Iranian Journal of Fisheries Sciences 4(2) 81-100 (2005)

# Using Computer Assisted Sperm Analysis (CASA) to Monitoring the Effects of Zinc and Cadmium Pollution on Fish Sperm

**M. Ebrahimi**

**Email:** ebrahimi@qom.ac.ir

Environmental Hormones Pollutants Research Center, University of Qom,

P.O.Box: 37185-438 Qom, Iran

**Abstract:** Since the quality of sperm is affected by water industrial or agriculture pollution, sperm motility was measured as a sensitive and accurate bioindicator of water quality. In this study, sperm motility was measured by a computer assisted sperm analysis after 24 hours exposure of sperm to different concentration of heavy metals) Cadmium and Zinc). The results show that the sperm motility was not related to the duration of exposure but to the metals concentration ration. The concentration of 1000ppm zinc or 2000ppm Cadmium could have adverse effect on sperm motility.

**Keywords:** Sperm motility, CASA, Heavy metals, Pollution

## Introduction

Since the quality of sperm is affected by water pollution where fish lives, measurement of sperm motility could provide a sensitive and accurate bioindicator of water quality. In previous studies, using copper, methylmercury, mercuric chloride and acid water, either fertilization rate or a subjective assessment of motility have been used to measure the effects of pollutants on sperm quality (Ramachandran *et al.*, 1997; Lawrence *et al.*, 1998; Rurangwa *et al.*, 1998; Au *et al.*, 2001 and Rurangwa *et al.*, 2001). Such studies, however, require a rapid and quantitative assessment of sperm motility. Spermatozoal motility is one of the most

widely used criteria to assess semen quality and viability (Alabi *et al.*, 1985; Au *et al.*, 2000 and Telisman *et al.*, 2000), and it has been also suggested that the motility of cryopreserved sperm might be used as a sensitive and readily available species-specific bioindicator of pollution (Kime *et al.*, 1996; Rurangwa *et al.*, 2001 and Jobling *et al.*, 2002).

Computer assisted sperm analysis (CASA) involves the electronic tracking of spermatozoa using sophisticated image analysis hardware and software (Ellington., 1993; Stachecki *et al.*, 1993; Toth *et al.*, 1995; Moore & Akhondi, 1996 and Kime *et al.*, 2001). A typical CASA system includes a video camera, a microscope equipped with phase optics and a warming stage, a microcomputer with hardware and software dedicated to motion analysis, a monitor and a printer. The image of the sperm cells under the microscope is transformed into discrete pixels producing a voltage proportional to the intensity of the light as it strikes the camera's CCD array (Auger, 1995; Au *et al.*, 2000). In general CASA provides a more reproducible (less variable), repeatable and accurate analysis than the manual microscopic method for assessing sperm count and motility (Stachecki *et al.*, 1993; Toth *et al.*, 1995; Kime *et al.*, 2001; Rurangwa *et al.*, 2001 and Xu *et al.*, 2001). This method has used in humans (Slott *et al.*, 1993; Clements *et al.*, 1995; Farrell *et al.*, 1995 and Stachecki *et al.*, 1995), rats (Moore & Akhondi, 1996 and Xu *et al.*, 2001), rabbits (Farrell *et al.*, 1995), bulls (Farrell *et al.*, 1995), dogs (Ellington *et al.*, 1993), cats (Stachecki *et al.*, 1993, Moore & Akhondi, 1996 and Rurangwa *et al.*, 2001), hamsters (Brandeis, 1993), zebra mussels (*Dreissena polymorpha*) (Mojares *et al.*, 1995) and fish (Toth *et al.*, 1995; Kime *et al.*, 2001 and Rurangwa *et al.*, 2001).

The development of CASA as a rapid and quantitative means of measurement of sperm activity in fish exposed to heavy metal pollutions was made possible by Hobson sperm tracker. Unlike mammalian sperm, that of teleost fish attains motility only after dilution in water or a hypotonic solution (Billard & Cosson, 1992; Perchec *et al.*, 1993; Perchec *et al.*, 1995 and Rurangwa *et al.*, 2001) and remains motile for only a few minutes in trout (Cosson *et al.*, 1989; Billard & Cosson, 1992 and Perchec *et al.*, 1995), carp and turbot, since the ATP content

declines rapidly (Billard & Cosson, 1992; Billard *et al.*, 1992and Perchec *et al.*, 1995). Fish sperm may, however, be kept immotile for 24 hours after partial dilution in an extender solution for fertilisation and will then attain motility after further dilution with water. To mimic sublethal effects of pollutants on the reproductive system, sperm could therefore, be incubated for 24 hours with pollutants in the extender (analogous to intratesticular exposure) or exposed to heavy metals only during final dilution on the microscope stage (analogous to direct aquatic pollution).

## Material and Methods

### Milt collection, storage and exposure

Eighteen male trout, (1057±310 (SD)g; GSI 1.2-3.4), were purchased from a local trout farm and kept in 1000 lit tanks of circulating freshwater at 10°C. Milt was collected by gently stripping the abdominal body walls. Milt samples from the testes were diluted 1:100 with extender (NaCl, 5.52 g/l; KCl 2 g/l, tris-HCl 2.42 g/l; glycine 3.75 g/l in distilled water, pH 7.5) at 4°C. Just before recording 1µl prediluted milt in extender was placed on the microscope slide and diluted with distilled water (19 µl: to give a final dilution of 1:2000) at room temperature. To prevent adhesion of sperm, slides were precoated by dipping in 1% polyvinyl alcohol solution (Average M.W. 30,000-70,000; Sigma) and dried at 60°C (Perchec *et al.*, 1995). The water was spread as tiny as possible on the microscope slide to limit vertical movement of the sperm. Plain slides rather those with a depression were used for the same reason.

### Experiment 1: Assessment of the effect of ageing during incubation

Milt from six mature males were stored in extender at 4°C for 0, 24 or 48 hours. It was subjected to the two step dilution technique of Billard and Cosson (1992) as described above and video recordings were taken immediately.

### Experiment 2: Incubation of sperm with zinc and cadmium

Milt from seven individual fish was diluted in the extender. Milt from the experimental groups was also diluted in the extender except that some of the NaCl was replaced by an equivalent molar amount of $ZnCl_2$, $CdCl_2$ or $ZnCl_2 + CdCl_2$ to

give zinc and cadmium concentrations of 1, 10, 50, 100, 200, 400, 500, 1000 and 2000 ppm (0.015-31 mmol/l zinc;0.009-17.9 mmol/l cadmium). Milt was incubated for 24 hours at 4°C in extender with or without pollutant before video recording.

**Experiment 3: Exposure of fresh sperm to zinc and cadmium**

Milt from six other fish was diluted first with extender (1:49) and stored at 4°C for up to 3 hours during a video recording session. Just prior to the video recording it was diluted further (1:1) in an extender containing double concentration of zinc, cadmium and zinc + cadmium to give final zinc and cadmium concentrations of 1, 10, 50, 100, 200, 400, 500, 1000 and 2000ppm. As described above, it was then activated by further dilution in distilled water (1:19) on the microscope slide.

**Computer assisted sperm analysis (CASA)**

Sperm movement was recorded for 2 minutes from the moment of final dilution using a CCD camera (ICD-290, Ikegami) attached to a BX 50 Olympus microscope with a phase-contrast objective lens (40 x 0.40) via an Olympus U-PMTVC adapter. Videotapes were analyzed using a Hobson sperm tracker (Hobson Tracking Systems Ltd., Sheffield, U. K.), with instrument parameters optimized to track catfish sperm (i.e. tracker picked up all sperm and a continues track line appeared), from 20 seconds after the mixing point (to allow for focusing and stabilization of water movement) for four successive 15 second periods. The parameters assessed were: VCL-curvilinear velocity (μm/sec), the sum of the incremental distances moved in each frame along the sampled path divided by the total time of the track; VSL - straight line velocity (μm/sec), the straight line distance between the start and end points of the track divided by the time of the track; VAP - angular path velocity (μm/sec), a derived path based on an average number of points and divided by the time of the track; % motility - % of motile sperm; ARE - area morphometry ($\mu^2$), the size of the sperm head obtained from the diameter averages per track in linear morphometry: LIN - the linearity (%)-the ratio of net distance moved to total path distance; MAD-mean angular displacement (°), average change in direction of the sperm head from frame to frame; MOC - motile concentration (millions/ml); STR-straightness (%), the ratio of net distance moved

to total path distance;ALH-amplitude of lateral displacement of the sperm head (μ) - the average deviation from the smoothed path, based on difference in linearity between the smoothed and real paths; BCF - beat cross frequency turning points of the sperm head (Hz), cycles per second of the sperm head across the mean path; and LIM - linear morphometry (μ), the diameter of each sperm head measured in two perpendicular directions in every frame. All parameters (except % motile sperm which was 100% due to technical problems) showed similar patterns (see 24h sperm storage figures) and hence only VAP, VCL and VSL are presented here. The optimal settings for the Hobson Sperm Tracker were obtained according to the manufacturer's instructions by analyzing the same video-recording of sperm motility. The settings for the image analysis at x 20 objective magnification were as follows: search radius = 8.50 μm; predict = off; video = pal; aspect = 1.49; refresh time = 1 second; threshold = +10 / -50; filter weightings 1= 2, 2 = 3, 3 = 3, 4 = 3 and immotile process = normal. Image capture rate of the Hobson Tracker was set up to 25 frames/second. Further details and validation by comparison with manual tracking is given in Moore and Akhondi (1996).

### Statistics

The data was analyzed using the paired-sample t-test for comparison between each concentration and its own group control (0ppm) and between each concentration of zinc + cadmium treated groups with the same concentration of the zinc or cadmium only treated group. For ageing studies the mean values of sperm motility were compared by an independent t-test with zero hour control. SPSS 6 for Windows software (SPSS Inc., 444 N. Michigan Avenue, Chicago, Illinois 60611, USA) was used for the analysis.

## Results

### The influence of storage at 4 °C in extender on sperm motility

Mean values of sperm velocities from six fish after 24 and 48 hours storage were compared with the velocities of the no-storage control group. None of the motility parameters were affected by 24 hours storage, but after 48 hours storage there was a significant ($P< 0.05$) decrease in velocity of all parameters (Fig.1).

**24 hour incubation of sperm with zinc and cadmium:**

Sperm movement decreased from the start of the recording and little movement was visible by 2 minutes post-dilution. Zinc had a significant effect on motility parameters only at 2000ppm ($P<0.0001$). Cadmium, however, decreased sperm motility even at 100ppm ($P<0.0001$) with almost complete cessation of movement at 200ppm and above. In the presence of zinc, cadmium had significantly less effect on motility and at 100ppm and above there was significantly more movement ($P<0.0001$) than with cadmium alone. At 100 and 200ppm zinc + cadmium, movement did not differ from control and zinc treated sperm, and was significantly greater than those with concentrations of cadmium alone. A similar effect was found for all parameters of sperm motility tested. Data for VAP, VCL and VSL are presented through figures 2 to 4.

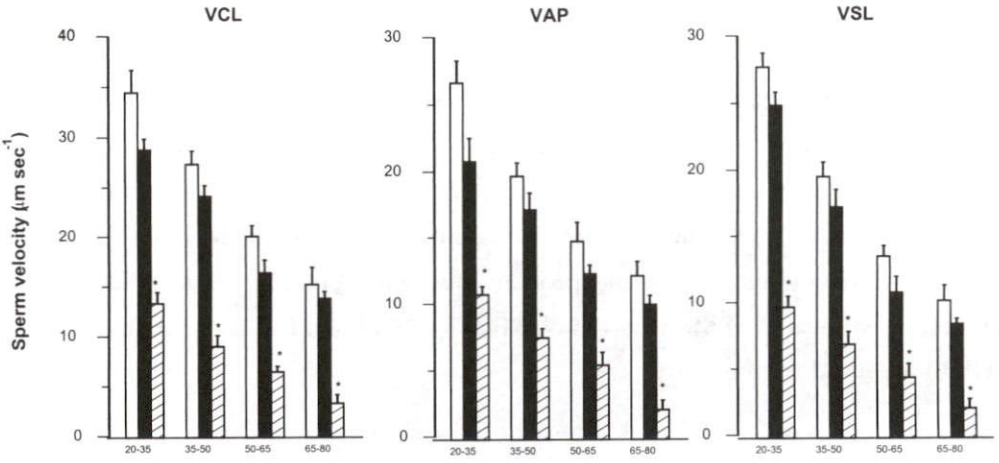


**Figure 1:** The effect of storage of sperm for 0, 24 and 48h on average path velocity (VAP), curvilinear velocity (VCL) and straight line velocity (VSL). Recordings were made 20-35, 35-50, 50-65 and 65-80 sec. after dilution. ☐ fresh sperm, ■ sperm stored 24h, ▨ sperm stored 48h. Vertical lines indicate SEMs. *indicates a significant difference ($P<0.05$) from fresh sperm.

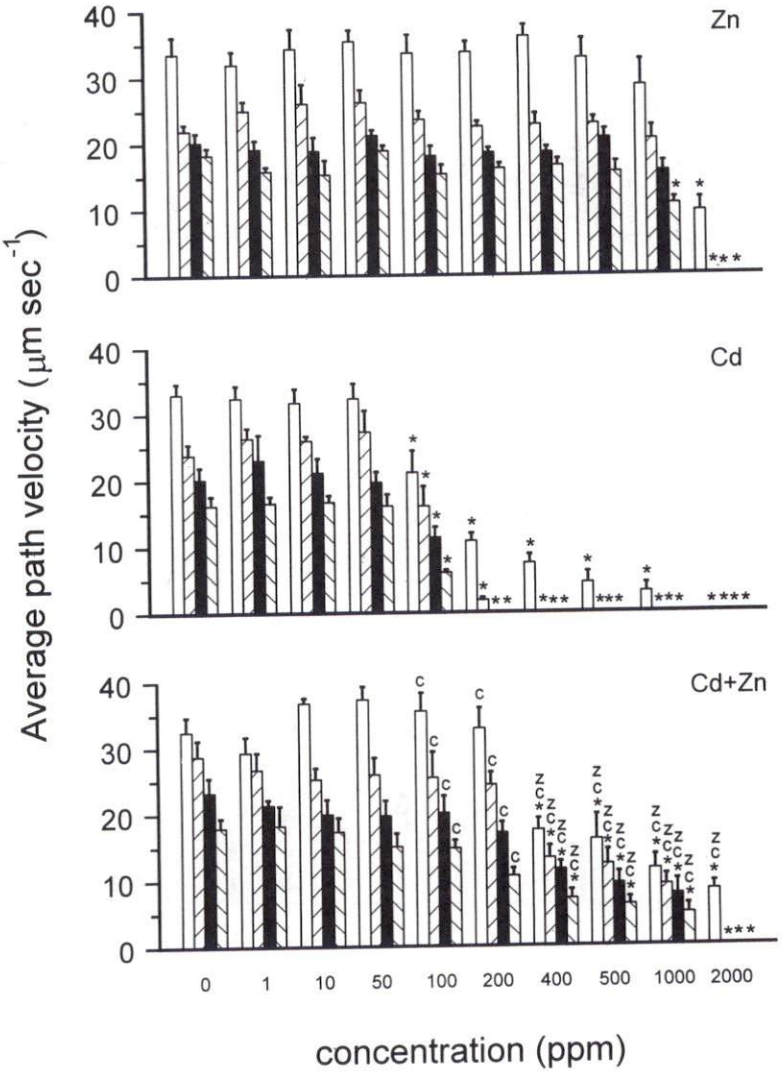


**Figure 2:** The effect of 24h exposure of catfish sperm to zinc, cadmium and zinc+cadmium on average path velocity (VAP, μm $s^{-1}$) at 20-35 □ , 35-50 ■ 50-65 ▨, 65-80 ▧ seconds. After final dilution, vertical lines indicate SEMs. *=significantly different from 0ppm; c=significantly different from Cd alone (P<0.0001); z= significantly different from Zn alone (P<0.05).

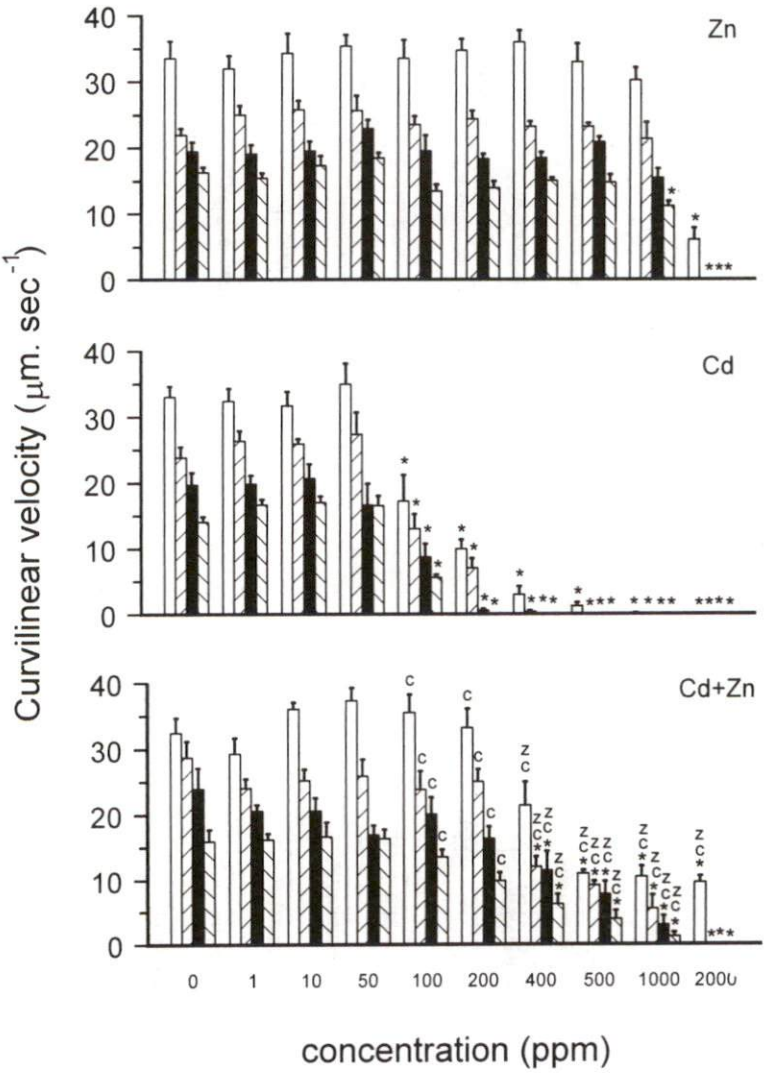


**Figure 3**: The effect of 24h exposure of catfish sperm to zinc, cadmium and zinc+cadmium on curvilinear velocity (VCL, μm $s^{-1}$) at 20-35 □, 35-50 ■, 50-65 ▨ and ▧ 65-80 seconds. After final dilution, vertical lines indicate SEMs. *=significantly different from 0ppm ($P<0.0001$); c = significantly different from Cd alone ($P<0.0001$); z = significantly different from Zn alone ($P<0.05$).

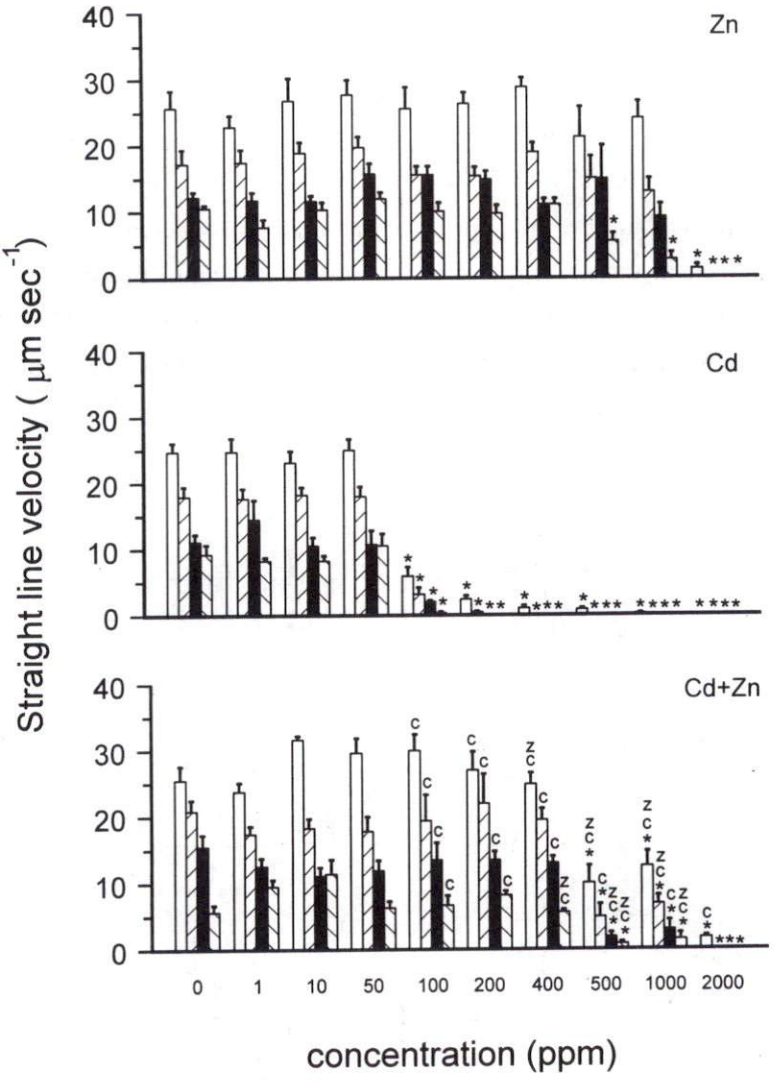


**Figure 4:** The effect of 24 h exposure of catfish sperm to zinc, cadmium and zinc+ cadmium on straight line velocity (VSL, µm $s^{-1}$) at 20-35 □ , 35-50 ■, 50-65 ▨ and 65-80 ▧ seconds. After final dilution. vertical lines indicate SEMs. *=significantly different from 0 ppm (P<0.0001); c=significantly different from Cd alone (P<0.0001); z= significantly different from Zn alone (P<0.05).

## Exposure of fresh sperm to zinc and cadmium

The results of exposure to the pollutant on the microscope stage were much less pronounced than for the 24h incubation period, but the effects were qualitatively similar. Sperm velocity was decreased only by concentration of 2000ppm of zinc ($P<0.002$), or 1000ppm of cadmium. As with the longer exposure periods of the incubations, the toxicity of cadmium was decreased in the presence of zinc. In fact, in the presence of concentration 2000ppm of zinc, 2000ppm of cadmium had no significant effect on motility parameters. Results for VAP, VCL and VSL are presented through figures 5 to 7. Similar effects were found for all other parameters of movement measured.

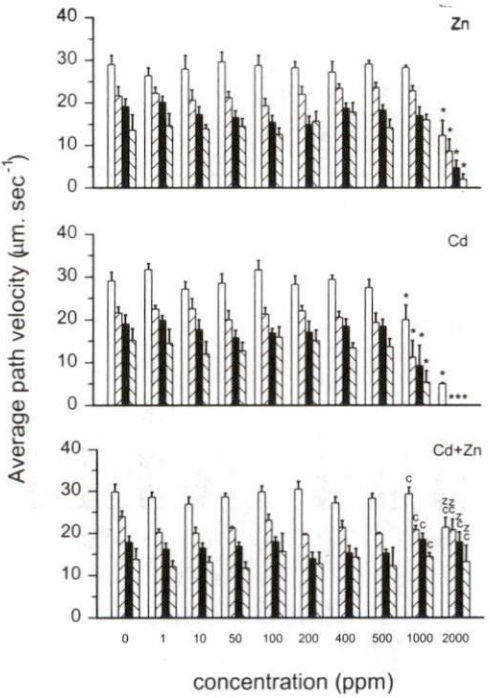


**Figure 5:** The effect of addition of zinc, cadmium and zinc+cadmium to fresh catfish sperm at final dilution on average path velocity (VAP, $\mu m\ s^{-1}$) at 20-35 □, 35-50 ■, 50-65 ▨ and 65-80 ▧ seconds. Vertical lines indicate SEMs. *=significantly different from 0 ppm ($P<0.002$); c=significantly different from Cd alone ($P<0.002$); z=significantly different from Zn alone ($P<0.05$).

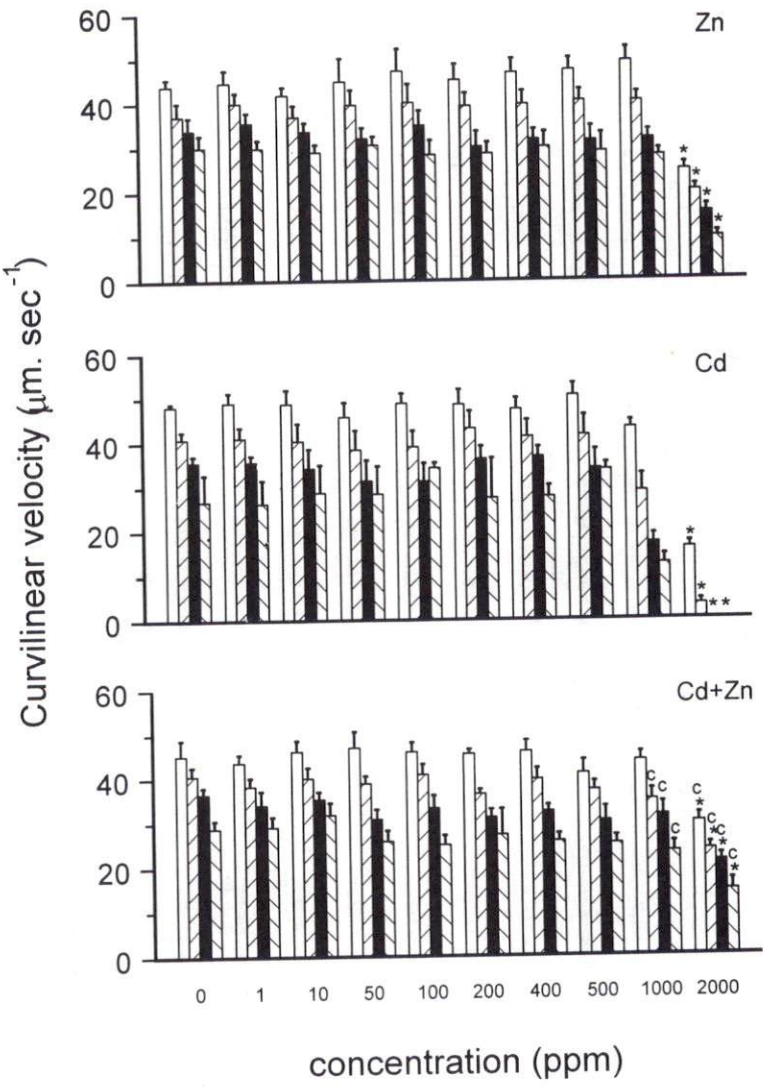


**Figure 6:** The effect of addition of zinc, cadmium and zinc+cadmium to fresh catfish sperm at final dilution on curvilinear velocity (VCL, µm $s^{-1}$) at 20-35 □ , 35-50 ■ , 50-65 ▨ and 65-80 ▧ seconds. Vertical lines indicate SEMs. *=significantly different from 0ppm ($P<0.002$); c=significantly different from Cd alone ($P<0.002$); z= significantly different from Zn alone ($P<0.05$).

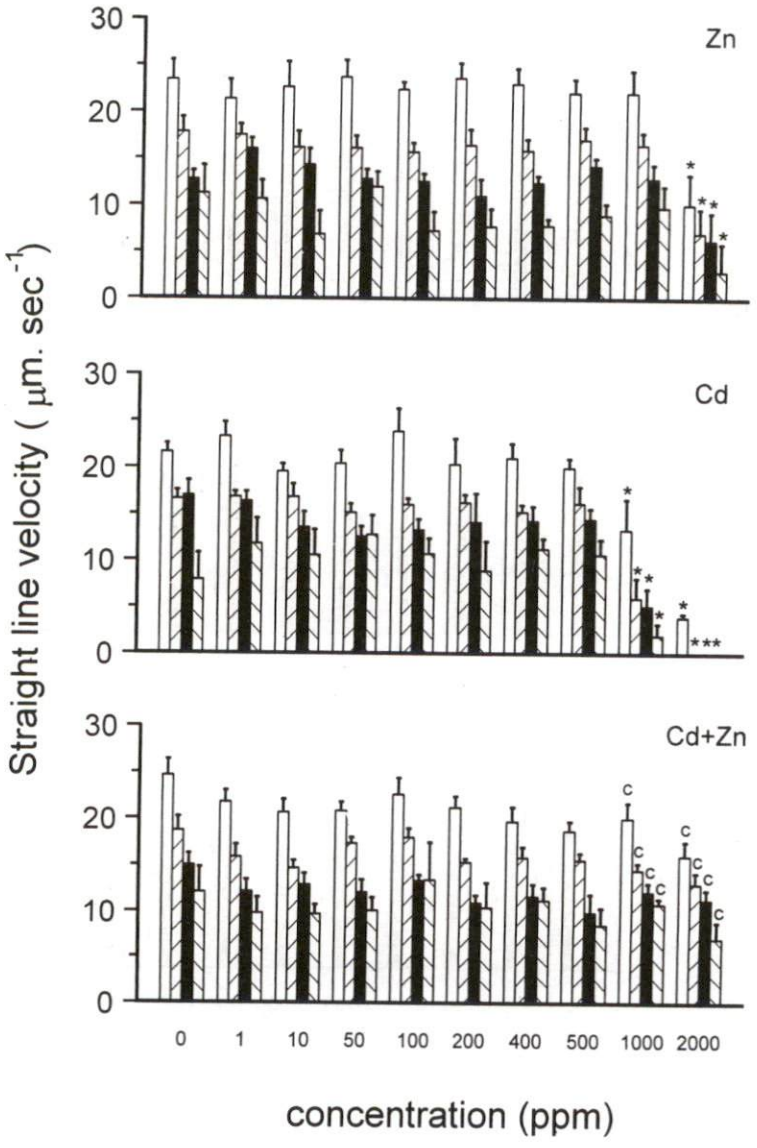


**Figure 7:** The effect of addition of zinc, cadmium and zinc+cadmium to catfish sperm at final dilution on straight line velocity (VSL, µm $s^{-1}$) at 20-35 □, 35-50 ■, 50-65 ▨ and 65-80 ▧ seconds. Vertical lines indicate SEMs. *=significantly different from 0 ppm ($P<0.002$); c =significantly different from Cd alone ($P<0.002$); z = significantly different from Zn alone ($P<0.05$).

## Discussion

A major problem encountered in previous studies (see Introduction) in which the effects of pollutants on the motility of fish sperm were examined, was the very short duration of movement. Unlike mammals in which sperm is motile for some hours after ejaculation, fish sperm ceases all movement after 1-2 minutes (Cosson *et al.*, 1989; Billard & Cosson, 1992; Perchec *et al.*, 1995). Fish sperm is immotile in the testes and sperm ducts, and motility is attained only after dilution in water in which osmolarity and ionic concentrations are correct for the particular species (Billard & Cosson, 1992). With the short duration of motility, it is essential that dilution is rapid and that observations begin at a consistent time after dilution. Billard & Cosson (1992) have described a two step dilution process, whereby sperm is initially diluted in an extender, in which it remains immotile, while the final dilution takes place on the microscope slide itself, thereby allowing consistency of measurements and observations. As a result of the very short duration of motility after dilution, sperm can be exposed directly to a pollutant for a maximum time of only 2 minutes. The use of an extender, in which sperm can be held for up to 24h, also permits the study of pollutant effects over a longer period, analogous to the exposure received during storage in the testes and its ducts. The use of video recording, rather than the more subjective direct operator observation, previously used, allows a permanent record to be made for later computer analysis. Such methodology is readily useable in both field and laboratory based studies since the recording equipment is portable and relatively inexpensive, while the relatively expensive interpretative hardware and software may be held in a central shared facility. The Hobson sperm tracker has the advantage, over other computer assisted trackers, in that it can follow more than 400 individual sperm simultaneously on video tape running at normal speed and is far more rapid in use than other trackers which follow single sperm frame by frame. Unfortunately the current computer programming, which was designed for mammalian use, does not permit analysis until drift resulting from the dilution step has completely ceased. For this reason analyses began 20 seconds after dilution, and for these initial studies collected data for four consecutive 15 sec intervals. The Hobson tracker produces a

large number of different parameters of motility (see Materials and Methods), but on advice from the manufacturer only three (VAP, VSL and VCL) parameters are reported since essentially similar results were obtained for all parameters. For routine use in studies of pollutant effects on sperm motility, the method could be simplified to analyze a single parameter for a 15 seconds interval (the minimum possible on the Hobson tracker) at 20 and 60 seconds after dilution.

The results show that computer assisted sperm analysis may provide a valuable tool to determinate the effect of pollutants on sperm motility and hence on fertilization rate and production of viable larvae. Use of a 24h incubation period, which does not decrease motility compared to fresh sperm, increases the sensitivity of the bioassay and mimics effects likely to occur due to long term incorporation of pollutants into the reproductive system. It is probable that the effects of exposure of sperm for a prolonged period during maturation could be even more pronounced. While the concentrations required to affect sperm motility in the aquatic environment during the first minute after milt ejection may be so high as to be lethal to the fish, bioconcentration of pollutants occurs particularly in the hepatic and gonadal tissue where levels may attain several orders of magnitude higher than those in the surrounding water. *Lepomis macrochirus*, for example, exposed to 0.0084ppm cadmium in the holding water had hepatic and gonadal concentrations of 0.52 and 9.5ppm respectively after 28 days (Cope *et al.*, 1994) while *Garra rufa* and *Cyprinion macrostomus* caught from water containing 0.14ppm zinc had muscle concentrations of 66 and 47ppm and hepatic concentrations of 483 and 607ppm respectively (Gumgun *et al.*, 1994). The cadmium and zinc concentrations (up to 53ppm and 900ppm respectively) found in gonads of cyprinids captured in industrial waters in England (Badsha & Goldspink, 1982) are comparable to the concentrations of these metals which resulted in decreased sperm motility after 24h exposure. The effects demonstrated suggest that fresh or cryopreserved sperm may provide a useful tool as a convenient biomonitor of pollution. The methodology described could be readily adapted for use as a standard toxicity test to assess the potential hazards of environmental pollutants on reproduction using fresh, or possibly cryopreserved, sperm from a defined species and strain of fish. This has

the advantage over many current such tests in that the test organism is a vertebrate, and therefore more relevant to human application, than the more commonly used invertebrates, is much more rapid than whole animal exposure, and if milt can be obtained without sacrifice (as in salmonids) obviates the use of animals for testing. It also has the added advantage that sperm may be used from different species for interspecific comparisons and direct comparisons made of the toxicities of different pollutants. Extrapolation of the results to the whole animal depend on the rate of incorporation into target tissues, exposure times and detoxification rates. Sea urchin sperm has previously been used as a standard toxicity test for marine waters (Dinnel *et al.*, 1989; Volpi_Ghirardini & Arizzi_Novelli, 2001), but it is difficult to relate the toxicities derived to individual species of fish. This assay, in addition to the quantitative aspects of CASA is amenable to use in species of particular interest in any location. It is not, however, clear how decreased sperm motility may relate to decreased fertility. Investigations in a number of mammalian species have demonstrated that indices of sperm motility (e.g. progressive velocity, lateral head displacement) are related to fertilizing capacity *in vivo* and *in vitro* (Holt *et al.*, 1994; Schulz *et al.*, 1994). Moreover, Moore and Akhondi (1996) have shown that the fertilizing capacity of rat spermatozoa is correlated with the decline in straight line velocity.

There has been no previous systematic comparison of the toxicities of zinc and cadmium on fish reproduction. Reproductive malfunction has been reported after exposure to water containing from 0.001 to 225ppm cadmium or 0.2 to 11ppm for zinc (Kime, 1995), for a wide range of species and exposure times. At a bioconcentration rate of 1000 fold for the gonads, as suggested by the studies of Cope *et al.* (1994) and Gümgüm *et al.* (1994), these values are broadly in agreement with those found in the present study. For carnivorous species, bioaccumulation from dietary sources may be even higher.

While the main aim was to use zinc and cadmium as test substances to assess CASA, the results provide some interesting new evidence that the toxicity of cadmium is decreased in the presence of zinc. Zinc and cadmium have similar chemical properties and may compete for binding sites on proteins or on

metallothioneins (Saxena *et al.*, 1989; Verbost *et al.*, 1989; King *et al.*, 1998). Zinc is an essential mineral and deficiency may cause reproductive malsfunction in mammals (Mills, 1988; Leno *et al.*, 1996). It is therefore possible that at least, some of the toxic effects of cadmium are due to displacement of essential zinc, a process which is reversed on addition of further zinc. A similar cause has been suggested for the cadmium inhibition of estradiol stimulated vitellogenesis in trout (Olsson *et al.*, 1989, Olsson *et al.*, 1995). Although zinc had a protective effect against cadmium in this case it had to be administered several days earlier to induce metallothionein synthesis. The decreased sperm counts and motility found when drinking water containing cadmium (50ppm) or cadmium + lead (25ppm) was administered to rats for 120 days was not found when zinc (50ppm) was administered simultaneously (Saxena *et al.*, 1989). The length of the treatment again suggests that the protection may be a result of induction of metallothionein synthesis. The rapidity of the protective effect in the present studies, which was noticeable after only 1 minute, precludes *de novo* metallothionein synthesis and suggests direct competition for existing protein binding sites. These results also demonstrate the importance of not considering the effects of individual pollutants in isolation since the effects of the cocktails of chemicals found in polluted environments may not simply be the sum of individual effects.

## Reference

Alabi, N.S. ; Whanger, P.D. and Wu, ASH , 1985. Interactive effects of organic and inorganic selenium with cadmium and mercury on spermatozoal oxygen consumption and motility in vitro. Biology Of Reproduction, **33(4):**911-919.

Au, D.W. ; Chiang, M.W. and Wu, R.S. , 2000. Effects of cadmium and phenol on motility and ultrastructure of sea urchin and mussel spermatozoa. Archives of Environmental Contamination and Toxicology, **38(4):**455-463.

Au, D.W. ; Lee, C.Y. ; Chan, K.L. and Wu, R.S. , 2001. Reproductive impairment of sea urchins upon chronic exposure to cadmium. Part I: Effects on gamete quality. **111(1):**1-9.

Auger, J. , 1995. Rappel Technologiques, Biais Method-ologiques Possibles Et comditions de Mesure Technology, methodological bias and guidelines for standard procedures. Andrologie, **5(1):**37-45.

Badsha, K.S. and Goldspink, C.R. , 1982. Preliminary observations on the heavy metal content of four species of freshwater fish in NW England. Journal of Fish Biology, **21:**251-267.

Billard, R. and Cosson, M.P. , 1992. Some problems related to the assessment of sperm motility in freshwater fish. Journal of Experimental Zoology, **261(2):** 122-131.

Billard, R. ; Weil, C. ; Bieniarz, K. ; Mikolajczyk, T. ; Breton, B. ; Epler, P. and Bougoussa, M. , 1992. Testicular and some hormonal changes during the first four years of life in the mirror carp, *Cyprinus carpio* L. Journal of Fish Biology, **41(3):**473-487.

Brandeis, V.T. , 1993. Importance of total motile oval count in interpreting the hamster ovum sperm penetration assay. Journal of Andrology, **14(1):**53-59.

Clements, S. ; Cooke, I.D. and Barratt, C.L.R. , 1995. Implementing comprehensive quality control in the andrology laboratory. Human Reproduction, **10(8):** 2096-2106.

Cope, W.G. ; Wiener, J.G. and Atchison, G.J. , 1994. Hepatic cadmium, metal-binding proteins and bioaccumulation in bluegills exposed to aqueous cadmium. Environmental Toxicology and Chemistry, **13(4):**553-562.

Cosson, M.P. ; Billard, R. and Letellier, L. , 1989. Rise of internal calcium accompanies the initiation of trout sperm motility. Cell Motility and the Cytoskeleton, **14(3):**424-434.

Dinnel, P.A. ; Link, J.M. ; Stober, Q.J. ; Letourneau, M.W. and Roberts, W.E. , 1989. Comparative sensitivity of sea urchin sperm bioassay to metals and pesticides. Archive of environmental contamination and toxicology, **18:**748-755.

Ellington, J. ; Scarlett, J. ; Meyers Wallen, V. ; Mohammed, H.O. and Surman, V. , 1993. Computer-assisted sperm analysis of canine spermatozoa motility measurements. Theriogenology, **40(4):**725-733.

Farrell, P. ; Trouern Trend, V. ; Foote, R.H. and Douglas Hamilton, D. , 1995. Repeatability of measurements on human, rabbit, and bull sperm by computer-assisted sperm analysis when comparing individual fields and means of 12 fields. Fertility and Sterility, **64(1):**208-210.

Gumgun, B. ; Tez, U.Z. and Gulsun, Z. , 1994. Heavy metal pollution in water, sediment and fish from the Tigris river in Turkey. Chemosphere, **29:**111-116.

Holt, W. ; Watson, P. ; Curry, M. and Holt, C. , 1994. Reproducibility of computer-aided semen analysis: Comparison of five different systems used in a practical workshop. Fertility and Sterility, **62(6 I):**1277-1282.

Jobling, S. ; Coey, S. ; Whitmore, J.G. ; Kime, D.E. ; Van_Look, K.J. ; McAllister, B.G. ; Beresford, N. ; Henshaw, A.C. ; Brighty, G. ; Tyler, C.R. and Sumpter, J.P. , 2002. Wild intersex roach (Rutilus rutilus) have reduced fertility. Biology of Reproduction, **67(2):**515-524.

Kime, D.E. ; Ebrahimi, M. ; Nysten, K. ; Roelants, I. ; Moore, H.D.M. and Ollevier, F. , 1996. Use of computer assisted sperm analysis (CASA) for monitoring the effects of pollution on sperm quality of fish; application to effects of heavy metals. Aquatic toxicology, In press.

Kime, D.E. ; Van_Look, K.J. ; McAllister, B.G. ; Huyskens, G. ; Rurangwa, E. and Ollevier, F. , 2001. Computer-assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. **130(4):**425-433.

King, L.M. ; Anderson, M.B. ; Sikka, S.C. and George, W.J. , 1998. Murine strain differences and the effects of zinc on cadmium concentrations in tissues after acute cadmium exposure. Archives of Toxicology, **72(10):**650-655.

Lawrence, Y. ; Ozil, J.P. and Swann, K. , 1998. The effects of a Ca2+ chelator and heavy-metal-ion chelators upon Ca2+ oscillations and activation at fertilization in mouse eggs suggest a role for repetitive Ca2+ increases. The Biochemical Journal, **335 (Pt 2):**335-42.

Leno, G.H. ; Mills, A.D. ; Philpott, A. and Laskey, R.A. , 1996. Zyper-phosphorylation of nucleoplasmin facilitates Xenopus sperm decondensation at fertilization. J. Biol. Chem., **271(13):**7253-7256.

Mills, C.F. , 1988. Zinc in human biology. Berlin, Springer. 307.

Mojares, J.J. ; Stachecki, J.J. ; Kyozuka, K. ; Armant, D.R. and Ram, J.L. , 1995. Characterization of zebra mussel (*Dreissena polymorpha*) sperm morphology and their motility prior to and after spawning. Journal of Experimental Zoology, **273(3):**257-263.

Moore, H.D.M. and Akhondi, M.A. , 1996. Fertilizing capacity of rat spermatozoa is correlated with decline in straight-line velocity measured by continuous computer-aided sperm analysis: Epididymal rat spermatozoa from proximal cauda have a greater fertilizing capacity *in vitro* than those from the distal cauda or vas deferens. Journal of Androalogy, **17(1):**50-60.

Olsson, P.E. ; Kling, P. ; Petterson, C. and Silversand, C. , 1995. Interaction of cadmium and oestradiol-17-beta on metallothionein and vitellogenin synthesis in rainbow trout (Oncorhynchus mykiss). Biochemical Journal, **307(1):**197-203.

Olsson, P.E. ; Zafarullah, M. and Gedamu, L. , 1989. A role of metallothionein in zinc regulation after estradiol induction of vitellogenin synthesis in rainbow trout, Salmo gairdneri. Biochemical Journal, **257(2):**555-560.

Perchec, G. ; Chauvaud, L. ; Suquet, M. ; Cosson, J. ; Andre, F. and Billard, R. , 1993. Changes in the movement characteristics and ATP content in the sperm of carp and turbot (teleost fishes). Comptes Rendus de l'Academie d'Agriculture de France, **79(6):**117-126.

Perchec, G. ; Jeulin, C. ; Cosson, J. ; Andre, F. and Billard, R. , 1995. Relationship between sperm ATP content and motility of carp spermatozoa. Journal of Cell Science, **108(2):**747-753.

Ramachandran, S. ; Patel, T.R. and Colbo, M.H. , 1997. Effect of copper and cadmium on three Malaysian tropical estuarine invertebrate larvae. Ecotoxicology and Environmental Safety, **36(2):**183-188.

Rurangwa, E. ; Roelants, I. ; Huyskens, G. ; Ebrahimi, M. ; Kime, D.E. and Ollevier, F. , 1998. The minimum acceptable spermatozoa to egg ratio for artificial insemination and the effects of heavy metal pollutants on sperm motility and fertilization ability in the African catfish (*Clarias gariepinus*, Burchell, 1822). J. Fish Biol., **53:**402-413.

Rurangwa, E. ; Volckaert, F.A. ; Huyskens, G. ; Kime, D.E. and Ollevier, F. , 2001. Quality control of refrigerated and cryopreserved semen using computer-assisted sperm analysis (CASA), viable staining and standardized fertilization in African catfish (*Clarias gariepinus*). Theriogenology, **55(3):**751-769.

Saxena, D.K. ; Murthy, R.C. ; Singh, C. and Chandra, S.V. , 1989. Zinc protects testicular injury induced by concurrent exposure to cadmium and lead in rats. Research Communications in Chemical Pathology And Pharmacology, **64(2):**317-330.

Schulz, R.W. ; Van Der Corput, L. ; Janssen Dommerholt, J. and Goos, H.J.T. , 1994. Sexual steroids during puberty in male African catfish (Clarias gariepinus): Serum levels and gonadotropin-stimulated testicular secretion in vitro. Journal of Comparative Physiology B Biochemical Systemic and Environmental Physiology, **164(3):**195-205.

Slott, V.L. ; Suarez, J.D. ; Poss, P.M. ; Linder, R.E. ; Strader, L.F. and Perreault, S.D. , 1993. Optimization of the Hamilton-Thorn computerized sperm motility analysis system for use with rat spermatozoa in toxicological studies. Fundamental and Applied Toxicology, **21(3):** 298-307.

Stachecki, J.J. ; Dresser, B.L. ; Pope, C.E. and Armant, D.R. , 1995. Stimulation of ejaculated domestic cat sperm motility with caffeine, pentoxifylline, and 2'-deoxyadenosine. Archives of Andrology, **34(2):**63-68.

Stachecki, J.J. ; Ginsburg, K.A. ; Leach, R.E. and Armant, D.R. , 1993. Computer-assisted semen analysis (CASA) of epididymal sperm from the domestic cat. Journal of Andrology, **14(1):**60-65.

Telisman, S. ; Cvitkovi , P. ; Jurasovi , J. ; Pizent, A. ; Gavella, M. and Roci, B. , 2000. Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men. Environmental Health Perspectives, **108(1):**45-53.

Toth, G.P. ; Christ, S.A. ; McCarthy, H.W. ; Torsella, J.A. and Smith, M.K. , 1995. Computer-assisted motion analysis of sperm from the common carp. Journal of Fish Biology, **47(6):**986-1003.

Verbost, P.M. ; Flik, G. ; Pang, P.K.T. ; Lock, R.A.C. and Bonga, S.E.W. , 1989. Cadmium inhibition of the erythrocyte calcium pump: A molecular interpretation. Journal of Biological Chemistry, **264(10):**5613-5615.

Volpi-Ghirardini, A. and Arizzi-Novelli, A. , 2001. A sperm cell toxicity test procedure for the Mediterranean species *Paracentrotus lividus* (Echinodermata: Echinoidea). **22(4):**439-445.

Xu, L.C.; Wang, S.Y. ; Yang, X.F. and Wang, X.R. , 2001. Effects of cadmium on rat sperm motility evaluated with computer assisted sperm analysis. Biomedical and Environmental Sciences: Bes, **14(4):**312-317.