucleotide (NAD+) and ATP, has been shown to induce cell death by either apoptosis or necrosis (Jagtap and Szabó, 2005). Duroquinone as a potent redox cycler (Lemaire and Livingston, 1997) was reported to induce ROS production in carp spermatozoa (Zhou et al., 2006) with a subsequent decline of spermatozoa motility.

In addition to negatively affecting spermatozoa motility, ROS have long been implicated in the lipid peroxidation of spermatozoa membranes because of their relatively high polyunsaturated fatty acid (PUFA) content (Li et al., 2009). The lipid peroxidation cascade is initiated when reactive oxygen species attack PUFA in the sperm cell membrane (Storey, 1997). As a consequence of lipid peroxidation, the plasma membrane loses the fluidity and integrity. In the present study, fish spermatozoa treated with duroquinone showed increased levels of LPO. Enhanced LPO may indicate an increased generation of oxygen free radicals, and has been associated with mid-piece abnormalities and decreased spermatozoa quality (Chitra et al., 2003).

Protein carbonylation is a result of protein oxidation. It is well known that different types of ROS induce damage in different cell structures (Dalle-Donne et al., 2003). Thus the nature of ROS determines the type of oxidative stress biomarker to be

used. However, the use of protein CO groups as markers may have some advantages over lipid peroxidation products, since the formation of protein-bound CO groups seems to be a common phenomenon of protein oxidation and because of the relatively early formation and stability of oxidized proteins (Dalle-Donne et al., 2003). Reactive oxygen species directly attack proteins and lead to the formation of carbonyl. The formation of CP produces structural changes and decreased catalytic activity in enzymes and ultimately results in, owing to increased susceptibility to protease action, breakdown of proteins by proteases (Almroth et al., 2008). In the present study, all tested concentrations of duroquinone significantly increased CP level after short-term exposure, and marked increases in CP level were readily observed at lower concentrations. Our results also suggested that CP was more sensitive than LPO as an indicator of oxidative stress in sterlet spermatozoa. This finding, as well as the absence of a correlation between TBARS level and CP, may suggest direct influence of DQ on spermatozoa proteins, not mediated by lipid peroxidation. Thus, CP may be one of the most sensitive biomarkers of DQ-induced oxidative stress in sterlet spermatozoa.

To counteract the damaging effects of ROS, a variety of antioxidant enzymes are present in spermatozoa (Chitra et al., 2003; Li et al., 2010). Superoxide dismutase is considered the first line of defense against deleterious effects of oxyradicals in the cell by catalyzing the dismutation of superoxide radicals to hydrogen peroxide and molecular oxygen (Li et al., 2009). In the present study a significant enhancement of SOD activity at the lowest concentration of DQ (25 μM) as well as significant correlation between spermatozoa velocity and SOD content was observed. It is likely that the enhancement of SOD activity was an adaptive response to toxicant stress and served to neutralize the impact of increased ROS generation.

The integrity of spermatozoa DNA is affected by ROS (Fraczek and Kurpisz, 2005; Menezo et al., 2007), which induce damage associated with poor semen quality, low fertilization rate, impaired implantation, increased abortion rate, and even an elevated incidence of disease in offspring (Lewis and Aitken, 2005). In general it is not only the DNA damage, that can affect spermatozoa, but DNA modifications can be generated by chemical reactions (Box et al., 2001) leading to mutations in the embryo (Kopejka et al., 2004). However, DNA strand breaks are usually considered the most damaging type of DNA modification. In the present study, using the comet assay, we

were able to demonstrate that the short-term exposure of sterlet spermatozoa to DQ caused a dramatic increase in DNA damage expressed by Olive moment and percent of tail DNA. Levels of CP were found to be significantly positively correlated with Olive moment and % Tail DNA. High concentrations of ROS were necessary to induce DNA damage (Figs. 5-8). With these data we can speculate that an antioxidant system is present in sterlet sperm that is capable of partially neutralizing ROS attack. High concentrations of uric acid, an important antioxidant in seminal plasma, have been reported in rainbow trout (*Oncorhynchus mykiss*), yellow perch (*Perca flavescens*), and common carp (*Cyprinus carpio*), potentially protecting spermatozoa against oxidative damage (Ciereszko et al., 1999). Further studies are needed to reveal mechanisms responsible for anti-oxidant defenses in sterlet sperm.

The results of the present study clearly demonstrated that duroquinone can induce oxidative stress in sterlet spermatozoa *in vitro*, resulting in accumulation of LPO and CP together with the inhibition of intracellular ATP content and DNA damage. These oxidative responses were associated with reduced spermatozoa quality, as measured by spermatozoa motility and velocity. The use of sperm *in vitro* assays may provide a simple and efficient means for evaluating the effects of pollutants on sturgeon in the aquatic environment. The data reported here showed for the first time that the most sensitive biomarkers of DQ-induced oxidative stress in sterlet spermatozoa are spermatozoa motility, protein carbonylation, and SOD activity. Compared to spermatozoa of common carp (Zhou et al., 2006), spermatozoa of sterlet showed lower sensitivity to DQ-induced DNA damage, but were highly susceptible to protein oxidation leading to significant motility reduction. Further research is needed to investigate the precise mechanisms involved and the relationship between motility and oxidative stress in sturgeon sperm as well as its consequences on fertilization.

Acknowledgements

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Figure legends

Figure 1. Sterlet (*A. ruthenus*) spermatozoa motility after *in vitro* exposure to duroquinone at nominal concentrations (25, 50, 100, and 150 μM). Data are presented as means ± SEM, n = 6. Different letters denote significant difference between treatments.

Figure 2. Sterlet (*A. ruthenus*) spermatozoa velocity (μm s^{-1}) after *in vitro* exposure to duroquinone at nominal concentrations (25, 50, 100, and 150 μM). Data are presented as means ± SEM, n = 6. Different letters denote significant difference between treatments.

Figure 3. *In vitro* effects of duroquinone on *Acipenser ruthenus* spermatozoa flagella. Beat frequency and number of waves along the flagellum decreased with increasing concentration of DQ. Different flagellar positions were observed during spermatozoa movement as well as when motility ceased. The white arrows indicate changes in flagellar shape, such as decrease in beat frequency and occurrence of a C-shape, with various concentrations of duroquinone compared to control.

Figure 4. Examples of results from a typical comet assay. Five levels of DNA damage in sterlet (*A. ruthenus*) spermatozoa: a: no damage, % tail DNA <5% (control); b: slight damage, % tail DNA <15% (25 μM of duroquinone); c: moderate damage, % tail DNA <50% (50 μM of duroquinone); d: higher damage, % tail DNA <60% (100 μM of duroquinone); e: highest damage, % tail DNA >60 % (150 μM Dur.).

Figure 5. Percent tail DNA (% DNA in tail measured in comet assay) after *in vitro* exposure of sterlet (*A. ruthenus*) spermatozoa to duroquinone at nominal concentrations (25, 50, 100 and150 μM). Data are presented as means ± SEM, n = 6. Different letters denote significant difference between treatments.

Figure 6. Olive Tail Moment of the spermatozoa after *in vitro* exposure of sterlet (*A. ruthenus*) spermatozoa to duroquinone at nominal concentrations (25, 50, 100, and 150

μM). Data are presented as means ± SEM, n = 6. Different letters denote significant difference between treatments.

Figure 7. Oxidative stress indices [TBARS (A) and CP (B)] following *in vitro* exposure of sterlet (*A. ruthenus*) spermatozoa to duroquinone at nominal concentrations (25, 50, 100, and 150 μM). Data are presented as means ± SEM, n = 6. Different letters denote significant difference between treatments.

Figure 8. Superoxide dismutase (SOD) activity levels following *in vitro* exposure of sterlet (*A. ruthenus*) spermatozoa to duroquinone at nominal concentrations (25, 50, 100, and 150 μM). Data are presented as means ± SEM, n = 6. Different letters denote significant difference between treatments.

Figure 9. Intracellular ATP content of sterlet (*A. ruthenus*) spermatozoa after 2 h exposure to duroquinone. Data are presented as means ± SEM, n = 6. Different letters indicate significant differences between treatments (ANOVA, $p < 0.05$).

Figure 1.

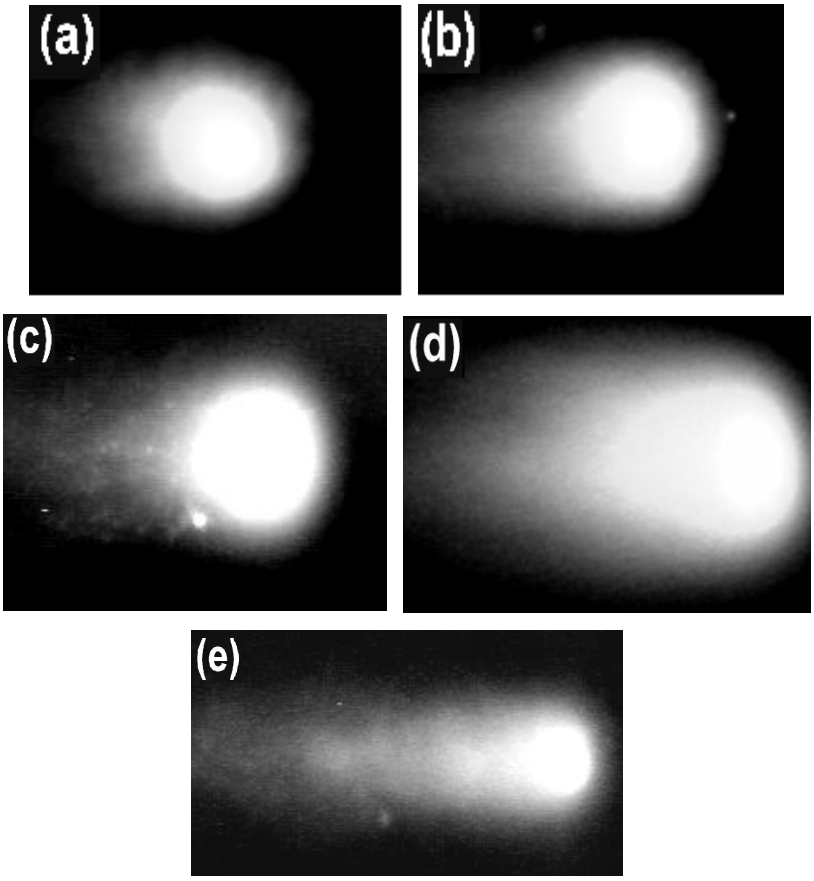


Figure 2.

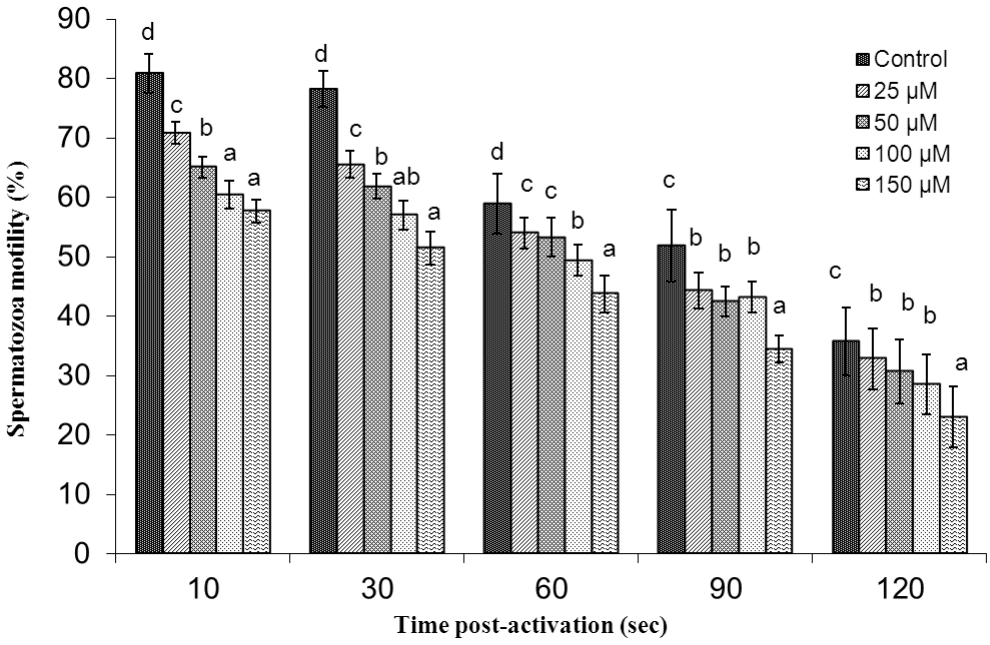


Figure 3.

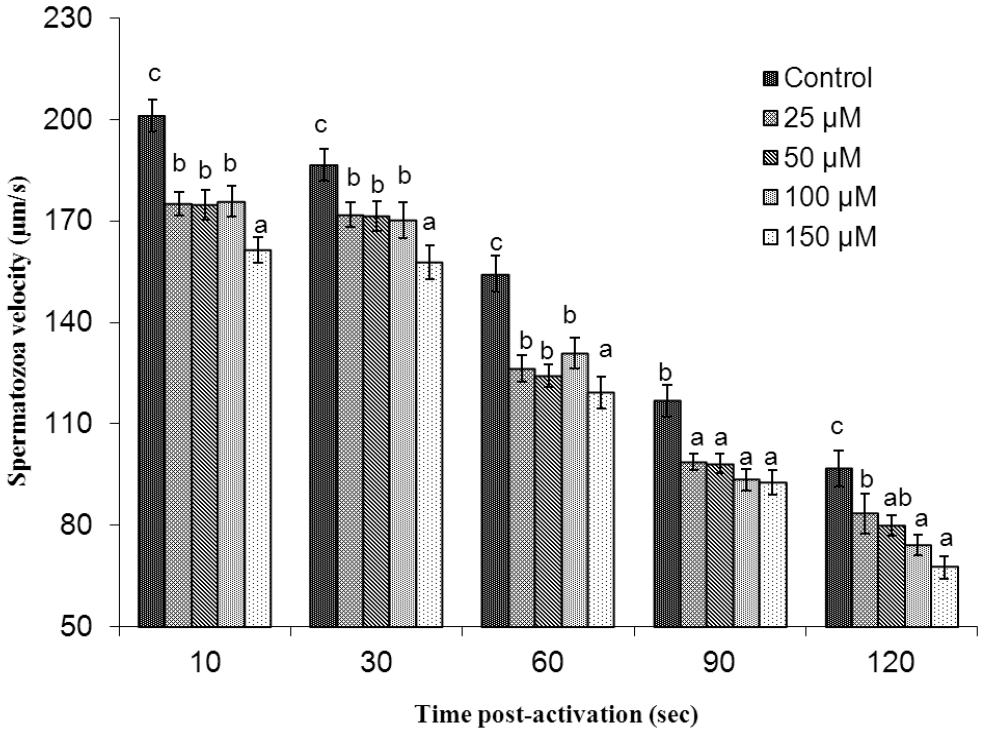

Spermatozoa velocity (µm/s)
230
200
170
140
110
80
50
Control
25 µM
50 µM
100 µM
150 µM
c
b b b
a
c
b b b
a
c
b b b
a
b
a a a a
c
b
ab
a
a
10
30
60
90
120
Time post-activation (sec)

Figure 4.

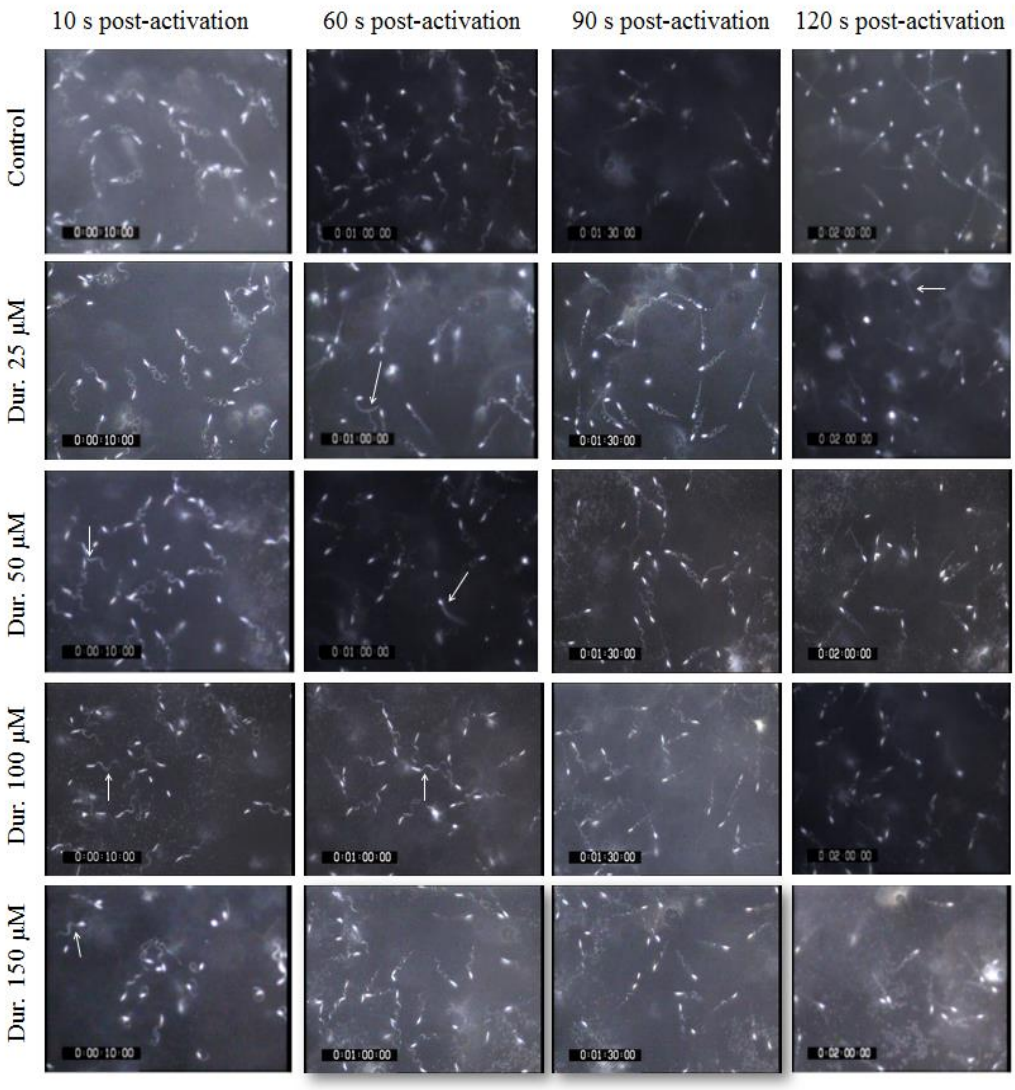

10 s post-activation
60 s post-activation
90 s post-activation
120 s post-activation
Control
Dur. 25 µM
Dur. 50 µM
Dur. 100 µM
Dur. 150 µM

Figure 5.

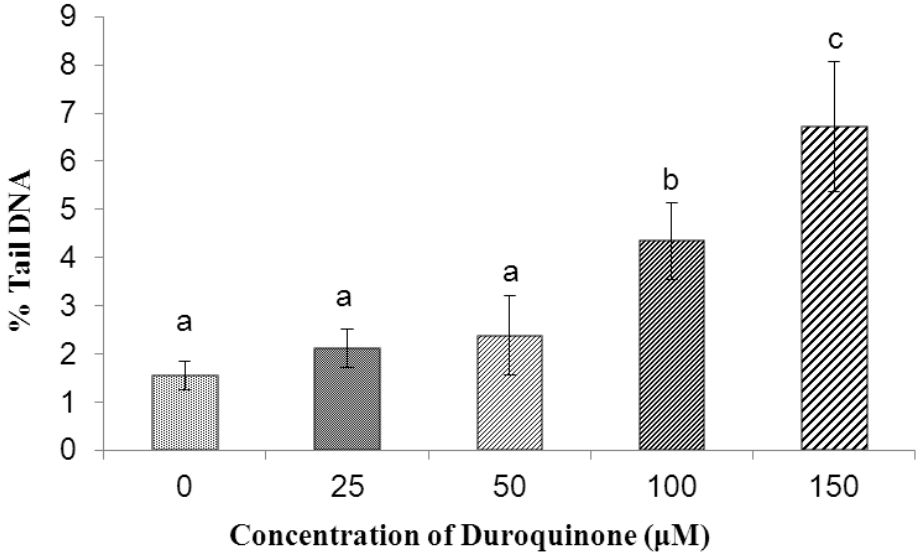


Figure 6.

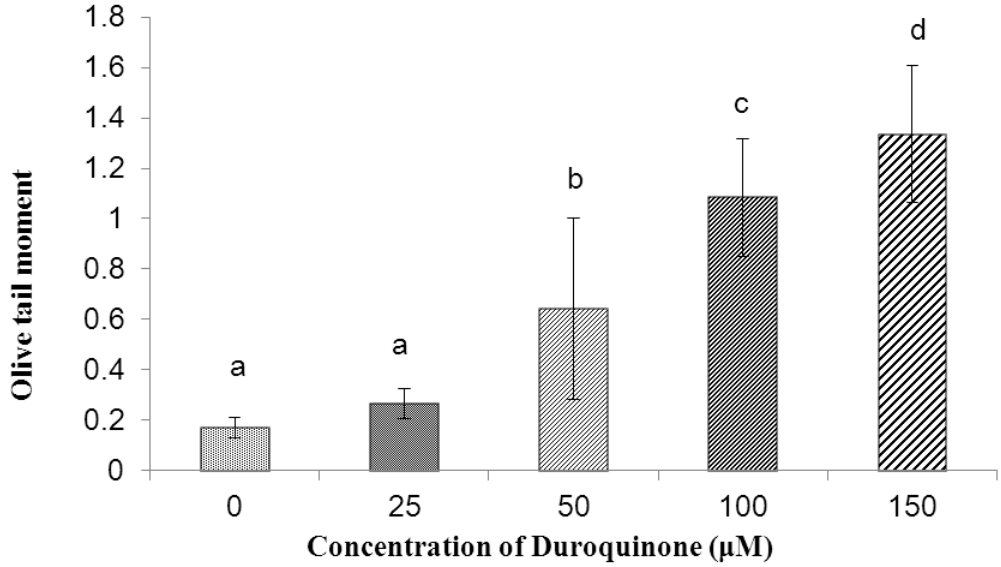


Figure 7. A

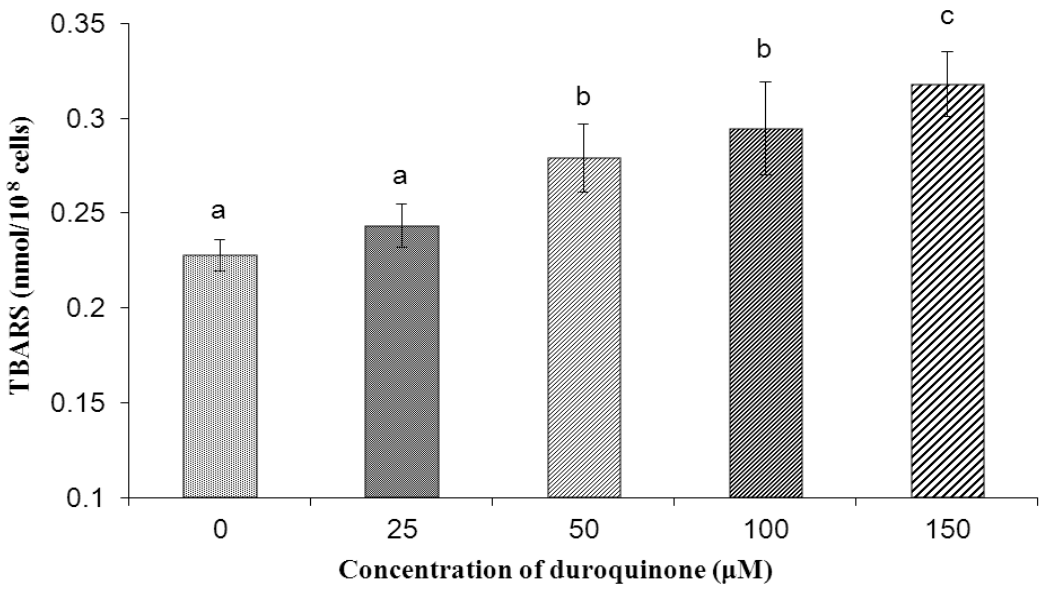
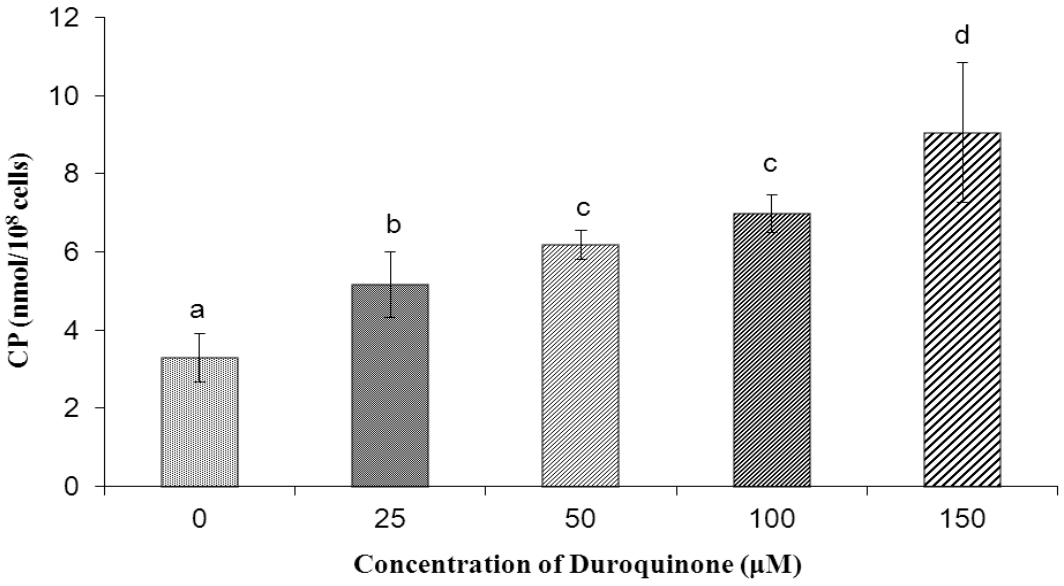


Figure 7. B



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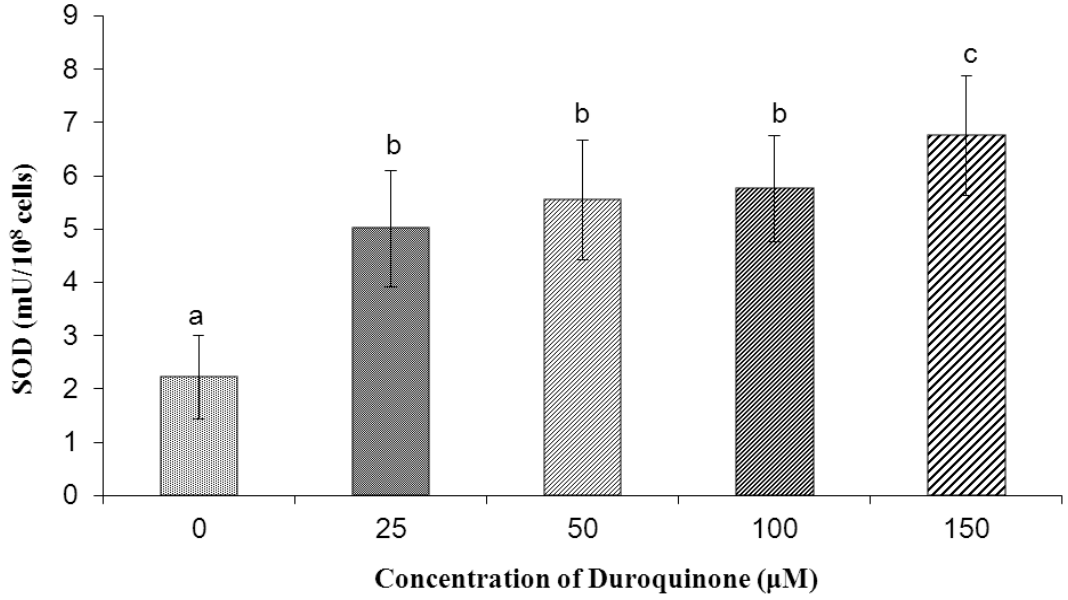
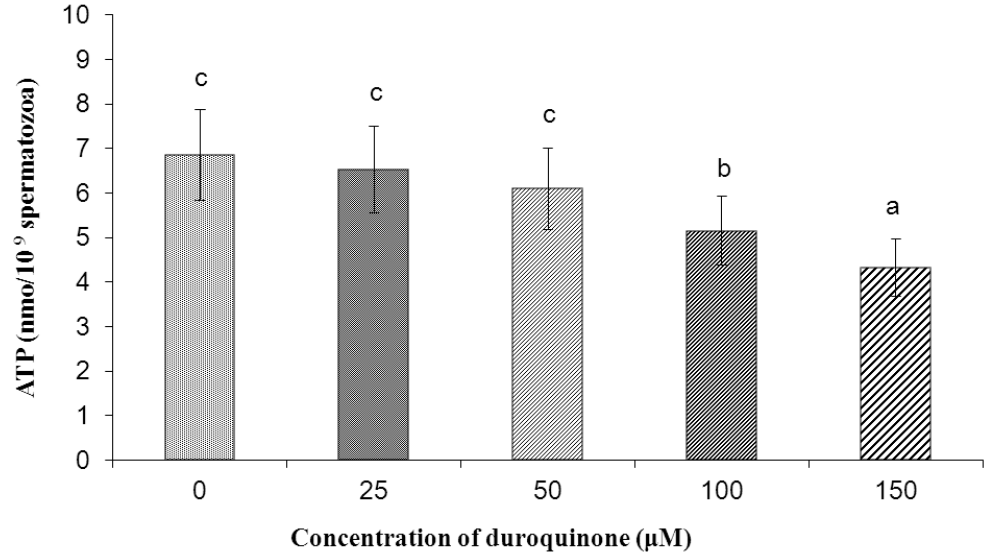


Figure 9.



9.3 Tetrabrombisphenol A and its effects on DNA integrity, oxidative stress indices and quality of spermatozoa of sterlet (*Acipenser ruthenus*)

This paper is in preparation to journal Cell Biology and Toxicology

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Abstract

The sperm of sterlet (*Acipenser ruthenus*) was used to investigate the effect of tetrabrombisphenol A (TBBPA) xenobiotic on sperm quality parameters (ATP content, spermatozoa motility and velocity), DNA integrity, and oxidative stress indices. Sperm was diluted to obtain the spermatozoa density of 5×10^8 cells/ml and then exposed for 2 h to final concentrations of TBBPA (0.5, 1.75, 2.5, 5 and 10 μg/l). The level of oxidative stress indices such as lipid peroxidation (LPO), carbonyl derivatives of proteins (CP) and antioxidant activity (SOD) increased significantly with increase in concentration of TBBPA. A significant decrease appeared in the intracellular ATP content in sperm samples, at concentrations 2.5 μg/l of TBBPA and above. Spermatozoa velocity and percentage of motile sperm were significantly decreased at each time post-activation in comparison with control. The level of DNA damage expressed by percentage of DNA in Tail and Olive Tail moment significantly increased, when spermatozoa were exposed to concentration 2.5μg/l of TBBPA and higher. The present study confirms that the application of fish spermatozoa for *in vitro* assays can have an important significance for monitoring of residual pollution in aquatic environment.

Key words: DNA damage, commet assay, reactive oxygen species, spermatozoa motility

1. Introduction

Since 1971, artificial propagation and rearing of sturgeon has been implemented along with the general trend to development of fish culture in several European countries (Chebanov and Billard, 2001). A major problem in the development of sturgeon culture is their later sexual maturation and their high sensitivity to the water quality. On the other hand wild stocks of sturgeons are highly endangered due to over-fishing, destruction of their natural environment and water pollution. That is why this species takes place in IUCN red list. Additionaly, the particularly long time of sexual maturation in wild sturgeons provides a longer period for xenobiotics accumulation in the reproductive organs and destroy them fractionaly (Gharaei et al, 2008 and Tashjian et al, 2006). Therefore, in the present study sterlet (*Acipenser ruthenus*) was used as a model organism because of its low price, high survival, small size, earlier sexual maturation and its freshwater status.

Nowdays more pollutants and toxic substances occur in the nature due to human activity and development of industry. Majority of these hazardous pollutants is soluble in water and accumulates in aquatic environment. One of the most common xenobiotics occurring in surface waters is tetrabromobisphenol A (TBBPA), which is a brominated flame retardant and derivative of bisphenol A. The annual consumption worldwide has been estimated as 119,600 tons in 2001, of which 11,600 tons were used by the European industry mainly in the production of plastics used in electrical and electronic applications. TBBPA emits from different processes to the environment and can be detected in trace concentration in the atmosphere, hydrosphere, soil, and sediments. It also occurs in sewage sludge and house dust (Sellström and Jansson, 1995). TBBPA has been the subject of an eight year evaluation under the EU Risk Assessment procedure which reviewed over 460 studies. The Risk Assessment

was published on the EU Official Journal in June 2008. TBBPA will now go through registration in REACH that is the acronym for the new chemical policy of the European Union, which is based on its content: registration, evaluation and authorization of chemical substances. TBBPA is in the process of being classified in the EU as an R50-53 substance, which means that it is toxic to aquatic organisms. In fish, TBBPA is

acutely toxic at low concentrations as shown in a number of studies. LC50 concentrations reported include 0.51 mg/l (0.9 μM) in bluegill sunfish, 0.40 mg/l (0.7 μM) in rainbow trout (WHO/ IPCS 1995), and a relatively high 3 mg/l (5.5 μM) in zebrafish (Lee et al. 1993). On the other hand, TBBPA is known to enhance production of reactive oxygen species (ROS) (Livingstone, 2001) that can attack a variety of biological macromolecules such as DNA, proteins and lipids, leading to oxidative damages (Kelly et al., 1998). Gametes of almost all fish species are particularly susceptible to oxidative injury, not only due to lack of protective cytoplasmic enzymes, but also due to high content of polyunsaturated fatty acids (PUFA) in the spermatozoa plasma membrane (Drevet, 2006). Since fish sperm are known for their extremely short period of motility, just 1-2 minutes (Cosson, 2010), any negative effect on its quality can significantly decrease the probability of successful fertilization (Zhou et al, 2006).

The purpose of this paper was to determine how physiology of sterlet spermatozoa was affected by short-term *in vitro* exposure (2 h) to environmentally relevant concentrations of TBBPA, by analysis of sperm functional parameters (spermatozoa motility and velocity), DNA integrity and intracellular ATP level. Additionally induction of oxidative stress in spermatozoa was assessed by measurement of lipid peroxidation (LPO), carbonyl derivatives of proteins (CP) and the antioxidant response was evaluated by superoxide dismutase activity (SOD).

2 Materials and methods

2.1 Broodstock handling and collection of gametes

Sperm of sterlet was obtained from six different males (age: 6–7 years old; body weight: 1.5 ± 0.2 kg; body length: 55 ± 9.3 cm), in the aquaculture facility of the Research Institute of Fish Culture and Hydrobiology at the University of South Bohemia, Vodnany, Czech Republic. Fish were maintained according to the EU harmonized animal welfare act and principles of animal care and national law 246/1992 "Animal welfare" were followed. Prior to hormonal stimulation, fish were kept in tanks with water temperature varying between 14 and 15°C. Spermiation of fish was stimulated by intramuscular injection of suspension of carp pituitary in physiologic saline solution (0.9% NaCl) at doses of 4–5 mg/kg of body weight, 48 h before sperm collection. Collection of sperm from the urogenital papilla was made by insertion of

plastic catheter (5-7 mm diameter) connected to a 20 ml syringe and collected sperm samples were kept on ice (0–4°C) until processing. Sperm contaminated by mucus, feces, or water was excluded. The sperm concentrations of all samples were estimated microscopically (Olympus BX 41) at 20× using a Burker cell hemocytometer.

2.2 Sperm dilution and exposure

Sperm from six individual males was centrifuged at 300×g, 4°C for 30 min to remove seminal plasma and then diluted in an immobilizing medium (20 mM Tris, 30 mM NaCl, 2 mM KCl, pH 8.5) to obtain spermatozoa density of 5 x 10^8 cells/ml. Tetrabrombisphenol A (TBBPA) [2,2',6,6'-Tetrabromo-4,4'-isopropylidenediphenol or 2,2-bis(3,5-dibrom-4 hydroxyfenyl) propane; empirical formula: $C_{15}H_{12}Br_4O_2$; MW: 543.85), (≥99%; Sigma-Aldrich, USA)] was dissolved in 96% ethanol and diluted in distilled water to obtain a stock solution of 0.1 g/l. The sperm sub-samples (n = 6) were then exposed for 2 h to environmentally related concentrations of 0.5, 1.75, 2.5, 5 and 10 μg/l of TBBPA at 4 °C. A group exposed to immobilizing medium with 0.5% of ethanol was used as a control. Each experimental condition was duplicated.

2.3 Sperm motility and velocity recording

Spermatozoa velocity (μm/s, measured only motile sperm) and percentage of motile sperm cells (%) were determined after triggering sperm motility under dark-field microscopy (Olympus BX 50, Japan) (20 x objective magnification). For triggering motility, sperm was diluted in activating medium (AM) (10 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris pH 8.5) at a dilution ratio of 1:5000. Pluronic 0.2% (w/v) (Sigma-Aldrich, USA) was added to the swimming solution to avoid sperm sticking to the microscope slide. Sperm motility was recorded with a CCD video camera (SONY DXC-970MD, Japan) mounted on the microscope, illuminated with the stroboscopic flash (ExposureScope®, Czech Republic). Record of sperm motility was done in triplicate for each sample using video-recorder (SONY DVD Recorder-1000MD, Japan).

2.4 Sperm motility and velocity evaluation

Velocity and motility were assessed at 10, 30, 60, 90 and 120 s after activation. The successive positions of the video recorded sperm heads were analyzed from video frames by means of Olympus MicroImage software (Version 4.0.1. for Windows with a special macro by Olympus C & S). Percentage of motile spermatozoa and velocity were calculated from sperm head positions on five successive video frames figured by three different colors (frame 1 red, frames 2–4 green and frame 5 blue) as described by Rodina et al. (2007). Twenty to 40 sperm were counted for each frame. Tracks of motile sperm were figured by three different colors of sperm heads whereas non-motile sperm was displayed by white color. Then we were able to calculate the percentage of motile sperm based on the number of white and red cells. Sperm velocity was calculated in μm/s based on length traces of sperm from blue to green and red heads, calibrated for magnification.

2.5 Assessment of DNA Damage

In present study DNA integrity was assessed using the Comet assay or single cell gel electrophoresis assay following the method described by Li et al, (2008). Unless otherwise stated, molecular grade DNAse-free reagents (Sigma Aldrich, USA) were used. Microscope slides (OxiSelectST; Cell Biolabs, INC. USA) were used for the assay and each slide was prepared in the following manner: the100 μl of sample ($5x10^8$ cells×ml^{-1}) were diluted in 5 ml of PBS (buffer solution; Ca^{2+} and Mg^{2+} free). Then the diluted samples (200 μl) were mixed with 700 μl of 0.8% NuSieve GTG low melting temperature agarose (OxiSelectST; Cell Biolabs, INC. USA). After that 50 μl of this mixture was added to the slide and allowed the agarose to solidify for 1 hour at 4°C. Lysis buffer (2.5M NaCl, 100mM EDTA, 10% of 10xLysis Solution [OxiSelect™ Comet Assay Kit], 10% DMSO pH 10) was used for immersion of slides for 1 hour. Finally, slides were subjected to enzymatic treatment with proteinase K (20 mM Tris-HCl, 1 mM $CaCl_2$ and 50% glycerol, pH 7.4) and incubated overnight at 32 °C. After the proteinase K solution was drained, the slides were immersed in a horizontal gel tank filled with running buffer and electrophoresis was carried out for 20 min at 35 V and 170 mA. Slides were transferred from the electrophoresis chamber to a clean container

with pre-chilled DI H_2O for 2 min. This washing procedure was repeated three times. Slides were drained well and dehydrated by dipping into absolute ethanol for 5 min and air-dried for storage. Prior to the analysis, 50 µl of Vista Green DNA Staining Solution (OxiSelectST; Cell Biolabs, INC. USA) was added to each agarose spot of the slide and analysed with an Olympus BX50 fluorescence microscope at 20x magnification. A total of 100 cells were scored for each sample and the captured images were analyzed using CometScore image analysis software (TriTek Corporation, USA). Tail length (measured from the middle of the head to the end of the comet tail) and % Tail DNA (content of DNA in comet tail) were measured. Percentage of DNA in tail and Olive Tail Moment were calculated using these two formulas:

Tail DNA% = 100 x Tail DNA Intensity/Cell DNA Intensity

Olive Tail Moment = Tail DNA% x Tail Moment Length.

2.6 Oxidative stress indices

The sperm samples were centrifuged at 13 000 x g at 4 ˚C for 10 min and the pellet in an ice bath was homogenized using a Sonopuls HD 2070 ultrasonicator (Bandelin Electronic, Berlin, Germany) with the potassium phosphate buffer (KPi-buffer). The homogenate was divided in two portions. First was for measurement TBARS and CP and the second was centrifuged at 12 000 x g for 30 min at 4 ˚C to obtain the post-mitochondrial supernatant for other studies of antioxidant enzymes activities. The TBARS method described by Li et al. (2010) was used to evaluate sperm lipid peroxidation (LPO). Concentration of TBARS was calculated by the absorption at 535 nm and a molar extinction coefficient of 156 mM/cm. The content of TBARS was expressed as nanomoles per 10^8 cells. Carbonyl derivatives of proteins (CP) were detected by reaction with 2, 4-dinitrophenylhydrazine (DNPH) according to the method described by Lenz et al. (1989). The amount of CP was measured spectrophotometrically at 370 nm using a molar extinction coefficient of 22 mM/cm and was expressed as nanomoles per 10^8 cells.

2.7 Superoxide dismutase activity (SOD)

Total SOD activity was determined by the method of Marklund and Marklund (1974). This assay depends on the autoxidation of pyrogallol. The prepared semen samples were diluted in phosphate buffer (50 mM KPi-buffer pH 7.0, 0.5 mM EDTA, 0.1 mM PMSF) to obtain a sperm density of $5x10^8$ cells×ml^{-1}. The homogenized samples were centrifuged at 12 000 ×g at 4°C for 30 min. The activity of SOD was assessed spectrophotometrically at 420 nm and expressed as the amount of enzyme per milligram of protein. One unit of SOD activity is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical per min.

2.8 Evaluation of ATP content

ATP content in spermatozoa was determined after 2 h of exposure to TBBPA using bioluminescence method described by Boryshpolets et al. (2009). Five ml of boiling medium containing 100 mM Tris-HCl, pH 7.5 and 4 mM EDTA were used to dilute sperm samples. After boiling for 2 min at 100 °C samples of the sperm suspension were centrifuged at 12 000 × g for 20 min. Before analysis we collected supernatant for storage at -80°C. A Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, Germany) was used for determination of ATP content. Luminescence was read with a multifunctional microplate reader Infinite M200 (Tecan, Austria). ATP content was expressed as nmol ATP/10^8 spermatozoa.

2.9 Statistical analysis

All measurements were conducted in triplicate. Normality and homogeneity of dispersions in studied values were tested using Shapiro–Wilk's and Levene test. Statistical comparison was made by analysis of variance (ANOVA) with subsequent post hoc Tukey's honest significant difference (HSD) test. The values were expressed as mean ± SEM (n = 6). The observed relationships among the parameters were confirmed and quantified according to the Spearman's test. All analyses were performed at a significance level of 0.05 using STATISTICA 9.0 software for Windows.

3 Results

3.1 Spermatozoa motility and velocity

Significant effect of TBBPA on sperm functional parameters (motility and velocity) after 2 h exposure to TBBPA *in vitro* was determined. Changes in sperm motility and velocity were observed after dilution with AM at 10, 30, 60, 90 and 120s post-activation (df = 4, $P < 0.05$, Fig. 1 A, 1 B). At 10 and 30 s post-activation, spermatozoa motility significantly ($P < 0.05$) decreased in all tested concentrations in comparison with control. At 120 s post-activation in control group 33.2% of spermatozoa were motile, while only 26.7% were motile in group exposed to 10 μg/l of TBBPA. On the other hand, no significant difference in spermatozoa velocity at 10 s post-activation was observed between the control group and the group treated with 0.5 μg/l of TBBPA (204.5 μm. s^{-1}), (Fig. 1 B). However, velocity decreased to 165.2 μm. s^{-1} for spermatozoa exposed to 10 μg/l of TBBPA. At 120 s post-activation the velocity of control group was 93.7 μm/s, whereas at 5 and 10 μg/l of TBBPA velocity significantly decreased up to 74.7 μm/s. Additionally we found a significant negative correlation (r=-0.35, $P < 0.05$) between percentage of motile spermatozoa and TBARS level (Table 1). A significant positive correlation (r=0.52, $P < 0.05$) was detected between ATP content and motility of spermatozoa. Figure 2 shows swimming behavior of sterlet sperm after activation in control or different TBBPA concentrations at 10, 60, 90 and 120 s post-activation. We observed the changes in flagella movement and C shaped flagella, which are typical for dead sperm, or sperm with very slow motility mostly at 90 and 120 s post-activation.

3.2 DNA fragmentation

An illustration of the outcome of this particular assay for DNA damage is presented in Figure 3. Obtained pictures of comets were used to evaluate following parameters: % of DNA in Tail (calculated by formula: % Tail DNA = 100 x Tail DNA Intensity/Cell DNA Intensity) and Olive Tail Moment (Olive Tail Moment = % Tail DNA x Tail Moment Length). The level of DNA fragmentation in spermatozoa exposed to different concentrations of TBBPA was measured by CometScore software (Figs. 4 A and B). Comet assay analysis indicated an increase of DNA fragmentation in spermatozoa

nuclei exposed to different concentrations of TBBPA (1.75-10 μg/l) compared to control. Fig. 4A shows the effects of *in vitro* exposure to TBBPA on percentage of DNA in tail. Sperm exposed to the highest concentration of TBBPA (10 μg/l) had a DNA fragmentation degree almost 3.8%, as compared with the control sample (1.5%). The level of DNA fragmentation, expressed as the mean Olive Tail Moment significantly increased ($df = 4$, $P < 0.05$) after *in vitro* exposure of sperm to concentration over 2.5μg/l of TBBPA as compared with control (Fig. 4B). Spearman's correlation test revealed significant correlation between % of DNA in Tail and Olive Tail Moment (r=0.83, $P < 0.05$) and CP level (r=0.29, $P < 0.05$) (Table 1).

3.3 Oxidative stress indices and antioxidant response

The level of lipid peroxidation (LPO) and carbonyl proteins (CP) level in all groups were measured in order to verify the presence of oxidative imbalance induced by TBBPA (Figs. 5 and 6). TBARS level was used as an indicator of LPO. The results showed that in the control group the measured level of TBARS was around 0.16 nmol/10^8 spermatozoa (Fig. 5). A significantly higher level of LPO was observed in spermatozoa exposed to TBBPA at concentrations 5 μg/l and higher. The highest concentration of TBBPA (10 μg/l) enhanced the level of TBARS to 0.33 nmol/10^8 spermatozoa. Treatment with TBBPA increased significantly ($P < 0.05$) the level of CP in all groups compared to control. However, there was no significant difference in CP level between spermatozoa groups exposed to 0.5 - 5 μg/l of TBBPA (Fig. 6) Significant ($P < 0.05$) increase in the level of CP was observed in the group exposed to concentration 10 μg/l. The maximum detected level of CP was 12.4 nmol/10^8 in spermatozoa exposed to 10 μg/l of TBBPA compared to control group, were the value was only 2.7 nmol/10^8. There was significant positive correlation between levels of TBARS and CP (r=0.37, $P < 0.05$) (Table 1). Also significant negative correlations were found between motility of spermatozoa and TBARS level (r=-0.35, $P < 0.05$) and CP level (r=-0.36, $P < 0.05$), as well as between TBARS level and ATP content (r=-0.39, $P < 0.05$) (Table 1).

The superoxide dismutase (SOD) activity was used as an indicator of antioxidant activity of fish spermatozoa during *in vitro* exposure to TBBPA. The result shows that the SOD activity increased significantly in all treatments as compared to the control

(df=4, P < 0.05, Fig. 7). It is worth to mention that SOD activity increased gradually and in dose-dependent manner.

3.4 Evaluation of ATP content

The ATP content was analysed to indicate the decline in quality of fish spermatozoa. Intracellular ATP content in the control group was 6.8 nmol ATP/10^8 spermatozoa (Fig. 8). Significant differences (df = 4, P<0.05) in intracellular ATP level were found upon exposure to 2.5, 5 and 10 μg/l of TBBPA. The lowest level of ATP in spermatozoa was found as 3.1 nmol ATP/10^8 spermatozoa for concentration 10 μg/l of TBBPA. ATP content in spermatozoa correlated significantly with spermatozoa motility (r=0.52, P < 0.05) and velocity (r=0.57, P < 0.05), TBARS level (r=-0.39, P < 0.05) and SOD activity (r=-0.36, P < 0.05) (Table 1).

4. Discussion

TBBPA, which is one of environmental xenobiotics, can induce reproductive dysfunction in both wildlife and humans (Grizard et al, 2007). The term xenobiotics is defined as substances foreign to an entire biological system, which had not exist in nature before and were synthesized by human. Toxicity screening using animal sperm has been taken as a simple and valid model during the past decades since its use does not require expensive sterile cell culture conditions (Rurangwa et al, 2002). In this study we tried to confirm and quantify a negative effect of TBBPA on sperm quality, DNA integrity, ATP metabolism, and the antioxidant defense system. Previous studies (Lahnsteiner et al, 2005) as well as our initial results suggested the impact of environmentally related doses of TBBPA on the reproduction of fish in nature.

It is already known that non-estrogenic as well as estrogenic organic compounds such as TBBPA, BPA or estradiol (Thomas et al, 1998) can bind to the sperm membrane receptors and reduce spermatozoa motility. The effect of TBBPA on sperm energetic is another option that could be considered. It is well known that ATP is essential energy resource required for axonemal beating in fish sperm (Perchec-Poupard et al, 1998; Rurangwa et al, 2002). ATP is one of the key factors of spermatozoa motility and decreased ATP level is associated with decreased spermatozoa movement. As have been

shown by de Lamirande and Gagnon (1992), spermatozoa motility may be the most sensitive indicator of oxidative stress, because high levels of ROS inhibit one or more enzymes of oxidative phosphorylation and/or glycolysis, limiting the generation of ATP. Thus, there is a possibility that evocation of stress, caused by dangerous chemicals, might inhibit ATP metabolism. Spermatozoa movement is primarily dependent on initial ATP content, plasma membrane potential and Ca^{2+} signaling for triggering the flagellar beating (Cosson, 2010; Li et al., 2009). Therefore any modification of these three key elements can significantly modify spermatozoa movement. Otherwise TBBPA was also capable to damage the sperm flagella and different forms of flagella were observed during spermatozoa movement as well as the motility period of sperm cells finished. In addition, our results showed that a positive significant correlation was found between intracellular ATP content and spermatozoa motility.

It has to be noted, that sperm DNA damage assessment gained a special attention as a sperm quality marker (Peknicova et al, 2002). Comet assay has been used for the evaluation of DNA damage associated with *in vitro* exposure of sterlet spermatozoa to TBBPA. Application of this method demonstrated that the short-term exposure of sterlet spermatozoa to TBBPA is resulting in dramatic increase in DNA damage expressed by two parameters: Olive moment and percentage of DNA in comet tail. Moreover, significant positive correlations have been found between level of CP and Olive moment. This is in accordance with other researches, which were made on human spermatozoa, showing that the DNA damage can be associated with oxidative stress and lipid peroxidation (Aitken et al, 1998, Chen et al, 1997). Except from DNA damage, such as strand breaks, DNA modifications also could take place in this study, because they are generated by many chemical reactions (Box et al, 2001). For example the oxidization of nitrogen bases without generating strand breaks and antioxidant responses can cause the mutation in the embryo (Kopejka et al, 2004). Environmental pollution with TBBPA can be a substantial problem, which is responsible for dramatic increase of DNA fragmentation in gametes of animals with external fertilization. It could be one of the key factors that would affect the future reproduction of sturgeon and their gradual disappearance from free waters.

As another step toward understanding, how xenobiotic TBBPA can affect sterlet spermatozoa, the oxidative stress indices and motility and velocity parameters were measured. Our results showed that the spermatozoa of sterlet were fully capable to be activated immediately after the transfer to swimming medium and 90 to 100% spermatozoa became motile. Changes of motility and velocity appeared after 2 h exposure of sperm to different concentrations of TBBPA and spermatozoa motility and velocity were significantly lower in all tested concentrations when compared to control. We could observe decreasing trend of both parameters (motility and velocity) with increasing concentration of xenobiotic substance. Our results are in accordance with study realized on brown trout (*Salmo trutta*), that showed lower spermatozoa motility and velocity in the sperm exposed to environmentally related concentrations of BPA either *in vivo* or *in vitro* (Hatef et al, 2010, 2012; Lahnsteiner et al, 2005). Key role of sperm motility for successful reproduction of fish and other animals is a constant fact, and still rehashing facts Lipid peroxidation (LPO) is important for aquatic organisms, because they normally contain higher amount of highly unsaturated fatty acids (HUFA) than other animals. HUFA is a major contributor to the loss of cell function under oxidative stress (Storey, 1996). Lipid peroxidation is usually indicated by TBARS in fish (Oakes and Van der Kraak, 2003). Our results showed a significantly increasing trend of the level of TBARS that appeared after 2 h incubation of sperm samples with concentrations of TBBPA higher then 5 μg/l.

The CP is a result of protein oxidation. In this study we observed alterations in CP in spermatozoa when different concentrations of xenobiotic TBBPA applied, namely the level of CP significantly increased with increasing concentrations of TBBPA.

It was demonstrated that TBBPA can induce production of ROS in cell cultures (Radice et al, 1998; Reistad et al., 2005). The effect of ROS on spermatozoa is well described and characterized in mammals. It may cause lipid peroxidation of spermatozoa membranes, damage of midpiece, axonemal structure and DNA, then malfunctions of capacitation and acrosomal reaction. It can lead to the loss of motility and infertility as well (Gagnon et al, 1991, Sikka, 2001). The toxic effect of ROS on fish spermatozoa has been reported for several species (Li et al, 2010; Dietrich et al, 2005). By means of measurement of lipid peroxidation, protein carbonylation and antioxidant response, we found indirect evidence that TBBPA may induce ROS production in fish spermatozoa.

A significant correlation was found between levels of DNA fragmentation and CP, meaning that DNA damage in spermatozoa exposed to TBBPA is caused by oxidative stress. Moreover, previous studies on zebrafish (*Danio rerio*) clearly showed that presence in environment of TBBPA in concentrations up to 1.5 μM is crucial for fish reproduction (Kuiper et al, 2007). It may be concluded, that TBBPA and compounds close to its, can induce ROS production and lead to oxidative damage, which may be one of the major causes of lower fish reproduction processes.

Thus it can be concluded that environmentally relevant concentrations of TBBPA have a potential to induce oxidative stress in spermatozoa leading to reduced motility, DNA integrity and ATP content. Though the antioxidant system apparently responds to increased lipid peroxidation and carbonylation of proteins, it seems that the antioxidant capacity of spermatozoa is not sufficient to prevent the cell damage. Based on our results, we hypothesize that the loss of spermatozoa motility and velocity during 2 h of sterlet sperm in vitro exposure to dangerous xenobiotic, was induced by oxidative stress that significantly interrupt cellular metabolism of spermatozoa. Present study showed that TBBPA desreases both motility and velocity parameters but the mechanism or mechanisms of action on spermatozoa are not known yet. There is a possibility of action through damage of plasma membrane, oraxonemal apparatus or depletion of ATP contents in spermatozoa.

Results of the present study demonstrated that sterlet spermatozoa are highly sensitive to the presence of small concentrations of brominated flame compounds such as TBBPA. Environmentally relevant concentrations of this xenobiotic are able to induce oxidative stress in spermatozoa leading to reduced motility, DNA integrity, and ATP content

In conclusion this study clearly demonstrated that sturgeon spermatozoa are highly susceptible to oxidative stress induced by environmental pollutants. Accumulation of products of lipid peroxidation as well as increased carbonylation of proteins in spermatozoa can serve as a proof of TBBPA-induced oxidative stress. As the consequence, we observed spermatozoa quality depression, as measured by decreasing in spermatozoa motility and velocity, DNA damage and inhibition of ATP formation. Based on the obtained results, the use of sperm in vitro assays may provide a novel and

efficiently means for evaluating the effects of human-made pollutants in aquatic environment on sturgeon. Further experiments are needed to investigate the exact influence mechanism and the relationship between motility and oxidative stress in sturgeon sperm as well as consequences of these effects on fertilization ability.

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Figure legends:

Figure 1. In vitro effects of Tetrabrombisphenol A (TBBPA) on spermatozoa motility (A) and velocity (B) after in vitro exposure of sterlet (*A. ruthenus*) sperm in nominal concetrations (0.5, 1.75, 2.5, 5 and 10 µg/L). Data are presented as means ± SEM, n=6. Superscript letters indicate significant differences among samples at the same time post-activation (ANOVA, $P < 0.05$).

Figure 2. Swimming behavior of *Acipenser ruthenus*. Flagella movement was recorded under dark field microscopy with stroboscopic lamp at 10, 60, 90 and 120 s post-activation of spermatozoa.

Figure 3. Example of figures from Comet assay of *Acipenser ruthenus* spermatozoa exposed to differing concentrations of tetrabrombisphenol A (a– control, b– 0.5 µg/l, c– 1.75 µg/l, d– 2.5 µg/l, e– 5 µg/l, f– 10 µg/l). The different degrees of DNA damage in sterlet (*A. ruthenus*) spermatozoa are arranged in six classes: a- no damage-control, % of DNA in Tail < 5%; b- small damage-%DNA in Tail < 15% (0.5 µg/l of TBBPA); c - smaller damage- % of DNA in Tail < 30% (1.75 µg/l of TBBPA); d-medium damage-% of DNA in Tail < 50% (2.5 µg/l of TBBPA); e- higher damage - % DNA in Tail < 50% (5 µg/l of TBBPA) and f- the highest damage- % of DNA in Tail >60% (10 µg/l of TBBPA).

Figure 4 A. Percentage of DNA in Tail (the % of DNA in Tail measured in comet assay) after in vitro treatment of sterlet (*A. ruthenus*) sperm in nominal concetrations (0.5, 1.75, 2.5, 5 and 10µg/l). Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.

Figure 4 B. Olive Tail Moment of the spermatozoa after in vitro treatment of sterlet (*A. ruthenus*) sperm in nominal concetrations (0.5, 1.75, 2.5, 5 and 10 µg/l). Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.

Figure5. Effect of tetrabrombisphenol A (TBBPA) on TBARS level in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SEM. Different letters indicate significant differences among samples (ANOVA, $P < 0.05$).

Figure 6. Effect of tetrabrombisphenol A (TBBPA) on CP level in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SEM. Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.

Figure 7. Effect of tetrabrombisphenol A (TBBPA) on SOD activity in *Acipenser ruthenus* spermatozoa. Data represent mean values ± SEM. Data are presented as means ± SEM, n=6. Different letters denote significant difference between treatments.

Figure 8. Effect of TBBPA on intracellular ATP content in sterlet (*A. ruthenus*) sperm after 2 h exposure to TBBPA. Data are presented as means ± SEM, n=6. Different letters indicate significant differences between treatments (ANOVA, $P < 0.05$).

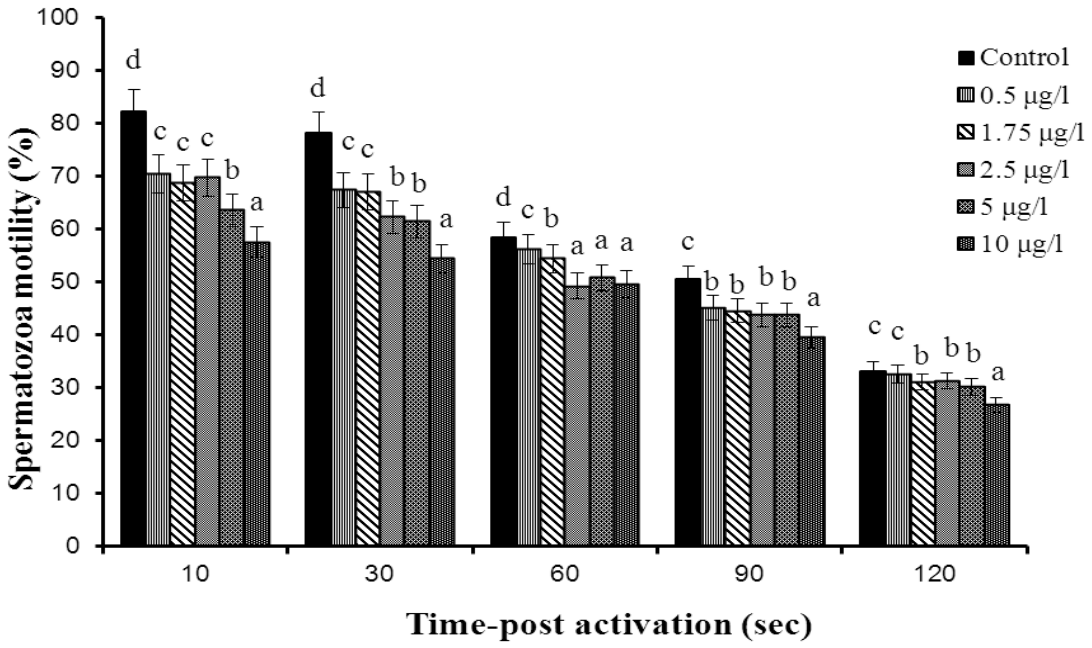
Table 1

	Motility	Velocity	Tail DNA	Olive tail moment	TBARS	CP	SOD	ATP
Motility	1							
Velocity	0.29*	1						
Tail DNA	-0.19	-0.02	1					
Olive tail moment	-0.13	-0.02	0.83*	1				
TBARS	-0.35*	-0.12	0.05	0.04	1			
CP	-0.36*	-0.01	0.29*	0.02	0.37*	1		
SOD	-0.19	-0.12	0.24	0.04	0.21	0.39*	1	
ATP	0.52*	0.57*	-0.14	-0.09	-0.39*	-0.26	-0.36*	1

* $P < 0.05$

Figure 1

A


Spermatozoa motility (%)
Time-post activation (sec)
Control
0.5 µg/l
1.75 µg/l
2.5 µg/l
5 µg/l
10 µg/l
10
30
60
90
120

B

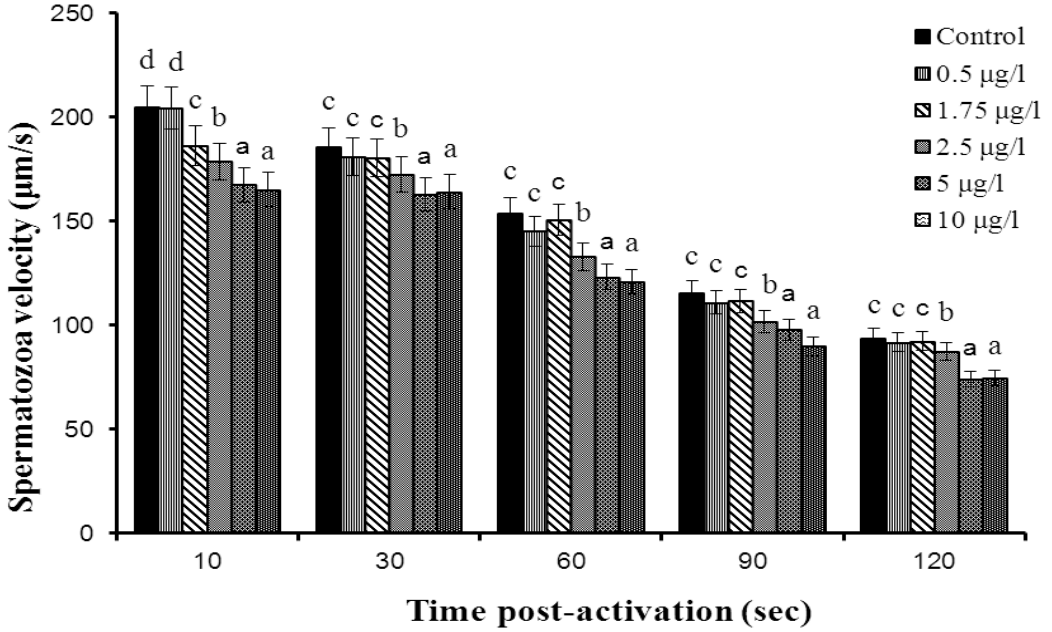

Spermatozoa velocity (µm/s)
Time post-activation (sec)
Control
0.5 µg/l
1.75 µg/l
2.5 µg/l
5 µg/l
10 µg/l
10
30
60
90
120

Figure 2

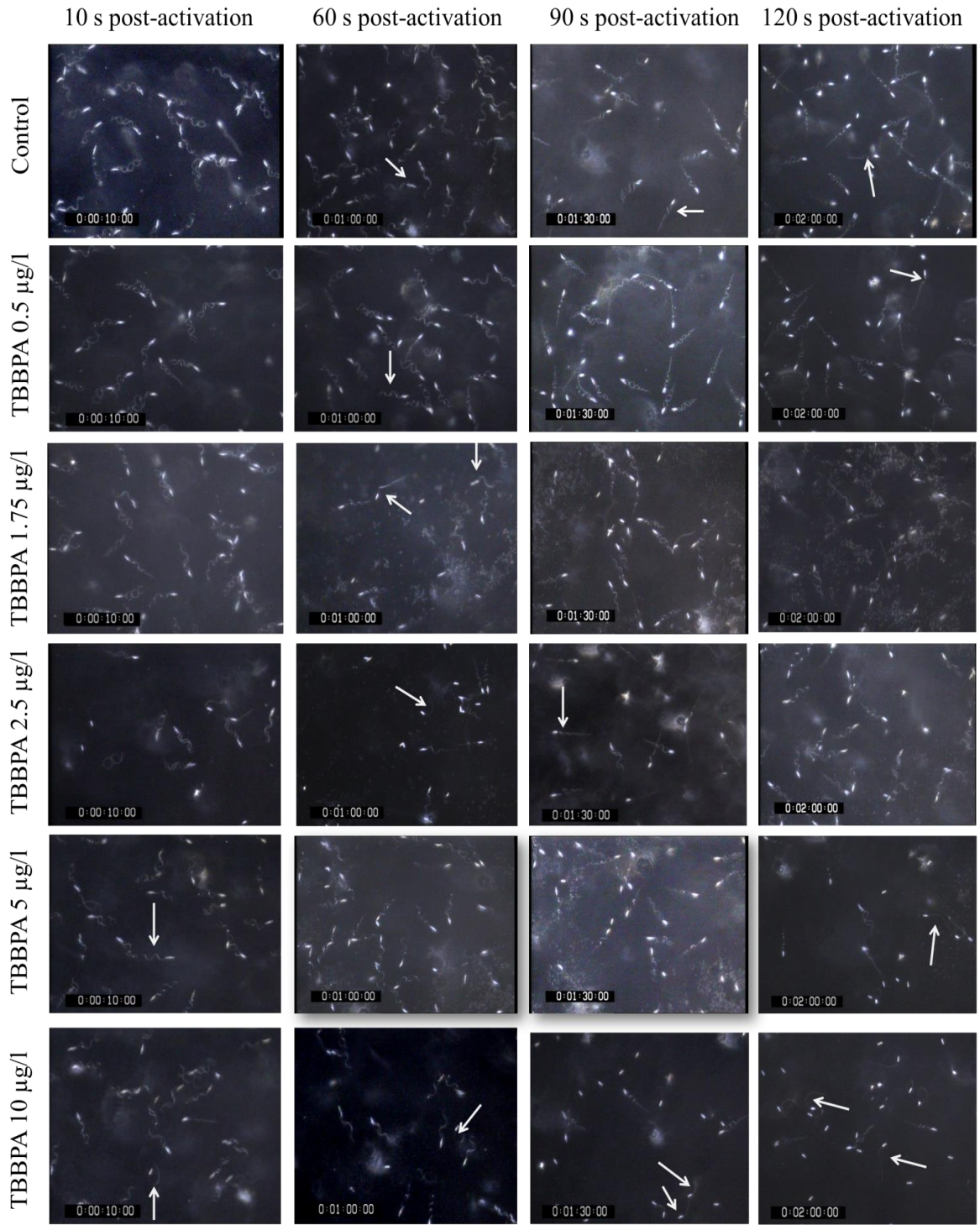

10 s post-activation
60 s post-activation
90 s post-activation
120 s post-activation
Control
TBBPA 0.5 µg/l
TBBPA 1.75 µg/l
TBBPA 2.5 µg/l
TBBPA 5 µg/l
TBBPA 10 µg/l
0:00:10:00
0:01:00:00
0:01:30:00
0:02:00:00

Figure 3

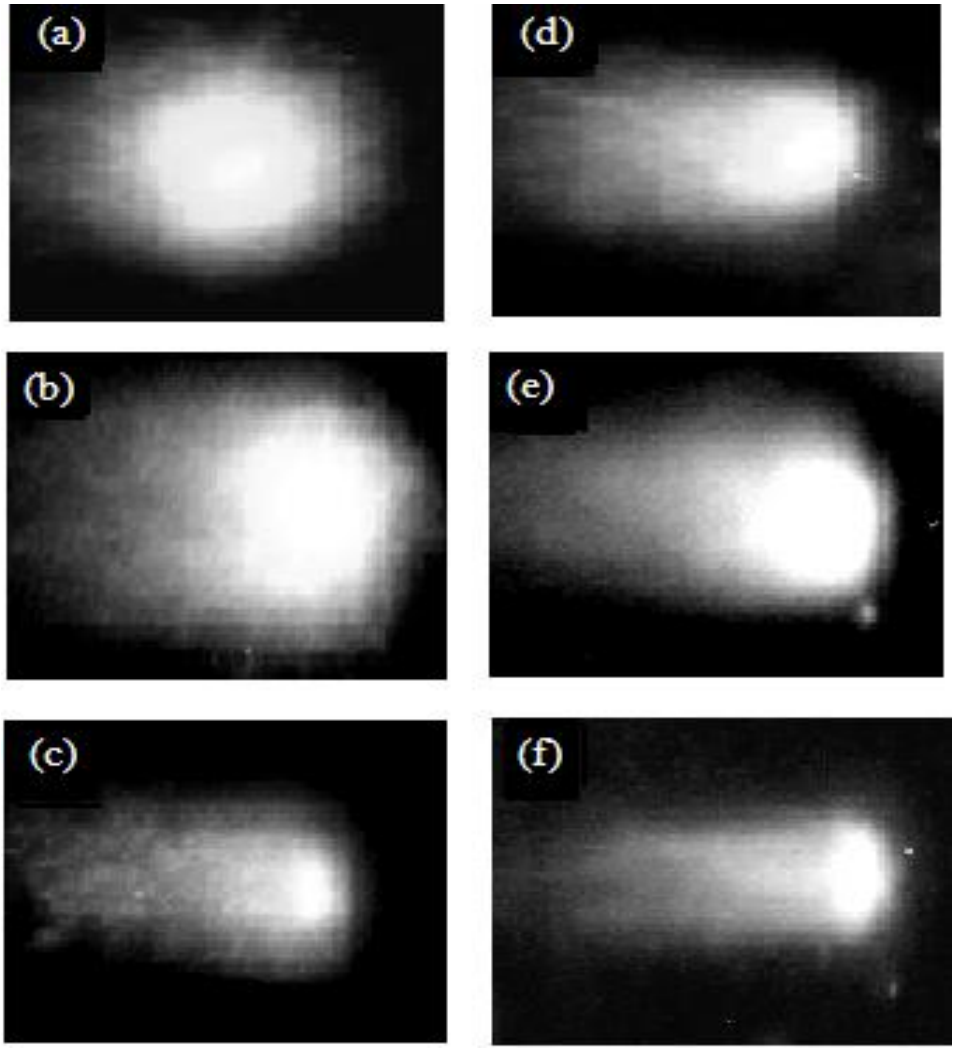
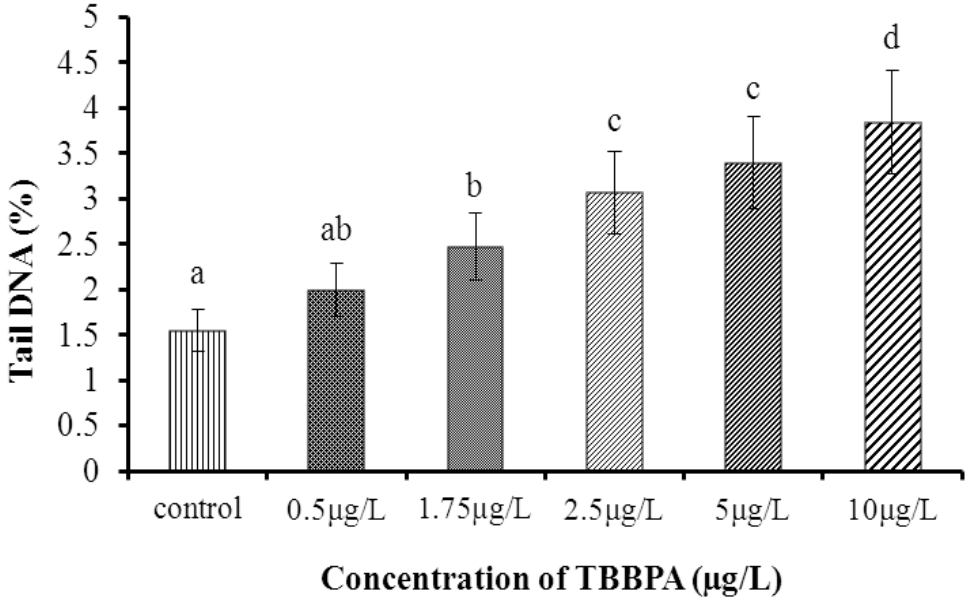


Figure 4

A


Tail DNA (%)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
ab
b
c
c
d
control
0.5μg/L
1.75μg/L
2.5μg/L
5μg/L
10μg/L
Concentration of TBBPA (μg/L)

B

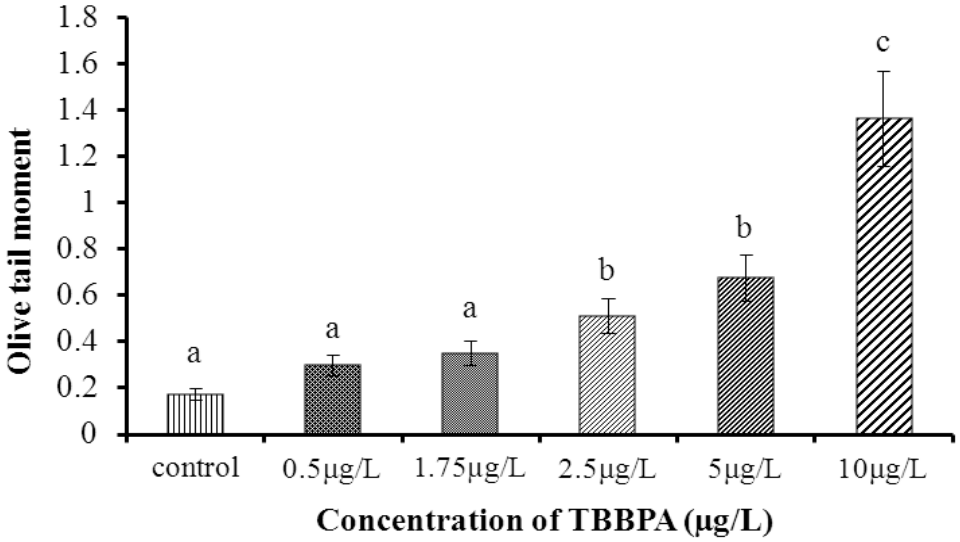

Olive tail moment
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
b
b
c
control
0.5μg/L
1.75μg/L
2.5μg/L
5μg/L
10μg/L
Concentration of TBBPA (μg/L)

Figure 5

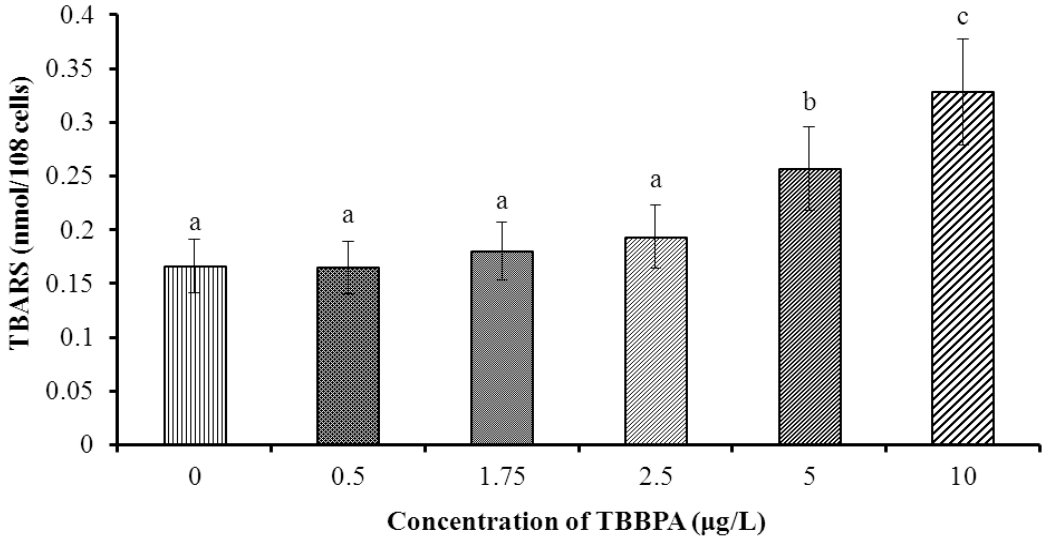


Figure 6

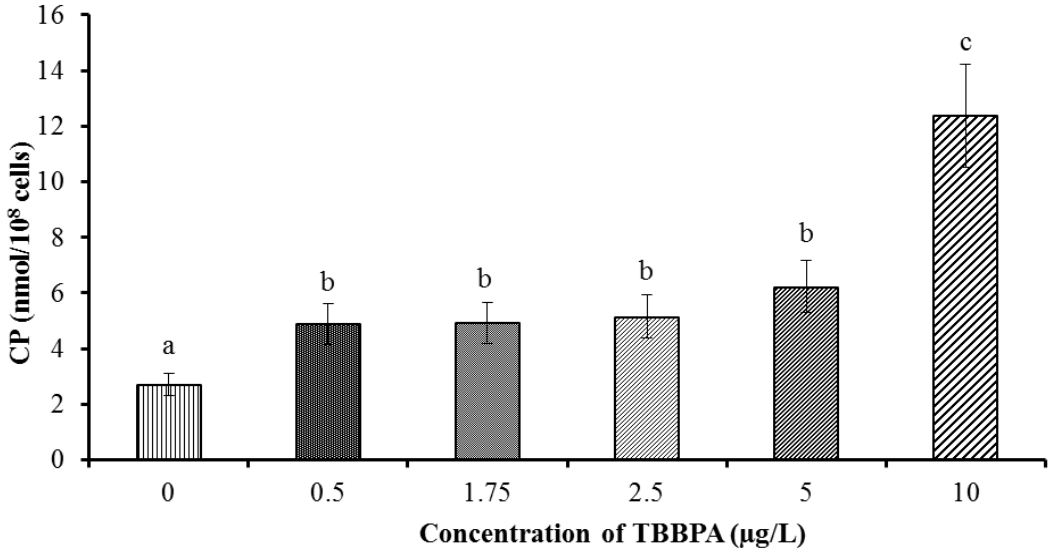


Figure 7

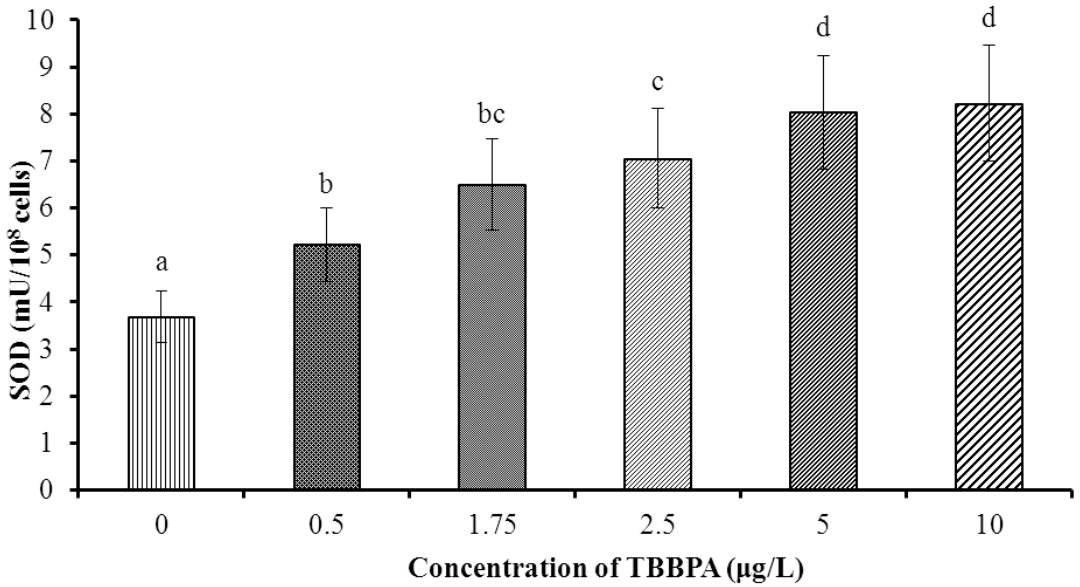
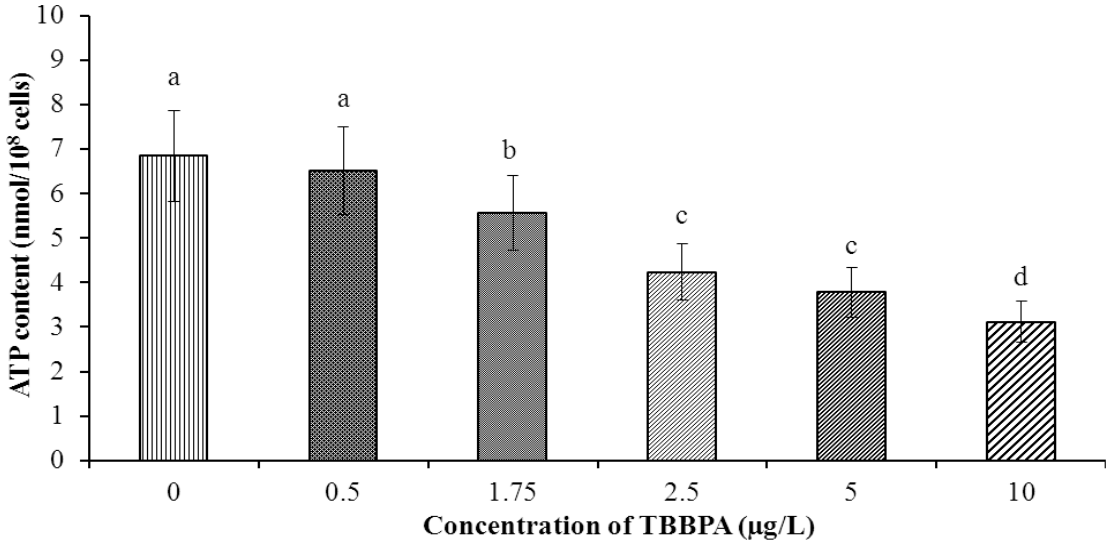


Figure 8



10 Souhrn

Znečištění vodního prostředí xenobiotiky se stává v posledních letech závážným problémem. V mé studii byl zkoumán účinek těchto xenobiotik: DQ, TBBPA, BPA a VIN a byly sledovány parametry kvality spermatu, DNA integrita a indexy oxidativního stresu u spermií jesetera malého (*Acipenser ruthenus*) a sivena amerického (*Salvenilus fontinalis*). Pro sledování *in vitro* testu pro motilitu spermií, byl použit systém Motion-Analysa podporovaný počítačem. Spermie ryb byly zředěny tak, abych získala hustotu buněk 5 x $10^8 \times ml^{-1}$, dále byla přidána xenobiotika o konečné koncentraci: DQ - 25, 50, 100 a 150 μM, TBBPA - 0.5, 1.75, 2.5, 5 a 10 μg/l, BPA - 0.5, 1.75, 2.5, 5 a 10 μg/l a VIN - 0.5, 1.75, 2.5, 5 a 10 μg/l a takto upravené vzorky byly ponechány v klidu po dobu 2 hodin při pokojové teplotě. Rychlost spermií a procento pohyblivých spermií byly významně sníženy se stoupající koncentrací xenobiotik v porovnání s kontrolou. Úroveň poškození DNA vyjádřené jako % DNA v ocásku a Olive Tail moment byly výrazně zvýšeny, když byly spermie vystaveny vyšším koncentracím xenobiotik. Úroveň oxidativního stres indexu lipidové peroxidace (LPO), karbonylových derivátů proteinů (CP) a antioxidační aktivity superoxid dismutázy (SOD) byl výrazně zvýšen s rostoucí koncentrací xenobiotik. Na druhé straně vnitrobuněčný obsah ATP ve vzorcích značně poklesl. Stručně řečeno, xenobiotika mohou vyvolat reaktivní formy kyslíku (ROS), které způsobí napětí v rybích spermiích a mohlo by to ohrozit integritu DNA ve spermatu, dále kvalituspermatu a antioxidační obranný systém spermií. Tato studie potvrzuje, že koncentrace xenobiotik vyskytujících se v přírodě, jsou schopné vyvolat oxidativní stres, což vede k poškození kvality spermií, fragmentaci DNA a ke snížení obsahu vnitrobuněčného ATP. Mé výsledky také naznačují, že sledování rybích spermií *in vitro* by mohla poskytnout efektivní data, která by sledovala hladinu zbytkových farmaceutických přípravků ve vodním prostředí.

11 Abstract

Pollution of the aquatic environment with xenobiotics has become a serious health concern in recent years. In the present study the effect of DQ, TBBPA, BPA and VIN on sperm quality parameters, DNA integrity and oxidative stress indices in sterlet (*Acipenser ruthenus*) sperm and sperm from brook trout (*Salvenilus fontinalis*) were investigated. To do this, an *in vitro* spermatozoa motility assay was used by a computer-aided Motion-Analysis system. The sperm of sterlet (*Acipenser ruthenus*) was diluted to obtain the spermatozoa density of 5×10^8 cells$\times$ml^{-1} and then exposed for 2 h to final concentrations of xenobiotics: DQ - 25, 50, 100 and 150 μM, TBBPA - 0.5, 1.75, 2.5, 5, 7.5 and 10 μg/l, BPA - 0.5, 1.75, 2.5, 5 and 10 μg/l and Vin - 0.5, 1.75, 2.5, 5 and 10 μg/l. Spermatozoa velocity and percentage of motile sperm were significantly decreased at each time post-activation compare to control. The level of DNA damage expressed as a % DNA in Tail and Olive Tail moment significantly increased when spermatozoa were exposed to higher concentrations of xenobiotics. The level of oxidative stress indices lipid peroxidation (LPO) and carbonyl derivatives of proteins (CP) and antioxidant activity of superoxide dismutase (SOD) increased significantly with increasing concentration of xenobiotics. On the other hand the intracellular ATP content in sperm samples had a significantly decreasing effect. In short, xenobiotics can induce reactive oxygen species (ROS) stress in fish spermatozoa, which could impair the sperm DNA integrity, quality and antioxidant defense system. The present study confirms that environmental concentrations of xenobiotics are capable to induce oxidative stress, leading to impaired sperm quality, DNA fragmentation and intracellular ATP content Obtained results also suggested that the use of fish spermatozoa *in vitro* assays may provide a novel and efficiently means for monitoring residual pharmaceutical in aquatic environment.