Research Journal of

Environmental Sciences

ISSN 1819-3412

Academic Journals Inc.

www.academicjournals.com

Research Journal of Environmental Sciences 2 (6): 474-482, 2008
ISSN 1819-3412

Effects of Butachlor on Density, Volume and Number of Abnormal Sperms in Caspian Kutum (*Rutilus frisii kutum*, Kamenskii 1901)

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Abstract: Morphological assessment of sexually mature *Rutilus frisii kutum* Kamenskii 1901 caught from the rivers (Shirud, Khoshkrud, Sepidrud and Chelavand Rivers) flowing in the southwest Caspian Sea region was conducted and sperm volume, total sperm count and sperm concentration of abnormal sperms were determined after exposing the spawners to 60% herbicide butachlor (machete). Spawners under study were maintained in tanks (1000 L) at the Shahid Ansari Teleost Fish Hatchery and exposed to two different concentrations (25 and 75% of its LC_{50} value) of butachlor. Results obtained indicate that exposure to high butachlor toxicity (75% of its LC_{50} value) decreased sperm volume to 0.61±0.42 cc in 2-3 year old fishes and to 0.55±0.42 cc in fishes above 3 years of age, while that in fish exposed to low butachlor toxicity (25% of its LC_{50} value) decreased to 1.55±0.42 cc in 2-3 year old fishes and to 1.28±0.42 cc in fishes above 3 years of age. The sperm volume under normal conditions in *R. frisii kutum* is 4.6±0.42 cc in 2-3 years old and 4.58±0.42 cc in fishes above 3 years of age. The total sperm count in *R. frisii kutum* is 39.74±2.5 billion spermatozoa cc^{-1} in 2-3 year old and 42.99±2.5 billion spermatozoa cc^{-1} in fishes above 3 years of age. When exposed to high butachlor toxicity, total sperm count dropped to 16.92±2.5 billion spermatozoa cc^{-1} in 2-3 year olds and to 15.98±2.5 billion spermatozoa cc^{-1} in fishes above 3 years of age. Similarly total sperm count in *R. frisii kutum* exposed to low butachlor toxicity was recorded as 23.6±2.5 billion spermatozoa cc^{-1} in 2-3 year olds and 29.4±2.5 billion spermatozoa cc^{-1} in fishes above 3 years of age. Under normal conditions, on the basis of morphology, spermatozoa showed only 10±1.92% of abnormal sperms. The number of abnormal sperms increased by 28.6±1.92% in fishes exposed to high butachlor toxicity, while that in fishes exposed to low butachlor toxicity increased by 19.7±1.92% in 2-3 year olds and 16.6±19.2% in fishes above 3 years of age. It is evident from the results obtained that increase in level of pollution caused a decrease in sperm volume but an increase in the percentage of abnormal sperms.

Key words: Spermatozoa, Caspian sea, herbicide, kutum

INTRODUCTION

Rutilus frissi kutum is the most important of teleosts inhabiting the Caspian Sea which comprises the maximum percentage of annual catch for bony fishes in the Caspian Sea (Nikou *et al.*, 2007). This species is an anadromous cyprinid which enters rivers in the south Caspian Sea for spawning (Bahmani *et al.*, 2007). During its spawning migration from the sea to the river, it remains in the estuary until it regulates the internal water and total solute concentrations of its body fluids to that

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of the freshwater (Bahmani *et al*., 2007). Spawners are caught from rivers in the southwest Caspian Sea region, 30-300 m from the river estuaries for semi artificial breeding programs. Spawners enter the rivers to attain complete sexual maturity of gonads in freshwater and are ready for spawning when they reach the spawning ground. Predicting the effects of environmental hazards on spermatozoa alterations can be useful in developing new technologies for the artificial breeding of this commercially valuable species of the Caspian Sea (Alavi *et al*., 2004).

It has been demonstrated that sperm motility, density, viability and quality is significantly affected by salinity, osmotic pressure, ionic composition and pollution (Bahmani *et al*., 2007; Carter, 2006; Alavi *et al*., 2004). The effects of pollution on sperm activity have also been studied in *Cyprinis carpio* and *Acipenser persicus* (Alavi, 2004). Butachlor is one of the most popular herbicides used to control weeds in transplanted rice paddy fields. With regard to the fact that most paddy fields are located on the banks of rivers, the run-off contaminated by this pesticide may affect the reproduction and physiological characteristics in fishes during their spawning migration (Esmaily Sari, 2002). Exposure to pollutants of any intensity may cause stress in aquatic organisms. Stress is the collective physiological response of an individual to maintain hemostasis (Pickering *et al*., 1982; Chen and Sontegard, 1984; Montpetit and Steve, 1998). The inhibiting effects of stress on fish reproduction cause adverse effects on the functioning of endocrine glands which result in decreased gamete and larval quality (Contreras-Sanchez, 1998). Apart from corticosteroids, sex steroids have also been recognized as primary messengers of stress response in fishes (Scherek *et al*., 2001). Stress is also a potent inhibitor of hormones such as gonadotropin, testosterone, 11 keto-testosterone and 17-β estradiol in fish (Kubukawa *et al*., 1999; Bayunova *et al*., 2002). Butachlor, N-butoxymethyl-2-chloro-2' 6'-diethyl acetanilide (Esmaily Sari, 2002) is the most commonly used herbicide, the biochemical composition of which is very close to estrogen (Esmaily, 2002). It is orange to brown in color with a molecular weight of 311.9 and a specific weight of 1.07 at 25°C. It has a melting point of 156°C and freezing point of < -5°C. Butachlor has a vapor pressure of 4.5×10^{-6} mm Hg at 25°C and decomposes at 165°C (Piri Zirkoohi and Ordog, 1997).

Butachlor is used for the control of weeds particularly in paddy fields. It is a selective herbicide that acts on soil and controls the growth of roots and also prevents emergence of weeds. It is particularly effective in controlling broad leaf arrowhead, *Sagittaria pigmaea* and the barnyard grass *Echinocloa crus* and *Alisma canaliculatum*. Butachlor is not very soluble in water and is sometimes mixed with propanil to control unwanted weeds. Exposure to hormones or biochemicals which are opposite to their sex hormones results in changes or termination of sexual axis (McCormick, 2007). Thus butachlor mimics estrogen and decreases testosterone levels in blood plasma. Growth and development of testes and development of sertoli cells affect the number, density and volume of spermatozoa (Schultz *et al*., 2003).

The effects of pollutants occurring in nature which are similar to estrogen in composition have been studied which include effects of natural estrogen in Zebra fish, *Danio rerio* (Anderson *et al*., 2003) and *Rutilus rutilus* (Liney *et al*., 2005). Similar studies have also been conducted on rainbow trout (Schultz *et al*., 2003). The effects of increasing osmolarity as a result of pollution on the number of spermatozoids were studied in *Rutilus frisii kutum* (Bahmani *et al*., 2007).

The minimum and maximum number of sperms in *Rutilus frisii kutum* are 32 billion and 48 billion sperms cc^{-1}, respectively (mean = 40 sperms cc^{-1}) and the sperm volume in kutum ranges from a minimum of 2 cc to a maximum of 6.4 cc (Azari Takami and Razavi Sayed, 1990). Malformed sperms with double heads, sperms bound to each other by their heads, or the head bound to the tail of other spermatozoa have been reported in kutum resulting from inappropriate maintenance of spawners, cold and/or hot shocks, toxicity and pollution and changes in acidity (Vladi *et al*., 2002). Spermatozoa with 10% anomalies have been reported in kutum (Bahmani, 2007). Based on Kortet *et al*. (2004) this is considered high sperm quality.

In the present study, fish under study were exposed to the herbicide butachlor in the hatchery and the effects of the herbicide were evaluated on spermatogenesis in terms of sperm volume, number of spermatozoids and number of abnormal spermatozoids.

With regard to the fact that chlorinated herbicides are capable of accumulating in steroid tissues (Esmaily Sari, 2002) the present study was conducted on two age classes to evaluate the changes in indices studied and thus offer recommendations for enhancement measures in the aquatic environment and reduction in the use of pollutants.

Considering the significance of *Rutilus frisii kutum* in the economy of the coastal dwellers and the need for suitable male spawners possessing good quality sperms in artificial breeding programs, the present investigation was carried out on this species to determine the physiological alterations in sperms caused by the extensive use of these herbicides in the study region, particularly during the spawning migration of the target species. These alterations in spermatozoa can be used as a model to predict environmental hazards and thus assist in developing suitable measures to protect these populations.

MATERIALS AND METHODS

Specimens and Experimental Set up

This study was conducted at the Shahid Ansari Hatchery from February 2007 through April 2007. Male spawners required for the study were randomly collected from the Sepidrud, Khoshkrud, Khaleh Sara and other rivers located in the southwest region of the Caspian Sea. Two doses, high dose (75% of LC_{50} = 0.3225 ppm) and low dose (25% of LC_{50} = 0.1075 ppm), were used for the herbicide butachlor that were based on the LC_{50} values for this herbicide (APHA, 1986; Simmons, 1993). The LC_{50} for butachlor for *Rutilus frisii kutum* was determined as 0.43 ppm which correlated with the previous findings of Piri Zirkohi *et al*. (2000). To study the effect of age, fishes under study were divided into two age classes-the first group with fishes belonging to the 2-3 year age class and the second group with fishes above 3 years age class. A control group was also studied simultaneously. The split plot design was used with three replicates for statistical analysis of results. The herbicide doses were applied one each per whole plot and different age classes were applied on each subplot. Fiber glass tanks (1000 L) equipped with aeration were used in a closed system at the Shahid Ansari Hatchery in Rasht. A total of 540 kutum fish caught from the rivers in the Gilan Province were stocked in these tanks. Age determination of fish was carried out using scale method. After stocking fish were exposed to the herbicide for six days. The toxic effects of the herbicide are neutralized within 6-10 days in clean water (Esmaily Sari, 2002).

Daily Experiments and Sampling

Dissolved oxygen concentrations, pH and water temperature were measured using the portable Multimeter and recorded. Fish are maintained in clean water for 6 days to neutralize the effects of 6-day exposure to herbicides and random samples are collected to measure total length (cm), total body weight (g) and weight of gonads in spawners prior to sperm collection.

Sperm Collection and Spermatological Experiments *in vitro*

Sperm were collected by applying gentle abdominal pressure to extrude the milt. Care was taken to avoid contamination with seawater or urine (Alavi *et al*., 2004). The milt was transferred to a glass vial and kept at 1-2°C until its use. The concentration of spermatozoa was determined using the haemocytometer method. In this procedure a 1:600 dilution (sperm:water) from each well-mixed sample was prepared (Kortet *et al*., 2004) and semen volume was determined using a wide mouthed pipette (Bahmani, 2007). The staining technique was used to differentiate abnormal spermatozoa using a light microscope (Sherek *et al*., 2001).

Statistical Analysis

The results obtained were analyzed using one way ANOVA and comparison of means was carried out using SPSS (Vladi *et al*., 2002).

RESULTS

Sperm volume and sperm count as well as percentage of abnormal spermatozoa were significantly affected by the dose of butachlor used at a 1% significance level. Sperm density and percentage of abnormal spermatozoa also showed significant correlations with total length and body weight at a 1% significance level. Although no correlations were detected between age and sperm volume, significant interactions were observed with different concentrations of the herbicide butachlor on age, total length, sperm density and percentage of abnormal spermatozoa at a 1% significance level (Table 1).

Comparison of means of results obtained using Duncan's Multiple Range Test (MRT) at a 5% significance level indicates that exposure to butachlor decreased sperm volume whereby lowest sperm volume (0.55 mL) was recorded in the high dose treatment. Sperm volume did not change significantly with age and decrease in sperm volume in both age classes studied was not significantly different from a single control (Table 2).

Comparison of means of sperm count and sperm density using Duncan's test indicate that high toxicity caused by the herbicide butachlor caused severe declines in sperm count in *Rutilus frissi kutum* resulting in 16.37 billion spermatozoa. Butachlor toxicity in 2 year old fish was greater and sperm count in this group declined to 26.7 billion spermatozoa. Exposure to butachlor also resulted in abnormal spermatozoa and highest percentages of these spermatozoa (28.6%) were recorded fish exposed to high dose of butachlor, while percentage of abnormal spermatozoa in 2 year-old fish was recorded as 17.98%.

Table 1: Mean-squares of characteristics and doses of herbicide butachlor in different age groups

Source of variation	df	Total length (cm)	Total weight (g)	Sperm volume (cc)	Sperm count (billion)	Abnormal spermatozoa (%)
Factor A						
Boutachlor	2	5.902**	9306.88**	27.386**	948.639ns	558.196**
Block	2	0.180ns	905.55ns	0.022ns	0.330ns	0.056ns
Error A	4	0.197	288.88	0.018	0.585	0.622
Factor B						
Age	1	141.120**	316808.00**	0.127**	031.680**	9.976**
A×B	2	013.122**	2384.66ns	0.530ns	018.362**	3.582**
Error B	6	0.191	494.44	0.055	0.448	0.060
CV		1.070	3.03	10.800	02.380	1.310

**Significant at 1 and 5% significance level

Table 2: Comparison of means of parameters in male *Rutilus frissi kutum* using Duncan's MRT at 5% significance level

Dose	Total length (cm)	Total weight (g)	Sperm volume (cc)	Sperm count (billion)	Abnormal spermatozoa (%)
Butachlor (0.3225 ppm)	41.02b	608.3b	0.556c	16.37c	28.6a
Butachlor (0.1075 ppm)	41.98a	675.0a	1.36b	26.51b	18.17b
Without butchlor	40.0c	605.3b	4.59a	41.37a	09.36c
Age					
(2-3 years)	38.2b	496.9b	2.25a	26.75b	19.47a
(up 3 years)	43.8a	762.2a	2.08a	29.41a	17.98b

Mean values with the different letter(s) significant different at 5% significant level

Table 3: Correlation coefficients detected between parameters studied in *Rutilus frissi kutum*

Parameters	Total length (cm)	Total weight (g)	Sperm volume (cc)	Sperm count (billion)	Abnormal spermatozoa (%)
Total length (cm)	1				
Total weight (g)	0.933**	1.0			
Sperm volume (cc)	-0.237	0.124	1.0		
Sperm count (billion)	-0.14	0.99	0.941**	1.0	
Abnormal spermatozoa (%)	0.30	-0.96	-0.907**	-0.983**	1

**Significant at 5% level

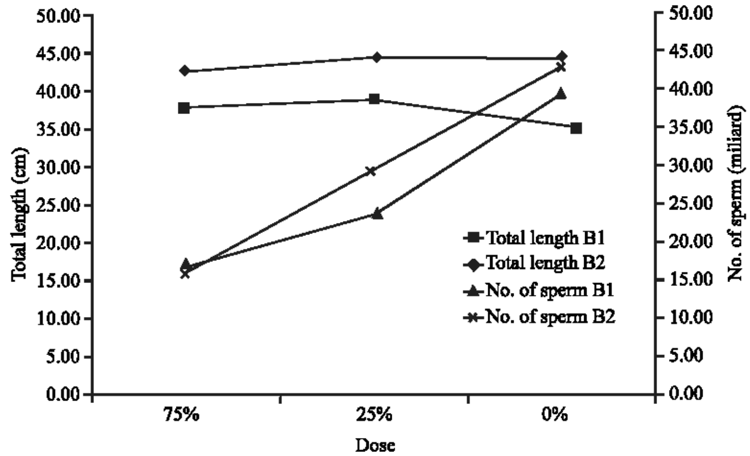

Fig. 1: Variations in total length and sperm count as a function of different doses of butachlor

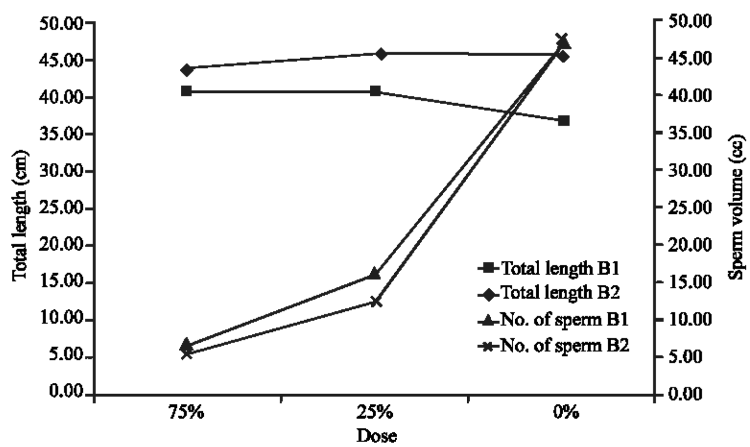

Fig. 2: Variations in total length and sperm volume as a function of different doses of butachlor

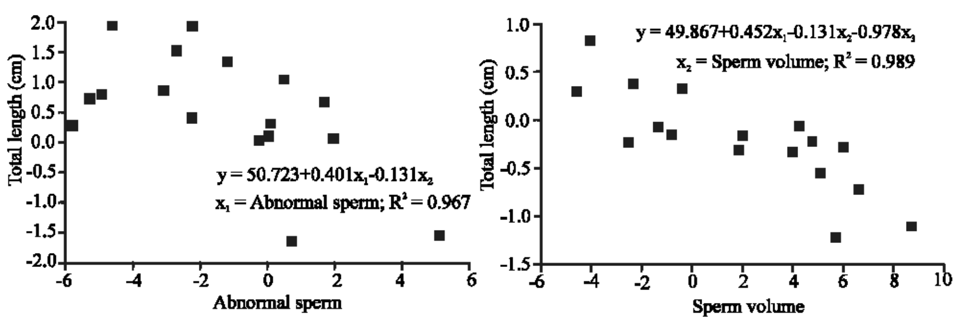

Fig. 3: Regression analysis using dependent and independent variables

Comparisons of correlations between parameters studied indicate linear correlations between total length and total weight at a 1% significance level. No correlations were found between sperm volume and percentage of abnormal spermatozoa, however sperm volume showed linear correlation with sperm count at 1% significance level and negative correlation with percentage of abnormal spermatozoa at 1% significance level. Negative correlations were also found to exist between sperm count and percentage of abnormal spermatozoa (Table 3).

Increase in butachlor concentration severely decreased sperm count (Fig. 1). Increase in butachlor concentrations also resulted in decrease in sperm volume (Fig. 2). Regression analysis using dependent and independent variables were also carried (Fig. 3). Step by step analysis indicate that the parameters studied showed strongest correlations with abnormal spermatozoa and last of all with sperm volume.

DISCUSSION

Understanding the ecology of different species of fishes in an aquatic ecosystem is an important factor in the conservation and rehabilitation of their stocks. Improving natural spawning areas and natural reproduction can be effective in meeting the increasing needs of aquaculture in the world (Moyle, 1991). Results obtained from morphological studies of spermatozoa in *Rutilus frissi kutum* indicate that they a round to oval shaped head with a cylindrical and elongated midpiece and long tail. This conformed to the sperm morphology reported in carps, trouts and pikes (Krasznai *et al*., 1995). Increased percentage in morphologically abnormal spermatozoa strongly affect the fertilization rate in fishes and result in the production of abnormal specimens. Careful handling and proper storage are primary factors in maintaining semen fertility. Cold and/or heat shocks and water pollution decrease stored sperm viability (Vladi *et al*., 2002). Percentage of abnormal sperms in *Rutilus frissi kutum* was reported as 10 % in the Shirroud, Tonekabon and Khoshkroud Rivers (Bahmani *et al*., 2007). After exposure to the herbicide butachlor the percentage of abnormal sperms increased to a maximum of 29.2% (mean = 20.6%) which is a physiological response of fish to stress following exposure. Reduced head, short or degenerated flagellum and/or flagellum coiled around the head were among the various morphological changes observed in spermatozoa. Kortet *et al*. (2002) regarded semen with 5-15% abnormal sperms as good quality semen. Based on the results obtained from the present study semen quality in fishes examined in this study is graded as average. Semen quality in the two year old age class was poorer and this may be related to the increased pollution uptake in testes in this age class. Sperm volume is also influenced by species, number of samples taken, age, weight, race and skill with which semen samples are taken. Azari Takami and Razavi Sayed (1990) reported the minimum and maximum sperm volume in *Rutilus frissi kutum* in the Hevigh River as 2 and 6.2 cc, respectively (mean = 4.2 cc). In another investigation carried out in the Shiroud, Tonekabon and Khoshkrud Rivers, mean sperm volume in this species was reported as 3.87 cc (Bahmani *et al*., 2007). In the present study mean sperm volume in *Rutilus frissi kutum* after exposure to herbicide butachlor decreased to a minimum of 0.55 cc. Mean sperm volume in the control group was recorded as 4.9 cc. The effect of age on semen volume was not significant. Sperm count in *Rutilus frissi kutum* in the Hevigh River ranged from a minimum of 32×10^{-6} to a maximum of 48×10^{-6} spermatozoa (mean = 40×10^{-6} spermatozoa) (Azari Takami, 1990), while that in specimens collected from the Shiroud, Tonekabon and Khoshkrud Rivers was reported as 33.61×10^{-6} spermatozoa (Bahmani, 2007). Toxicity caused by high dose of butachlor resulted in severe decrease in sperm count (16.37×10^{-6} spermatozoa) in this species. This decrease was more pronounced in the two year old age class. Sperm count in the control group was reported as 41.3×10^{-6} spermatozoa. Most of the recent investigations on the effects of pollution on reproduction aim at determining the maximum tolerable concentration (MTC) of the contaminant because they are of the opinion that some of the stages of life can be more sensitive (Esmaily Sari, 2002). Most of these investigations are designed to determine the MTC for different fish species during different stages of growth by focusing on the physiological responses associated with exposure to pollution (Esmaily Sari, 2002). Some pollutants directly affect sexual maturity and levels of sex hormones such as testosterone and ultimately on spermatogenesis. The toxic effects of cadmium have been documented in several shown to cause variations in testosterone levels in blood plasma. This has been documented in minnows (*Phoxinnus phoxinus*) as well as in carp and rainbow trout. Decline in testosterone levels was obvious after 3-4 weeks exposure to PCBs . It has been proved that the herbicide butachlor is similar in structure to estrogen (Esmaily Sari, 2002) and its entry into aquatic ecosystems can result in physiological changes in aquatic organisms. There are different pathways for the entry of natural estrogen into the ecosystem. Some of the major pathways through which estrogens can enter into the ecosystem are industrial effluents from paper and paint production, industrial detergents and agricultural effluents (Johnson *et al*., 2005). Of the estrogen like compounds, 17 α-ethinyl estradiol (EE2) has received considerable attention (Johnson *et al*., 2005). Estrogenic activity is strongly associated with sludges produced during wastewater treatment (Anderson *et al*., 2003). Estrogen and its analogue can strongly inhibit sperm production. Similar results have also been documented in human (Groves and Batten, 1986). Estrogen-like compounds have also been reported to effect gonad tissue (Van Der Van *et al*., 2003) and have suggested a possible link between estrogen-like compounds and reproduction indices such as sperm count, gonad weight and gonadal indices in male fish (Nielson and Baatrup, 2006). Inhibited development of testes and the formation of sertoli cells may be related to exposure to estrogens (Dalgaard *et al*., 2002). Decrease in production of sertoli cells decreases sperm production as a result of estrogen activity (Atanassova, 2005). Stress is one of the adverse effects of pollution in fish which alters hemostasis (Jensen, 1983). Physiological change in the endocrine system is considered a primary response to stress (Nikou *et al*., 2007). Nikou *et al*. (2007) reported a significant decrease in testosterone levels in *Rutilus frissi kutum* from 18.9 mg mL^{-1} at the time of catch to 4.8 mg mL^{-1} one hour after catch subjected to stress during handling and transport. Exposure to herbicide butachlor results in estrogenic effect and also reduced testosterone levels in fish. All fish have both testosterone and estrogen in their blood that are bound by receptors which influence growth (Takahashi, 1975; Mayer, 2004), development and behavior and regulate reproductive cycles and exert a positive feedback on the reproduction axis (McCormick, 2007). But if these hormone levels are perturbed, they exert a negative feedback on the hypothalamus and inhibit the secretion of GnRH (Nagahama, 1983) and spermatogenesis of sertoli cells and thus stop sperm production and cause variations in sperm volume (Nielson and Baatrup, 2006). The effects of estrogen mimicking compounds have been evaluated in rainbow trout (Shultz *et al*., 2003). The results of the present study show that exposure to the herbicide butachlor, which is an estrogen mimicking compound results in a decrease in sperm volume and sperm count and an increase in the percentage of abnormal spermatozoa. These findings are in line with results of Carter (2006) which state that low toxicity influences reproduction and directly affects free gametes such as sperms. Based on the theory of 'sperm competition' males occupying disfavoured mating roles compensate by having larger gonads for their body size (Stockley *et al*., 1997) and by producing sperms with increased sperm velocity, rather than longevity (Kortet *et al*., 2004). The effects of herbicide butachlor in water goes even beyond this because its estrogenic effects results in significant decreases in steroid hormones particularly testosterone during stress (Bayunova, 2002). We may therefore conclude that treatment with lower concentrations may produce sublethal effects which are sometimes difficult to discern but, nevertheless, important from a behavioral or ecological standpoint. Although chemicals may be accumulated in the fish without causing their death, there are many possible chronic effects on the living community. Careful evaluation of chemical use cannot therefore be overemphasized. Efforts should be taken to regulate pesticide use in order to prevent their entry into rivers at least during the migratory season of anadromous fish.

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