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THE EFFECTS OF NITRITES (NO_2^-) AND NITRATES (NO_3^-) ON SPERM MOTILITY OF COMMON CARP *IN VITRO*

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ABSTRACT. The effects of nitrogenous compounds on carp sperm were investigated. The freshly collected carp sperm was incubated for 2 hours at 4°C in the presence of different concentrations of nitrites (NO_2^-) and nitrates (NO_3^-). Sperm motility was determined with the use of computer assisted sperm analysis - CASA. The parameters of sperm movement assessed in the experiments were: VCL - curvilinear velocity (μm s^{-1}), VSL - straight line velocity (μm s^{-1}), VAP - average path velocity, (μm s^{-1}), MOT - percent of motile sperm.

Analysis of these parameters showed that nitrites (NO_2^-) did not significantly change VCL, VSL and VAP parameters at any of the tested concentrations. Significant differences ($p<0.05$) were only observed in the percent of motile sperm (MOT) after the incubation with nitrites at the concentration of 2000 mg N l^{-1}. Nitrates (NO_3^-) at the concentration of 2000 mg N l^{-1} significantly diminished VCL, VSL, VAP and MOT parameters of sperm movement. We conclude that sperm movement can be negatively affected by relatively high concentrations of nitrites and nitrates, the latter being more harmful to motility. Additional studies are needed to clarify if the long term exposure of fish to nitrites and nitrates in lower concentrations will affect the sperm motility.

Key words: CARP, NITRITES, NITRATES, SPERM MOTILITY, CASA,

INTRODUCTION

Eutrophication of aquatic systems caused by high levels of nitrites (NO_2^-) and nitrates (NO_3^-) leads to progressive ecosystem degradation. The major origin of this degradation is the agricultural contamination (e.g. fertilisers). Both compounds of nitrogen (nitrites and nitrates) are the fundamental elements of nitrogen cycle within ecosystems and in the nitrogen balance in animals. They are taken up directly from the environment or with the food (Jensen 1995a).

It is believed that the uptake of nitrites and nitrates in freshwater fish occurs through HCO_3^-, Cl- exchanger at the gill epithelia by the substitution of chloride ions (Bath and Eddy 1980, Williams and Eddy 1986). The nitrogenous compounds are concentrated in the fluids of extracellular space (Margiocco et al. 1983). In different fish species there are differences in the uptake and the accumulation of nitrites and nitrates

(Jensen 1995b). There are data demonstrating changes in the blood caused by nitrogenous compounds, such as haemolytic anaemia and methaemoglobinaemia (Scarno and Saroglia 1984, Williams and Eddy 1988a, 1988b, Jensen 1990, Jensen et al. 1987), however very few papers deal with the problems of the effects of nitrogenous compounds on fish reproduction, and they are primarily related to the effects of ammonia or ammonium ions (Penaz 1965) or nitrites and nitrates. Bieniarz et al. (1996a) have demonstrated the toxic effects of nitrosamines on the maturation of carp oocytes *in vitro* and on the embryonic development of fertilised carp eggs. The process of carp reproduction was also disturbed by the highly eutrophic pond conditions: lower fecundity, lower volume of sperm, decreased survival rate of embryos and adult fish were observed. Also significantly lower percentage of hatching was found in NO_2^- concentrations of l, 10 and 20 mg l^{-1} as well as in NO_3^- concentrations of 15, 150 and 500 mg l^{-1}) in comparison with control incubations (Bieniarz et al. 1996b). Fertilisation *in vitro* was performed in the presence of the nitrogenous compounds which could affect both the eggs and the sperm and so far there were no information on the influence of nitrites or nitrates on the biological properties of carp sperm. Therefore, the purpose of the present pilot work was to determine the effects of different concentrations of nitrites (NO_2^-) and nitrates (NO_3^-) on different parameters of sperm motility (which may affect the reproductive success of this species) with the use of computer assisted sperm analysis - CASA (Toth et al. 1995, Kime et al. 1996, Ebrahimi et al. 1996).

MATERIALS AND METHODS

The experiments were conducted on ten 4-5 year old male carp *(Cyprinus carpio* L.) raised from one parental couple originating from the Fisheries Station of the University of Agriculture, Kraków, Poland. Before sperm sampling fish were acclimated in 400 l basins at 20°C for 7 days. The pilot trials with the use of different concentrations of nitrites and nitrates showed that decreased sperm motility starts from 50 mg l^{-1} of these compounds. In the present experiments this concentration was set as the lowest. The solutions of the nitrites and nitrates were prepared of $NaNO_2$ and $NaNO_3$ respectively, at the concentrations from 0 to 2000 mg l^{-1} (Table l).

Milt was collected with l ml syringe after gentle stripping and stored on ice. Then milt was diluted according to the two-step dilution method described by Billard and Cosson (1992), where the initial, 100-fold dilution (at 400 m0sm kg^{-1}) was keeping the sperm immobile, whereas the second dilution (20-fold) was responsible for the entire

TABLE 1

Nitrites and nitrates concentrations used in the experiment

Solution number	Nitrites concentration (mg N l^{-1})	Nitrates concentration (mg N l^{-1})
1 - control	0	0
2	50	50
3	100	100
4	250	250
5	500	500
6	1000	1000
7	2000	2000

and homogenous activation of spermatozoa. Briefly, 10 μl of sperm was mixed in 10-ml polypropylene tube with 990 μl of extender solution (200 mM KC1, 30 mM Tris-HCI, pH 8.0), except the sperm subjected to influence of nitrate and nitrite, where some of the KCl was substituted with equivalent molar amounts of $NaNO_3$ or $NaNO_2$ in order to give the concentrations of nitrates and nitrites as stated in Table l. Ten sperm samples, originating from different fish, were used for each concentration. The mixtures were then incubated in 4°C for 2 hours in the presence of air. The second dilution was made on a microscope slide precoated with 1% polyvinyl alcohol (Sigma, St. Louis, MO, USA, MW 30000-70000 Da) to prevent adhesion of sperm: l μl of sperm was diluted further in 19 μl of distilled water to give the final 2000-fold dilution. Sperm movement was then immediately tape recorded for two and half minutes at 20°C from the moment of dilution, with a video camera (JVC, TK 1280E, Japan) attached to a phase-contrast inverted microscope under the magnification of 100X, video cassette recorder (Samsung VQ 336, South Korea) and monitor (Sony KX 1410QM, United Kingdom). Video recordings were analysed using a Hobson sperm tracker (Hobson Tracking Systems Ltd., Sheffield, U. K.) according to Kime et al. (1996). The recordings started to be monitored 20 seconds after the second dilution (to allow the stabilisation of water movement) for four successive 15 second periods (marked as l, 2, 3 or 4).

The parameters of tracker were adjusted for carp sperm. Sperm motility was analysed by assessing following parameters:

VCL - curvilinear velocity (μm s^{-1}), the sum of the incremental distances moved in each frame along the sampled path divided by the total time of the track (VCL l, VCL2, VCL3, VCL4);

VSL - straight line velocity (μm s^{-1}), the straight line distance between the start and end points of the track divided by the time of the track (VSL1, VSL2, VSL3, VSL4);

VAP - average path velocity, (μm s^{-1}), a derived path based on an average number of points and divided by the time of the track (VAP l, VAP2, VAP3, VAP4);

MOT - percent of motile sperm (MOT1, MOT2, MOT3, MOT4).

Data was subjected to the analysis of variance and for determination of the significant differences between the groups the Duncan's test was applied.

RESULTS

NITRITES (NO_2^-)

There were no significant differences in VCL, VSL and VAP parameters in all the tested concentrations of nitrites in comparison to control incubation.

Significant differences ($p<0.05$) were observed in the percent of motile sperm -MOT (Fig. l):

- at the second period of measurement (MOT2) the highest concentration (2000 mg N l^{-1}) decreased sperm motility in comparison with the motility observed at the control,
- at the third period of measurement (MOT3) the concentration of 2000 mg N l^{-1} caused significantly lower sperm motility compared with that found at 50, 100, 250 and 500 mg N l^{-1},
- at the last period of measurement (MOT4) the concentration of 2000 mg N l^{-1}, caused significantly lower sperm motility compared with all other concentrations.

NITRATES (NO_3^-)

VCL parameter of sperm motility showed that at the first three periods of measurement (VCL l, VCL2 and VCL3) the motility of sperm at the concentration of 2000 mg N l^{-1} was the lowest in comparison with the motility at all other nitrogen concentrations (Fig. 2). Moreover, at the last period of measurement (VCL4) at this concentration no sperm movement was found.

VSL parameter of sperm motility showed that the nitrogen concentration of 2000 mg N l^{-1} significantly lowered the sperm motility at the first three periods of measurement (VSL l, VSL2 and VSL3) in comparison with all other concentrations (Fig. 3). The last period of measurement (VSL4) demonstrated the lack of sperm

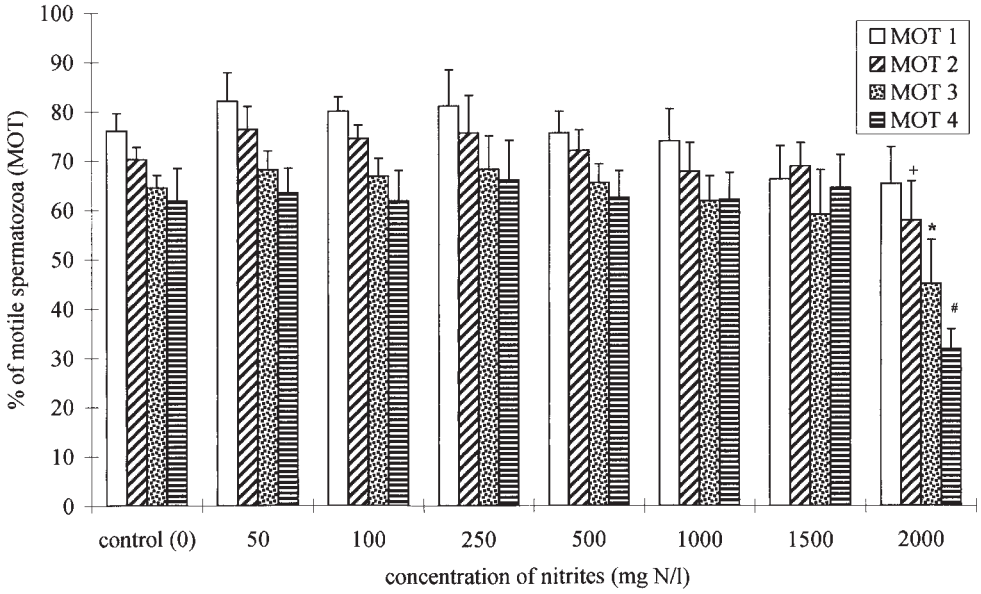

Fig. l. The effects of different concentrations of nitrites on the percentage of motile spermatozoa (MOT) in carp. Values are expressed as mean+SEM. + - significantly different vs. control group ($p<0,05$); * - significantly different vs. groups: 50, 100, 250 and 500 ($p<0.05$); # - significantly different vs. all other groups ($p<0.05$)

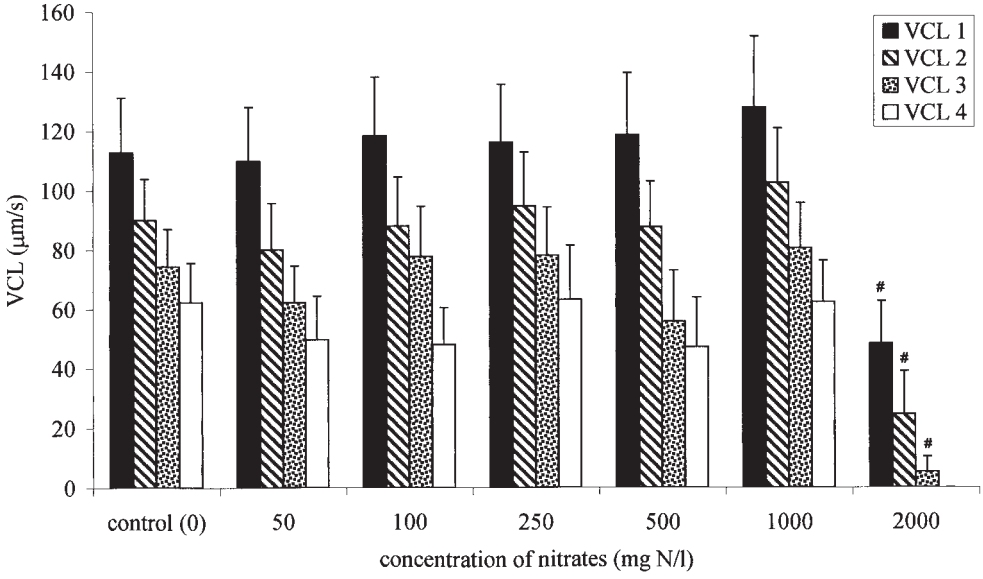

Fig. 2 The influence of different concentrations of nitrates on carp sperm motility expressed as VCL parameter. Values are expressed as mean+SEM. # - significantly different vs. all other groups ($p<0.05$)

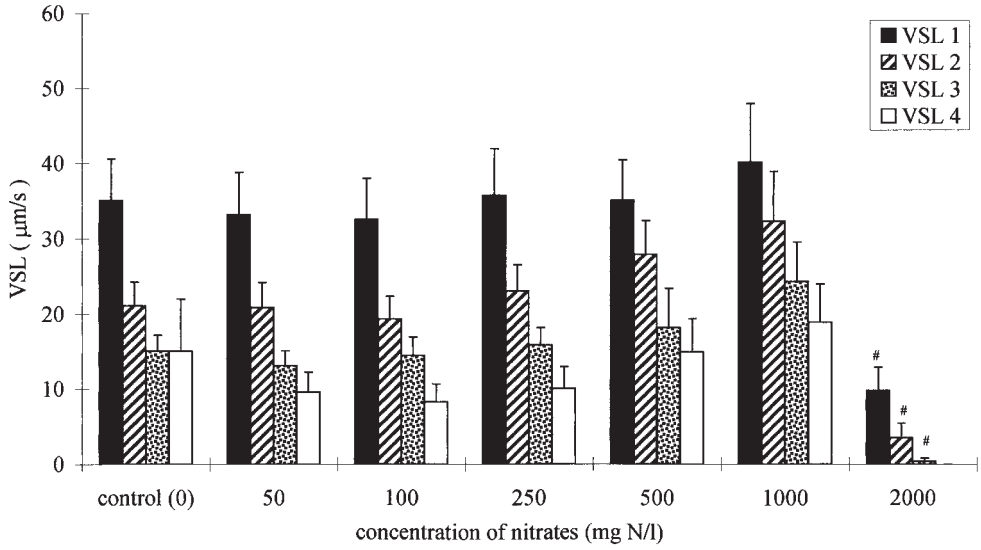

Fig. 3. The effects of different concentrations of nitrates on carp sperm motility expressed as VSL parameter. Values are expressed as mean+SEM. # - significantly different vs. all other groups ($p<0.05$)

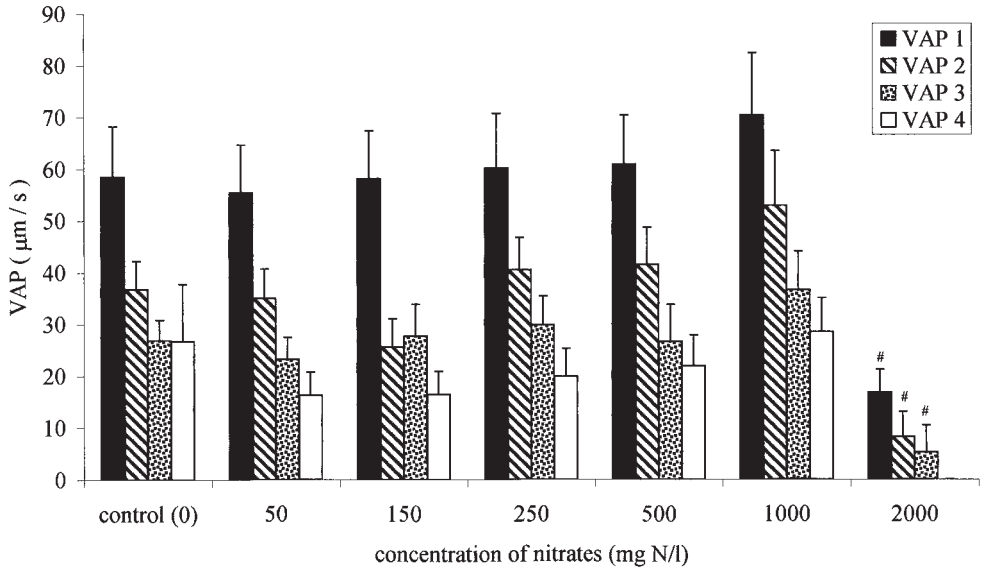

Fig. 4 The effects of different concentrations of nitrates on carp sperm motility expressed as VAP parameter. Values are expressed as mean+SEM. # - significantly different vs. all other groups ($p<0.05$)

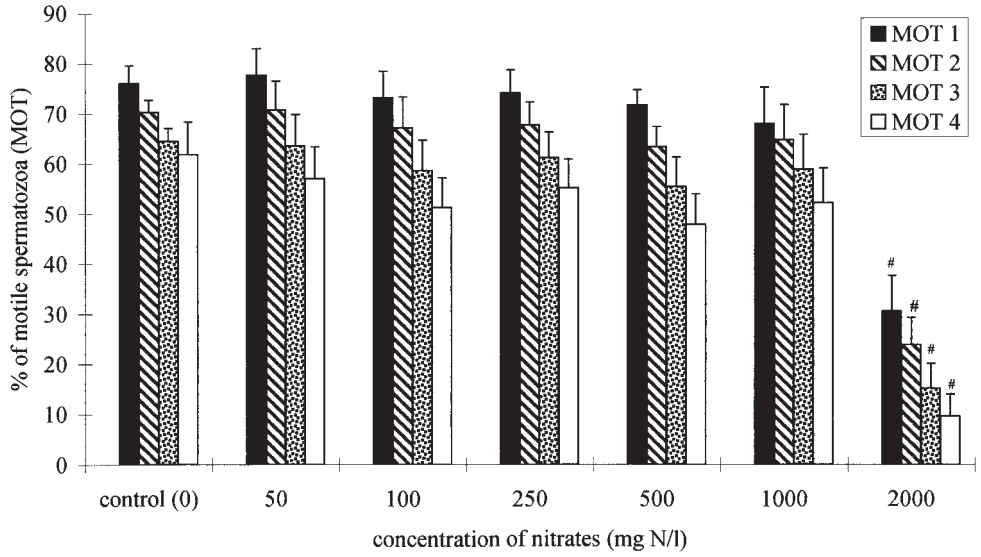

Fig. 5. The influence of different concentrations of nitrates on the percentage of motiie spermatozoa (MOT) in carp. Values are expressed as mean+SEM. # - significantly different vs. all other groups ($p<0.05$)

motility in the highest concentration of 2000 mg N l^{-1}. No significant differences between the sperm motility in all other groups were observed.

VAP parameter of sperm motility demonstrated that the highest nitrogen concentration (2000 mg N l^{-1}) caused significant decrease of sperm motility in comparison with that observed in all other nitrate concentrations (Fig. 4).

The percentage of motile spermatozoa (MOT) was also decreased in all measurement periods in comparison with control solution and other nitrate concentrations (Fig. 5).

DISCUSSION

Experiments on the influence of highly eutrophic pond conditions (with the high content of nitrites and nitrates) on carp reproduction (Bieniarz et al. 1996b) have demonstrated their toxic effects: significantly lower gonadosomatic index, lower percentage of mature oocytes in the ovary, lower relative fecundity and lower percentage of hatching were observed. In males eutrophic waters decreased volume of sperm. Water nitrate concentration span from the micromolar to millimolar range. The primary source of elevated nitrate levels in aquatic ecosystems and in groundwater is agricultural use of fertilizers (Bouchard et al. 1992). Other minor sources include

combustion of fossil fuels which emit nitrogen oxides (NO and NO_2^-) that are oxidized to nitric acid in the atmosphere and return to the surface as H^+ and NO_3 in acid rain (Mason 1989). The WHO guidelines for maximum nitrate concentrations in drinking water is 10 mg NO_3 N l^{-1}. This concentration is exceeded in many agricultural areas (Bouchard et al. 1992). In the investigations on long lasting (3 years) and short effects (5 days) of nitrites and nitrates on carps cultured in eutrophic ponds the dose of 0.5 kg N m^{-3} was applied, and on embryonic development and hatching: 0, l, 10, 20 mg l^{-1} and NO_2 and 0, 15, 150, 500 mg l^{-1} NO_3 respectively were used (Bieniarz et al. 1996b).

Although the recent literature supplies the growing data concerning the influence of nitrogen-containing compounds on many aspects related to fish reproduction, up to our knowledge there was no investigation about the effects of these compounds on sperm motility. One of the major difficulties in such investigations is the very short term of spermatozoa motility, reaching 45 s for the rainbow trout and about 90's for the carp (Billard and Cosson, 1992), moreover the subjective microscopic observations of different aspects of sperm motility would be often inaccurate. Therefore for these studies we have used a computer assisted sperm analysis (CASA) - a technique, which permits to analyse different parameters related to sperm motility. For the clarity in the results we have incorporated only 4 of these parameters, which refer to spermatozoa velocity (VCL, VSL, VAP) and to percent of spermatozoa being motile during a given time period (MOT). Moore and Akhondi (1996) showed that in the rat the capacity of sperm to fertilise the eggs is related to one of above parameters - straight line velocity, however it is possible that the decreasing percentage of motile sperm may also be related to diminution of the fertilisation success but the data from the literature is scarce.

According to Jenssen (1992) higher uptake of NO_2^- than NO_3^- in rainbow trout is the reason of lower nitrates toxicity. However the results presented in our paper show that the effects of nitrates on sperm motility is much more pronounced than of nitrites, because only the highest dose of nitrites (2000 mg N l^{-1}) resulted in total movement cessation. The tested concentrations of nitrates and nitrites are extremely high and very rarely can occur in aquatic environment. This indicates that the concentrations observed in the eutrophied environment do not affect the movement ability of sperm, however 3 years lasting action of eutrophied waters caused in male carp significant decrease of sperm volume (Bieniarz et al. 1996b). It seems that nitrogenous compounds affect oocytes more than the sperm - it is known (Bieniarz et al. 1996b) that reproduction of carp females is more susceptible to the toxic effects of nitrates and nitrites, which in much lower concentrations lowered reproductive success of carp females. Also it is

important to remember that the sperm incubated with pollutants was finally diluted 20 times with water, so the final dilution of nitrates and nitrites was 20 times lower. However, since the effects of these pollutants is exhibited after their incorporation into the spermatozoa, it is unlikely that the final dilution with water can decrease the harmful effect of nitrates and nitrites. On the other hand it is possible that 2 hour period of sperm incubation in the presence of pollutants could be insufficient to evoke the changes in sperm motility in lower concentrations of nitrogen-containing compounds. Therefore, it would be necessary to verify if the long term accumulation of above compounds in living fish could decrease the sperm motility in much lower concentrations corresponding to the levels of nitrites and nitrates in eutrophied waters.

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STRESZCZENIE

WPŁYW AZOTYNÓW (NO_2^-) I AZOTANÓW (NO_3^-) NA RUCHLIWOŚĆ SPERMY KARPIA *IN VITRO*

Celem niniejszej pracy było zbadanie wpływu związków azotowych na ruchliwość plemników karpia. Świeżo pobraną spermę karpia inkubowano przez 2 godziny w temperaturze 4 C w obecności różnych stężeń azotanów i azotynów (od 0 do 2000 mg N l^{-1}) przy użyciu dwustopniowej metody rozcieńczeń (Billard i Cosson 1992). Pomiary ruchliwości plemników dokonano za pomocą komputerowej metody analizy plemników -CASA. Przeprowadzono pomiary następujących parametrów charakteryzujących ruchliwość plemników: VCL, VSL, VAP i MOT.

Analiza powyższych parametrów wykazała, że azotyny nie miały istotnego wpływu na ruchliwość plemników mierzoną parametrami VCL, VSL i VAP w przypadku żadnego z użytych stężeń. Zaobserwowano istotne ($p<0.05$) obniżenie procentu ruchliwych plemników mierzonego za pomocą parametru MOT w przypadku koncentracji 2000 mg N l^{-1}. Azotany użyte w stężeniu 2000 mg N l^{-1} spowodowały istotne obniżenie ruchliwości plemników mierzonej za pomocą parametrów VCL, VSL i VAP a także obniżenie procentu ruchliwych plemników MOT.

Otrzymane wyniki sugerują, że ruchliwość plemników, kluczowa dla efektywnego zapłodnienia, może być zaburzona w obecności wysokich koncentracji azotanów i azotynów. Azotany wydają się w większym stopniu hamować ruch plemników, chociaż w obydwu przypadkach zastosowane stężenia są relatywnie wysokie. W celu wyjaśnienia tego zjawiska niezbędne są dodatkowe badania nad wpływem długotrwałych hodowli ryb oraz inkubacji plemników karpia *in vitro* w obecności azotanów i azotynów na ruchliwość plemników.

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