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EVALUATION OF THE FERTILIZATION CAPABILITY OF *PARACENTROTUS LIVIDUS* SEA URCHIN STORAGED GAMETES BY THE EXPOSURE TO DIFFERENT AQUEOUS MATRICES

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Abstract. Several experiments were performed to evaluate the fertilization capability of *Paracentrotus lividus* sea urchin gametes, which are usually used in fertilization tests, but which, until now, were employed only for few hours after spawning.

The aim of this work is to evaluate the possibility of performing the bioassay for longer periods, employing 'conserved' gametes. As far as the method, the sperm was stored 'dry' at 4 °C; eggs were conserved in an antibiotic solution at 4 °C, and they were exposed both to copper solutions as reference toxicant and to aqueous matrices.

The use of conserved gametes in the fertilization bioassay lead to EC50 values which were acceptable for about 3 days. Moreover, these experiments permitted to identify the limiting factor: the male gamete.

Keywords: antibiotics, bioassay, copper, gametes storage, *Paracentrotus lividus*, sea urchin, aqueous matrices

1. Introduction

In Italy the use of biological methods for the environmental quality assessment is becoming very important, in order to integrate the physical-chemical approach. To this purpose, Italian law has recently established the need for short and long term toxicity bioassays to assess water quality (Legislative Decree n. 152, 1999).

Because of its great assurance as bioindicator, the sea urchin sperm cell test has been included in the International Council for the Exploitation of the Sea list as one of the most reliable bioassay for environmental pollution monitoring and quality assessment (ICES, 1997). Indeed, this method combines rapidity of response with high sensitivity and ecological relevance, it is easy, not expensive to perform and discriminates between different risk levels (Dinnel *et al.*, 1988; Lambertson *et al.*, 1992; Pellegrini *et al.*, 1997).

In addition, the reproductive success of sea urchins depends mostly on the environmental conditions, having an external fecundation (Dinnel *et al.*, 1988; Kobayashi, 1984; Pagano *et al.*, 1986; Pinto *et al.*, 1995) and furthermore, sea urchin gametes, due to their high sensitivity towards toxicants, can be used as indicators of pollutants in marine waters (Chapman, 1995).

Regarding the fertilization test, one factor which may affect its sensitivity is the age of the sperm used for testing (Dinnel *et al.*, 1987), and, for this reason, this kind of bioassay is usually performed using just collected gametes (Chapman, 1995; Marin *et al*., 2001; Volpi Ghirardini and Arizzi Novelli, 2001); moreover, once spawned, the fertilization capability of the sperm decreases (Dinnel *et al.*, 1987) and the eggs are degraded by bacteria (mostly *Vibrio*) (De Angelis, 1976; D. Epel, personal communication; C. Falugi, personal communication).

The purpose of the present work is to evaluate the fertilization capability of *Paracentrotus lividus* (Lamarck, 1816) sea urchin storaged gametes, in order to perform the sea urchin sperm cell test for longer periods.

In the past, many authors had considered the problem of sea urchin gametes conservation in biochemical and medical studies, and they proposed the use of antibiotic solutions to preserve eggs cultures from spoilage by contaminating bacteria. The most used device to reduce marine bacterial contamination in eggs culture of *P. lividus*, was an addition of penicillin and streptomycin into sea water (De Angelis, 1975; C. Falugi, personal communication; Mackintosh and Bell, 1970; Nemer and Infante, 1967). In addition, it was recognized for many years that, in order to allow the sea urchin sperm to be held longer without losing viability, it had to be collected 'dry' (D. Epel, personal communication; C. Falugi, personal communication; USEPA, 1995).

In accordance with literature, eggs were treated with antibiotics (penicillin and streptomycin) and sperm was conserved dry at 4 °C. The results obtained from these experiments allowed to perform the sperm cell bioassay not only immediately after gametes spawning but also for longer periods, without significant differences between 'fresh' (just collected) or 'conserved' gametes.

2. Materials and Methods

2.1. Tested solutions

2.1.1. *Dilution Water*

Dilution water, also used as negative control, was 1-μm-filtered natural seawater (USEPA, 1995). Organisms and water were collected at the same source, far from any kind of pollution.

2.1.2. *Reference Toxicant*

Sperm cell toxicity bioassays were performed using an atomic absorption standard copper solution, $Cu(NO_3)_2 x 3H_2O$ (1000 mg/l) (Fluka, Switzerland) as reference toxicant (Volpi Ghirardini and Arizzi Novelli, 2001). The concentrated standard solution was diluted 1:1000 by adding double-distilled water (Milli-Q system, Millipore) and this second solution was then diluted with dilution water to reach the following concentrations: 0.016, 0.024, 0.032, 0.040, 0.048 mg/l;

2.1.3. *Antibiotic Solution*

The antibiotics employed were: streptomycin sulphate (1.000.000 UI) (Bristol-Myers Squibb) and crystallizzed benzilpenicillin potassic (1.000.000 UI) (Bristol-Myers Squibb).

Firstly, each drug was diluted in 5 ml of natural filtered seawater and then the two solutions were mixed together, in order to reach the final solution of 10 ml. Secondly, the antibiotic solution was stored at –20 °C and used for at least 2–3 times; in fact, the processes of freezing and thawing out affect the antibiotic molecular structure (C. Falugi, personal communication). This solution was used since it seemed to be the less toxic among the antibiotics which were tested for maintaining eggs of *P. lividus* and, furthermore, it reduced bacterial contamination in eggs cultures without inducing a great number of anomalies during the development of the embryos (De Angelis, 1976).

2.1.4. *Sediments Collection*

Sediments for elutriate tests were collected in February from two sites along the coastal marine area of the Northern Tyrrhenian Sea: one site, in the inner part of Leghorn harbour, was a polluted location chosen on the basis of previous toxicological data, and the other was an offshore site (43°39′50″N 10°15′08″W) chosen as reference (Pinto *et al*., 1995).

Harbour sediment sample was collected with a 8-L van Veen grab (at 11–12 m depth), whereas, unpolluted offshore sediment was collected with a gravity corer (at 13 m depth) and used as reference sediment.

The sediment samples were stored at 4 °C in capped glass vessels from the time of collection until the preparation of the elutriates; for the chemical analysis they were immediately frozen in decontaminated vessels.

2.1.5. *Elutriates*

Elutriates were prepared by combining four volumes of dilution water with one volume of wet sediment in 1–l borosilicate glass beakers, and mixing on a shaker table at about 200 oscillations/min for 1 h. Then, the aqueous fractions were isolated by centrifugation at 2.500 *g* for 30 min at 4 °C (Ankley, 1991) and subsamples of the supernatant were collected in plastic tubes and frozen. Regarding the freezing process, it did not affect significantly the qualities of nutrients (NO_3 e PO_4) of the liquid phase (Clementson and Wayte, 1992), and furthermore, an experiment performed by Carr and Chapman (1995) demonstrated that significant differences did not exist between the toxicity of fresh or frozen aqueous matrices.

The unpolluted elutriate was tested both undiluted (100%) and diluted at dilution values of 25% and 50%, whereas the polluted elutriate was tested both undiluted (100%), and diluted at values of 95% and 90%. The dilutions of the polluted sample seemed necessary to understand if little variations in sample concentration could influence fertilization rates through time.

2.1.6. *Chemical Analysis*
Metal concentrations were determined following the procedure described by Pinto *et al.* (1995). Sediments were digested in Teflon bombs with a mixture of concentrated hydrochloric, nitric, and hydrofluoric acids. The digestion was performed by a high performance microwave digestion unit (MILESTONE MLS 1220 MEGA). Cd, Pb and As analysis were performed by graphite furnace atomic absorption spectrophotometer (VARIAN SPECTRA AA-220 Z). Al, Cr, Cu, Ni and Zn analysis were performed by Inductively Coupled Plasma-Atomic Emission Spectrometry (VARIAN LIBERTY AZ ICP-AES). Hg was determined by cold vapor atomic absorption spectrophotometer after reduction by stannous chloride (BACHARACH, Coleman Model 50 B Mercury Analysis Accessory System). The analytical performance of the systems was assessed by using certified reference materials (PACS-2 bulk sediment, MESS-3 reference sediment, National Research Council, Canada). Heavy metals concentrations were determined on wet sediments and the results reported in dry weights.

2.2. Organisms collection

To ensure sexual maturity, adult sea urchins were collected from September to May (Fenaux, 1968), at a rocky site far from anthropic pollution (industrial or urban discharges). All urchins (40–50) were gathered at depths ranging from 1 to 3 m. During sampling, organisms were collected in large plastic tanks partially filled with seawater coming from the sampling site; once at the laboratory, the animals were stored in a 300-l glass aquarium containing filtered seawater from the sampling site, and continuously aerated. Furthermore, specimens were fed with macroalgae (*Ulva* sp.) (Volpi Ghirardini and Arizzi Novelli, 2001), shrimp and carrots (Chapman, 1995), and maintained at a salinity of 35 $\pm$1‰ and at a temperature of 16 $\pm$ 1°C (Marin *et al.*, 2001). Sea urchins were kept under these conditions for at least one week before the tests were performed.

2.3. Test procedure

2.3.1. *Gametes Collection And Counts*
Adult sea urchins (generally 3 males and 3 females) were induced to spawn by injecting 1 ml of 0.5 M KCl solution into the coelom through the peristome, as suggested by Tyler (1949). To ensure a 'good quality' of gametes (viable sperm and mature eggs), only gametes released during the first 30 min after the injection were collected (Chapman, 1995).

Sperm was collected dry, directly from the surface of the sea urchins using a Pasteur pipette, and conserved in a small test tube at 4 °C. Indeed, as suggested by several authors, (D. Epel, personal communication; C. Falugi, personal communication; USEPA, 1995), the dry collection technique allows the sperm to be held longer without losing viability and decreases the sensitivity of a test because

semen is more viable at the beginning of the exposure period (Anderson *et al.*, 1990; Chapman, 1995; Cherr *et al.*, 1987). In order to determine sperm concentration, 50 μl of semen were diluted and fixed in 9.95 ml fresh water and sperm count performed on a hemocytometer (Thoma chamber) under a microscope at 40×. Then, once the dilution of semen necessary to provide a 15.000:1 sperm/egg ratio per test chamber was determined, the necessary aliquot of semen was accordingly diluted (Giambartolomei, 1990, thesis; Giuliani *et al.*, 2002, in press; Kozinkova *et al.*, 2003).

Eggs from each female were shed into 50-ml beakers previously filled with seawater. After spawning, they were collected with a 2 ml pipette and examined under a microscope to determine their maturity. Secondly, mature eggs were pooled and decanted into a 1-l beaker and washed with natural filtered seawater. Decanting, rinsing and settling processes were repeated several times to remove damaged eggs, which tend to float, and to reduce the amount of egg jelly, which could interfere with fertilization (Chapman, 1995). Thirdly, eggs concentration was determined by counting subsamples under a microscope at 4× and a final solution of 300 eggs/ml was prepared. Fourthly, subsamples of 35–40 ml were placed into 60-ml sterile flasks (Corning Tissue Culture treated Flasks), and 2 μl of the antibiotic solution, previously defrosted and diluted with dilution water, were added to each ml of stock solution, until the final concentration of 80 UI/ml (De Angelis, 1976). Finally, flasks were conserved at 15 °C.

2.3.2. *Test Execution*

The procedure developed by Dinnel *et al.* (1987) was modified, according to the standard procedures described by the United States Environmental Protection Agency (1991, 1994, 1995) and by the American Society for Testing and Materials (1995). As a consequence, semen was collected dry, as described in USEPA (1995) and ASTM (1995) procedures, and 0,1 ml of diluted sperm solution was added in 10 ml of test solution, in accordance with all the methods mentioned above. But even if sperm/egg ratio (15.000:1; 300 total eggs) was different from those found in most of literature, it was in accordance with the methodologies previously employed by Italian authors working with *P. lividus* (Giambartolomei, 1990, thesis; Giuliani *et al.*, 2002, in press; Kozinkova *et al.*, 2003).

In addition, following the results of several experiments performed in our laboratory with *P. lividus*, the acceptability of test results was fixed at a fertilization rate in the control ranging from 80 to 90% and at an EC50 value ranging from 0.026 mg/l to 0.043 mg/l. The range of the EC50 was calculated as two standard deviations from the EC50 mean values obtained after two years of periodic monitoring (Kozinkova *et al*., 2003).

In these experiments three replicates were set for each sample and 0.1 ml of sperm solution was added to each test chamber. Test tubes were maintained at 16 ± 1 °C for 1 hour; then 1 ml of natural filtered seawater containing 300 eggs was added and after 20 min, time allowed for eggs fertilization, the test was stopped

by the addition of a fixative, as described by ASTM (1995), USEPA (1995) and Dinnel *et al.* (1987); we used to preserve samples by adding 1 ml of 40% formalin. After that the eggs were settled, samples were concentrated by pipetting off most of the overlying solution and a subsample of the concentrated eggs was placed into a counting slide. At least 100 eggs from each tube were examined and scored for the presence or absence of a fertilization membrane. The decrease in fertilization rates, with respect to a control of natural filtered seawater, was evaluated.

2.3.3. *Statistical Analysis*

The Abbott's formula was applied, in order to consider the number of unfertilized eggs in the control (Finney, 1971) and according to it, the relative percentage of unfertilized eggs in each treatment was compared and normalized to that in the control. Then, the adjusted data were used to obtain toxicity values as EC50, using the Trimmed Spearman-Karber statistical method (Hamilton *et al*., 1978). Moreover, the analysis of variance (ANOVA) was applied to determine the interactions between the different treatments, between the time of treatment and between these variables. In addition, data were normalized prior to statistical analysis and Tukey test was performed to compare the couples of variables when the ANOVA resulted significant ($p < 0.005$).

3. Results and Discussion

3.1. Chemical analysis

Metals concentrations recorded are given in Table I. These data were in agreement with the results of Pinto *et al.* (1995) and confirmed the different quality of the sediments.

3.2. Effect of copper

In Figure 1 fertilization rates of a first set of experiments performed to understand how longer conserved gametes could be employed in sperm cell bioassay are shown.

TABLE I
Metal concentrations recorded in sediments

Samples	Al%	As	Cd	Cr	Cu	Hg	Pb	Ni	Zn
Unpolluted	3.9	38.2	0.03	61.5	8.5	0.05	14.8	23.2	34.0
Polluted	5.6	40.8	1.07	98.9	75.2	0.23	176.9	42.9	217.6

Concentrations are given in mg/l, except Al which is given in %.

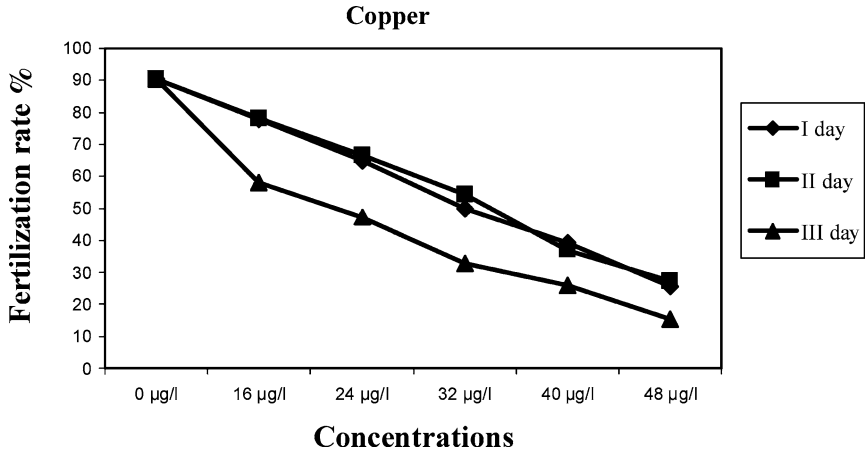

Figure 1. Variation in sperm fertilization capability. The conserved gametes were exposed to the reference toxicant (copper) for 3 consecutive days.

First of all, gametes were exposed to various concentrations of the reference toxicant for three consecutive days. It was noticed that fertilization rates decreased with the increasing of copper concentration, as recognized for many years (Dinnel *et al.*, 1987) and the lowest values were registered on the third day. As far as the collected data, the EC50 values resulted 0.033 mg/l on the first day, 0.035 mg/l on the second day and 0.022 mg/l on the third day, respectively. As a result, fertilization rates of the negative control resulted acceptable, ranging from 85 to 90%, and the EC50 values were in agreement with those found in literature (Chapman, 1995). Only the third value of EC50 (0.022 mg/l) resulted not acceptable, not belonging to the range of acceptability for *P. lividus*, which ranges from 0.026 mg/l to 0.043 mg/l (Kozinkova *et al.*, 2003). These data provided information on the fact that experiments performed with conserved gametes lead to acceptable values of EC50 within 2 days, and it is important to underline that standard deviations relative to the third day were the highest observed (Figure 2).

Nevertheless, fertilization rates of the control did not decrease and resulted acceptable (80%) within 6 days. This result was in agreement with those described for the sea urchin *Strongylocentrotus purpuratus* in the protocol of Epel lab (D. Epel, personal communication).

Secondly, to determine which gamete was responsible for the decrease in fertilization rate, two series of experiments were performed in parallel. Both types of tests were applied for three consecutive days using eggs maintained in antibiotics, but in the first set of experiments sperm preserved at 4 °C was used (Figure 3a), whereas in the second, sperm just collected was used (Figure 3b).

A higher fertilization rate stability was obtained with just collected sperm. Indeed, fertilization rates obtained day after day for each copper concentration were

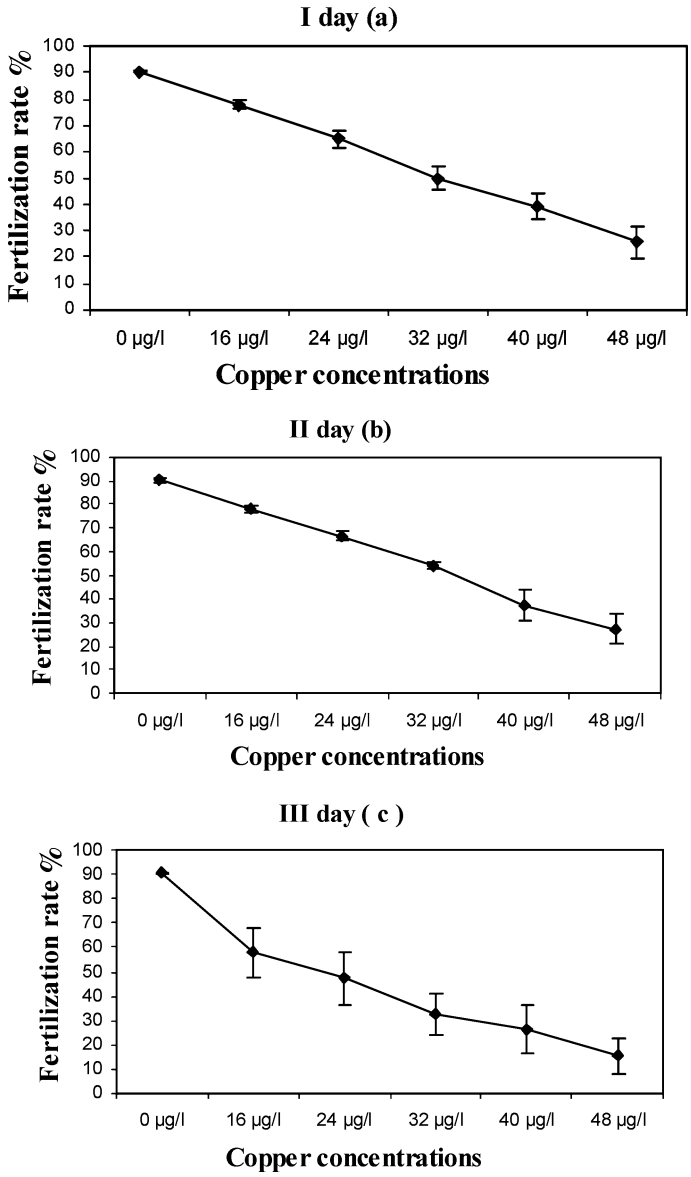

Figure 2. Fertilization rates and standard deviations ($n = 6$) relative to various copper concentrations for the entire period of bioassay performance (3 days).

quite similar and EC50 values ranged from 0.034 to 0.038 mg/l. On the other hand, when conserved sperm was used, there were some variations in fertilization rates, which tended to decrease through time, and EC50 values remained acceptable until the third day, but decreased from 0.041 mg/l (first day) to 0.028 mg/l (third day). At this time, the test was set also on the fourth day and it was noticed that, when sperm

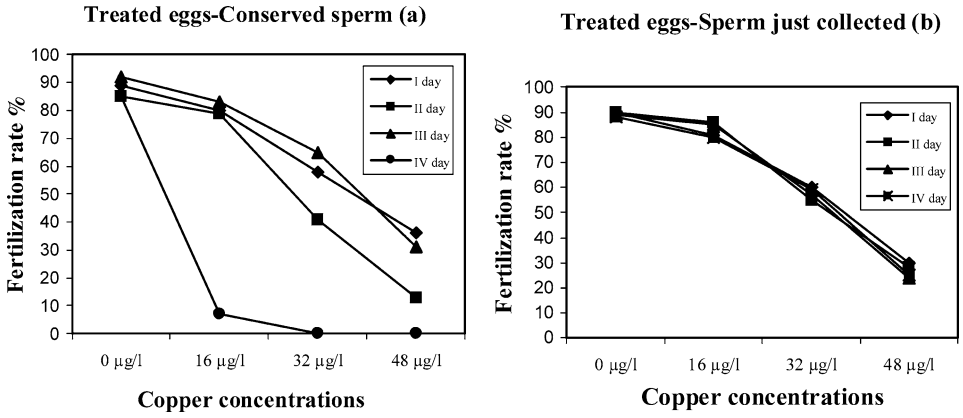

Figure 3. Tests performed in parallel using conserved sperm (a) and just collected sperm (b). In both cases, eggs were treated with an antibiotic solution.

just collected was employed, EC50 value remained in the range of acceptability (Figure 3b); whereas, when sperm conserved was employed, EC50 value was not acceptable (0.011 mg/l) (Figure 3a).

Summarizing, statistical analysis confirmed what observed: when fresh sperm was used, differences between data resulted not significant ($p = 0.203$); whereas, when conserved sperm was used, differences between data resulted significant ($p = 0.012$).

In addition, fertilization rate of the control remained acceptable in both series of experiments within the 6th day, showing that sperm cells did not lose their fertilization capability, but they became more sensitive towards the reference toxicant.

The decrease in fertilization rate was not caused by the exposure of the sperm to antibiotic solution; in fact, even if in all the experiments sperm cells were exposed to these compounds, this relatively short exposition (20 min) did not affect sperm cells quality (C. Falugi, personal communication). Moreover, the quality of treated eggs was not affected by the storage in antibiotic solution and it was possible to perform the bioassay also on the fourth day if fresh sperm was used (Figure 3b).

The good condition of eggs stored in antibiotic solutions has already been demonstrated by Epel (personal communication), who described the possibility to store eggs of *S. purpuratus* for at least one week and eggs of *Lytechinus pictus* for three weeks; in both cases the fertilization rates obtained were excellent (around 80%).

These data demonstrated that the limiting factor was the male gamete, which lost in viability after its collection. As widely accepted for many years, dry sperm collection and storage at low temperature, provides sperm which remain viable for long period of time, from few days (Chapman, 1995) up to a week (if kept in a small

container with less air space) (D. Epel, personal communication). The decreasing in sperm vitality resulted in anomalous and unacceptable values of EC50.

3.3. Effect of aqueous matrices

Natural samples are important because they are generally used to evaluate the environmental risk assessment (Ankley, 1991; Marin *et al.*, 2001; Arizzi Novelli *et al.*, 2001). Basing on this consideration, a third series of experiments was performed in order to evaluate variations in sperm fertilization capability exposed to environmental samples. Moreover, these experiments could be useful to determine if the process of gametes storage can influence fertilization capability of sperm exposed to complex aqueous matrices in a different way respect to the reference toxicant. For the experiment, samples were represented by elutriates, deriving from a polluted

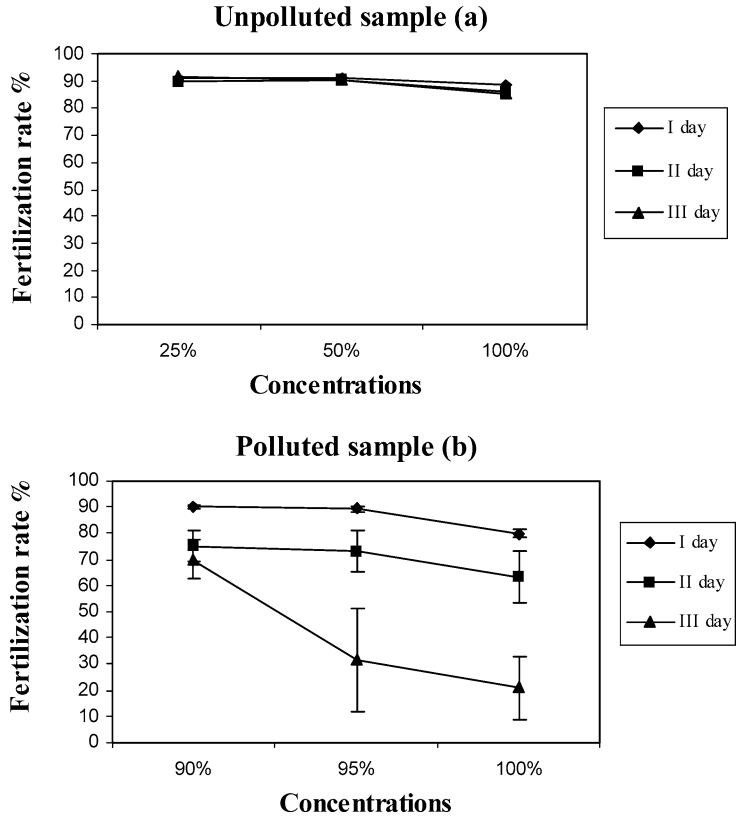

Figure 4. Variation in sperm fertilization capability. The conserved male gametes were exposed to different aqueous matrices: a polluted and an unpolluted one. For the polluted sample (b) standard deviations ($n = 6$) are shown. For the other sample (a) standard deviations resulted in any case ≤ 2. Fertilization rate of the negative control ranged from 90% to 87%.

and an unpolluted site. Fertilization rates of the unpolluted sample remained acceptable for 3 days, as well as the control, with values ranging from 90 to 87% (Figure 4a), whereas fertilization rates of the polluted sample resulted high on the first day and decreased though time (Figure 4b). Moreover, a great variation in fertilization rates occurred even for slight variations in samples concentration. In addition, standard deviations relative to the unpolluted sample remained near to 1% for the entire period of bioassay performance; whereas the ones relative to polluted sample resulted high (10–12%) starting from the second day.

These results demonstrated that conserved gametes could be employed to evaluate the toxicity of an environmental sample only if it was an unpolluted one and, in this case, any variation appeared in fertilization capability during the period of test performance (2–3 days). On the other hand, if a polluted sample was used, the fertilization capability was reduced and decreased through time, with the increasing of pollutants concentration, as previously underlined for the reference toxicant.

4. Conclusion

The analysis performed in the present work, concerning the sperm cell fertilization bioassay, showed that comparable results can be obtained with conserved or fresh gametes for at least two days. After the second day in fact, fertilization rates changed in accordance with the quality of the biological matrices: if the quality of gametes was good, EC50 values were acceptable until the third day; further the sensitivity of bioassay increased and made the reference parameter not acceptable. These results were mainly influenced by the male gamete, which became the limiting factor of the bioassay. Indeed, fertilization capability of conserved sperm relied on the quality of gametes and it was possible to perform the bioassay for longer periods (3 days) only if the initial quality of the biological matrices was good. Nevertheless, fertilization rate of the control remained suitable for at least 6 days, lasting period of the eggs treated with antibiotic solutions.

As far as the antibiotic treatment, it causes morphological alterations in egg cells and in pluteous developing from treated eggs (De Angelis, 1976), so that they could not be used in embryo toxicity bioassays. On the other hand, treated eggs could be used in fertilization bioassays; in fact, even if eggs lost their perfect sphericity, it is possible to discriminate a fertilized egg from a unfertilized one because of the presence of the fertilization membrane.

These outcomes on gametes storage resulted interesting. An extensive use of gametes on a national context, instead of adult organisms, is fundamental since it allows to perform bioassays in different times and places, being the handling and the transfer of gametes through laboratories easier. Moreover, it permits to work with the same pool of gametes, maintaining similar conditions and limiting the variability relied on the employment of different populations of sea urchin. For this reason, results obtained through this research are very useful for the standardization,

in a national context, of the sperm cell fertilization bioassay with *P. lividus*. Finally, it gives the opportunity to reduce the waste of fresh biological material.

References

Anderson, S., Hoffman, E., Steward, D. and Harte, J.: 1990, Ambient Toxicity Characterization of San Francisco Bay and Adjacent Wetland Ecosystem, *Report*, LBL-29579, Lawrence Berkeley Laboratory, University of California, pp. 1–85.

Ankley, G. T.: 1991, 'Predicting the toxicity of bulk sediments to aquatic organisms with aqueous test fractions: Pore water vs. Elutriate', *Environmental Toxicology and Chemistry* **10**, 1359–1366.

Arizzi Novelli, A., Volpi Ghirardini, A., Giuliani, S., Falugi, C., Pagano, G. and Iaccarino, M.: 2001, 'Valutazione della tossicità di acque e sedimenti costieri con gameti e embrioni di specie autoctone di ricci di mare utilizzando metodiche differenziate', *Biol. Mar. Medit.* **8** (2), 41–59.

ASTM: 1995, 'Standard guide for conducting static acute toxicity tests with echinoid embryos', E 1563-95, American Society for Testing and Materials, Philadelphia, pp. 1029–1046.

Carr, R. S. and Chapman, D. C.: 1995, 'Comparison of methods for conducting marine and estuarine sediment porewater toxicity tests: Extraction, storage and handling techniques', *Arch. Environ. Contam. Toxicol*. **28**, 69–77.

Chapman, G. A.: 1995, 'Sea urchin sperm cell test', in: G.M. Rand (ed.) *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate and Risk Assessment*, 2nd Edition, Bristol, PA, Taylor and Francis, pp. 189–205.

Cherr, G. N., Shenker, J. M., Lundmark, C. and Turner, K. O.: 1987, 'Toxic effects of selected bleached kraft mill effluent constituents on the sea urchin sperm cell', *Toxicol. Chem.* **6**, 561–569.

Clementson, L. A. and Wayte, S. E.: 1992, 'The effects of frozen storage of open-ocean seawater samples on the concentration of dissolved Phosphate and Nitrate', *Wat. Res*. **26** (9), 1171–1176.

De Angelis, E.: 1975, 'The problem of bacterial contamination during sea urchin egg culture', *Pubbl. Staz. Zool.* **40**, 19–25.

De Angelis, E.: 1976, 'The effects of antibiotics on sea urchin egg culture', *Pubbl. Staz. Zool.* **40**, 26–33.

Decreto legislativo no. 152: 1999, 'Disposizione sulla tutela delle acque dell'inquinamento e recepimento della direttiva 91/271/CEE concernente il trattamento delle acque reflue e della direttiva 91/676/CEE relativa alla protezione delle acque dell'inquinamento provocato dai nitrati provenienti da fonti agricole', *Gazzetta Ufficiale*, 29 Maggio 1999, pp. 1–125.

Dinnel, P. A., Link, J. M. and Stober, Q. J.: 1987, 'Improved methodology for a sea urchin sperm cell bioassay for marine waters', *Arch. Environ. Contam. Toxicol* **16**, 23–32.

Dinnel, P. A., Pagano, G. and Ossida, P. S.: 1988, 'A sea urchin test system for marine environmental monitoring', in: R. D. Burke, P. V. Mladenov and R. L. Parsley (eds.), *Echinoderm Biology. Proceedings of the Sixth International Echinoderm Conference*, Balkema, Rotterdam, pp. 611–619.

Fenaux, L.: 1968, 'Maturation des gonads et cycle saissonier des larves chez *Arbacia lixula, Paracentrotus lividus*, et *Psammechinus microtuberculatus* (Echinedes) à Villefranche-sur-Mer', *Vie Milieu* **19**, 1–52.

Finney, D. J.: 1971, *Probit Analysis*, Cambridge University Press, London, UK.

Giambartolomei, F. M.: 1990, 'Nuove acquisizioni sul test di fertilità dello sperma di riccio di mare *Paracentrotus lividus*', *Thesis*, University of Padova, Italy.

Giuliani, S., Ennas, C., Pellegrini, D. and Kozinkova, L.: 2002, 'The bioassay with sea urchin *Paracentrotus lividus*: Different end-points to be applied to different environmental studies', *Fres. Environ. Bull.* **11** (10), 806–809.

Giuliani, S., Lera, S., Kozinkova, L., De Ranieri, S. and Pellegrini, D.: 2004, 'Sensibilità del saggio di fecondazione con gameti di *Paracentrotus lividus* (Lamarck) al variare di alcune condizioni sperimentali', *Biol. Mar. Medit.*, in press.

Hamilton, M. A., Russo, R. C. and Thurston, R. V.: 1978, 'Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays', *Environ. Sci. Technol.* **12**, 714–720.

ICES: 1997, *Report of the ICES Advisory Committee on the Marine Environment*.

Kobayashi, N.: 1984, 'Marine ecotoxicological testing with echinoderms', in: G. Persoone, E. Jaspers and C. Claus (eds.), *Ecotoxicological Testing for the Marine Environment*, State Univ. Ghent and Inst. Mar. Scient. Res., Bredene, Belgium, pp. 341–405.

Kozinkova, L., Ennas, C., Giuliani, S. and Pellegrini, D.: 2003, 'Valutazione della qualità dei gameti di *Paracentrotus lividus* (Lamarck) utilizzando $Cu(NO_3)_2 \cdot 3H_2O$ come tossico di riferimento su animali stabulati e non stabulati', *Biol. Mar. Medit*. **10** (2), 1101–1104.

Lambertson, J. Q., Dewitt, T. H. and Swartz, C.: 1992, 'Assessment of sediment toxicity to marine benthos', in: G. A. Burton (ed.), Lewis Publishers, *Sediment Toxicity Assessment*, pp. 183–211.

Mackintosh, R. F. and Bell, E.: 1970, 'Reversible response to puromycin and some characteristics of the uptake and use of aminoacids by unfertilized sea urchin eggs', *Biol. Bull.* **139**, 296–303.

Marin, M. G., Da Los, L., Moschino, V. and Campesan, G.: 2001, 'Sediment elutriate toxicity testing with embryos of sea urchin (*Paracentrotus lividus*)', *Aquatic Ecosystem Health and Management* **4**, 215–221.

Nemer, M. and Infante, A.: 1967, 'Ribosomal ribonucleic acid of the sea urchin egg and its fate during embryogenesis', *J. Mol. Biol.* **27**, 73–86.

Pagano, G., Cipollaro, M., Corsale, G., Esposito, A., Ragucci, E., Giordano, G. G. and Trieff, N. M.: 1986, 'The sea urchin: Bioassay for the assessment of damage from environmental contaminants', in: J. Cairns (ed.), C*ommunity Toxicity Testing*, ASTM STP 920, Philadelphia, PA, pp. 66–92.

Pellegrini, D., Giuliani, S., Onorati, F., Bigongiari, N., De Ranieri, S. and Meschini, P.: 1997, 'Impiego di due saggi biologici per la valutazione della tossicità di sedimenti del porto di Piombino', *Biol. Mar. Medit.* **4** (1), 490–492.

Pinto, B., Pellegrini, D., Gabellini, M. and Ausili, A.: 1995, 'Harbour and coastal sediments chemistry and toxicology: a preliminary study in dredging activities', *J. Aquat. Ecosyst. Health* **4**, 1–17.

Tyler, A.: 1949, 'A simple non-injurious method for inducing repeated spawning of sea urchins and sand dollars', *Collecting Net* **19**, 19–20.

U.S EPA:1991, Earl-Standard Operating Procedure Conducting the Sea Urchin *Arbacia punctulata* Fertilization Test, Environmental Research Laboratory, Narragansett, RI, pp. 125–131.

U.S. EPA: 1994, Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms, 600-4-91-003, Cincinnati, Ohio.

U.S. EPA: 1995, Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Water to West Coast Marine and Estuarine Organisms, 600/R-95/136, Cincinnati, Ohio.

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