Environmental Monitoring and Assessment (2006) **122:** 101–109
DOI: 10.1007/s10661-005-9167-4

STANDARDIZING THE METHODOLOGY OF SPERM CELL TEST WITH *PARACENTROTUS LIVIDUS*

S. LERA*, S. MACCHIA and D. PELLEGRINI
ICRAM – Central Institute of Marine Research, Via di Casalotti 300, Rome, Italy
*(*author for correspondence, e-mail: s.lera@icram.org)*

(Received 14 October 2004; accepted 20 December 2005)

Abstract. The sperm cell toxicity test with *Paracentrotus lividus* is widely used in Italy to assess the quality of complex aqueous matrices, but at present there is a shortage of standardized methodologies for the bioassay performance. In this work several critical points were considered, in order to verify the reliability of this bioassay and to improve its standardization.

In particular, we have studied the differences in EC50 values at different sperm: egg ratios and with different quantities of gametes; the influence of sperm and eggs on fertilization rate and the influence of different recipients materials (plastic or glass). At the same time, it was evaluated if the obtained EC50 values were in accordance with those reported in literature.

Experiments performed yielded EC50 values for copper ranging from 16.54 μg/l to 69.59 μg/l, with a total mean value $\pm$ SD of 39.79 μg/l $\pm$ 11.17. These data fit with those found in literature for other echinoid species. According to the same authors, poor fertilization was probably due to the effects on sperm and not on eggs, and moreover the linear regression analysis performed on each experiment suggested that the best amount of eggs to use in the test was 1000.

Results from the comparison between the performance of the test in plastic or glass tubes showed an higher success in fertilization rate using borosilicate glass tubes.

Keywords: copper, gametes, *Paracentrotus lividus,* plastic or glass tubes, sea urchins, sperm cell test, sperm: egg ratio, standardizing

1. Introduction

Toxicity bioassays using echinoderms have been developed worldwide and their reliability is well recognized (Kobayashi, 1984; Bay *et al.*, 1993). The most widespread use of sea urchin sperm cell test appears in regulatory programs dealing with the evaluation of seawater quality and marine or estuarine sediments toxicity (USEPA, 1994).

In toxicity testing, especially for regulatory purposes, the conditions and mechanics of the test must be suitably standardized, in order to guarantee good quality controls and the accuracy of the decisions to make. To these aims, standard procedures using fertilization and embryo development are available in the US using several marine or estuarine animal species (ASTM, 2004; USEPA, 1991), and tests using fertilization as endpoint have been also developed in Canada, with both sea urchins and sand dollars (EC, 1992). In Italy standardized toxicity bioassays are available for freshwater, but at present there is a lack in the regulation

(Legislative Decree n.152) concerning coastal marine environments. One of these non-standardized bioassays is the sea urchin sperm cell test, in which differences between methodologies are both related to the final sperm concentration both to the sperm: egg ratio, factors which play an important role in test results.

Indeed, sperm longevity depends mostly on its concentration, according to a process termed "respiratory dilution effect", described by Chia and Bickell (1983). These authors found out that the amount of oxygen consumed by a spermatozoon over its lifetime is fixed. Since diluted sperm consumes more oxygen than concentrated one do, concentrated sperm maintains its longevity over a longer period of time if compared to a diluted one. Pennington (1985) also demonstrated that a high percentage of fertilization rate was achieved only with relatively dense sperm suspensions.

On the other hand, the use of non-standardized sperm: egg ratios in bioassays can affect test results (Giuliani *et al.,* 2004). Experience has shown that the optimum sperm: egg ratio is species dependent and that the optimum ratio can vary from spawn to spawn within a single population (Dinnel *et al.*, 1982). Moreover, the sensitivity to toxicants is inversely related to the sperm: egg ratio (Dinnel *et al*, 1987).

In addition, there is a third factor which may affect test results, that is the kind of test-vessels. Indeed, this bioassay can be set both in glass test tubes and polystyrene plates, as suggested by international literature (ASTM, 2004; Dinnel *et al.,* 1987; US EPA, 1995), but these different materials have not yet been tested for the species *P. lividus.*

For all these reasons, the aims of the present study were to:

- Monitor the EC50 values, in order to evaluate if they are in good agreement with those found in marine acute tests.
- Perform the test using different sperm: egg ratios and different quantities of total gametes, to evaluate if exists a combination of factors which determines good levels of precision and repeatability.
- Evaluate if the success of fertilization is mainly influenced by sperm or eggs concentration.
- Evaluate if the use of different test vessels (plastic or glass tubes) 'do affect' test results.

2. Materials and Methods

2.1. Organisms collection

Adult sea urchins were collected from an intertidal rocky site along the coast of Leghorn (Italy), far from industrial or agricultural discharges. A suitable container partially filled with seawater from the sampling site was used to transport the organisms to the laboratory, where animals were stored in glass aquaria containing

aerated and filtered seawater from the sampling site. Animals were kept under these conditions for at least one week (Volpi Ghirardini and Arizzi Novelli, 2001). Salinity (36–38‰), pH (7.8–8.2), ammonium, and water temperature ($16 \pm 22\,^{\circ}$C) were checked every day.

2.2. Gametes collection

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).

Sperm from each male was collected dry and conserved in a small test tube at $4\,^{\circ}$C. Sperm density was determined by fixing $50\,\mu$l of semen in 9.950 ml of fresh water and, later on, sperm count was performed on a hemocytometer (Thoma chamber) under a microscope at $40\times$. Then, dry sperm was diluted into filtered seawater to reach the desired sperm densities (Kozinkova *et al.,* 2003).

Eggs from each female were shed into 50-ml beakers previously filled with seawater. Eggs from different females could be pooled, but before they were examined under a microscope to determine their maturity. After that, eggs were decanted into a 1-l beaker and allowed to settle. Decanting, rinsing, and settling processes were repeated three times to remove damaged eggs, which tend to float. Eggs concentration was determined by counting subsamples under a microscope $4\times$ and three final concentrations of 300, 1000, and 2000 eggs/ml were prepared. Eggs were stored at $16\,^{\circ}$C.

2.3. Test execution

The methodology adopted in this work has been described in Lera and Pellegrini (2004, in press). The procedure is a derivation of the methodology originally described by Dinnel *et al.* (1987), in accordance with the standard procedures adopted by the United States Environmental Protection Agency (1991, 1994, 1995), by the American Society for Testing and Materials (1991, 1995) and by the Environmental Protection Series (1991, 1992). The cited procedures were modified, in order to match with other methodologies previously employed by Italian authors working with the species *P. lividus* (Giambartolomei, 1990, thesis; Giuliani *et al.,* 2002, 2004; Kozinkova *et al.,* 2003; Volpi Ghirardini and Arizzi Novelli, 2001).

A set of 4 toxicity tests were conducted from February to May 2003. Sperm cell toxicity tests were performed using a control of natural filtered seawater and an atomic absorption standard copper solution, $Cu(NO_3)_2 \times 3H_2O$ (1000 mg/l) (Fluka, Switzerland) as reference toxicant (Lera and Pellegrini, 2004, in press; Volpi Ghirardini and Arizzi Novelli, 2001) with concentrations of 0.016, 0.032, 0.048, 0.060, 0.072, 0.084 mg/l. Three replicates were set for each concentration and the final volume of each tube was 10 ml. The tests were performed both in

glass test tubes (16×100 mm) and in polystyrene tissue culture plates, with 6 wells and flat bottom (35×20 mm). 0,1 ml of sperm solution was added to each test chamber and test tubes were maintained at $16 \pm 1\,^{\circ}$C for 1 hour. Then, 1 ml of eggs suspension was added to each test tube and after 20 min (time allowed for eggs fertilization) the test was stopped by adding 1 ml of 40% formalin. Eggs were subsequently pipetted from each test tube and at least 100–200 eggs were examined and scored for the presence or absence of a fertilization membrane.

To evaluate which sperm: egg ratio was able to determine precision and intral-aboratory reproducibility of the method, several combinations of different ratios and total eggs were tested. In particular the sperm: egg ratios used were 1:20000, 1:15000, 1:10000, as suggested by Giuliani *et al.* (2004) and the total number of eggs for each test chamber were 300, 1000 and 2000.

2.4. Statistical analysis

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% (Kozinkova *et al*., 2003). In order to consider the number of unfertilized eggs in control, the Abbott's formula was applied (Finney, 1971) and according to it, the relative percentage of unfertilized eggs in each treatment was compared and normalized to the one in the control. Then the adjusted data were used to calculate EC50 values, by the Trimmed Spearman-Karber statistical method (Hamilton *et al.*, 1978).

The analysis of variance (ANOVA) was applied on raw data to determine the interactions between the factors involved in the experiments (number of total eggs, treatment with copper, sperm: egg ratio). The relationships between the factors were considered significant when $p < 0.05$. Consequently, a linear regression analysis was applied for each combination of sperm: egg ratio and number of eggs to determine the interaction which lead to a straight line with the highest and comparable R^2 values.

3. Results and Discussion

The experiments performed in this study yielded EC50 values ranging from 16.54 μg/l (300 eggs, sperm: egg ratio 10000:1) to 69.59 μg/l (2000 eggs, sperm: egg ratio 20000:1), with a total mean EC50 value $\pm$ SD of 39.79 μg/l $\pm$ 11.17. The values of EC50 obtained for copper are in good agreement with those found in literature for various echinoid species, as well as for *P. lividus*: the mean EC50 ranges from 12 μg/l for *Arbacia punctulata* (Nacci *et al.*, 1986) to 59 μg/l for *Strongylocentrotus droebachiensis* (Dinnel *et al.*, 1989). Existing data concerning toxicity effects of copper on male gametes of *P. lividus* were reported by Volpi Ghirardini and Arizzi Novelli (2001). They took into account a sperm: egg ratio

of 1:20000 with 2000 eggs for each test chamber, with a mean EC50 value of 55 μg/l. Other data concerning the same species were provided by Kozinkova *et al.* (2003) with EC50 values ranging from 16.76 to 42.93 μg/l (1:15000, 200 eggs), by Giuliani *et al.* (2004) with EC50 values similar to those found in this work, and by Lera and Pellegrini (2004, in press) with EC50 values ranging from 22.25 to 41.86 μg/l (1:15000, 300 eggs).

Coefficients of variations CVs (standard deviation of the EC50s/mean EC50) ranged from 11.6% to 40.9%, with a median value of 28.3% (n = 47) (Table I).

The data obtained in this work were in good agreement with those obtained in the US EPA laboratories for tests with common marine species. In particular, these laboratories conducted several series of intralaboratory precision tests with variations of the *Arbacia* sperm cell test protocol. The largest test series, with copper as reference toxicant, gave CVs ranging from 22.6% to 48% in five different precision checks. Moreover, Morrison *et al*. (1989) published results of inter and intralaboratory tests with common marine and freshwater species. For marine short-term chronic tests (including the *Arbacia* sperm cell test), the CVs for copper ranged from 1.8% to 46.4% with a median value of 24.1%. For marine acute tests, the CVs ranged from 22% to 104% with a median value of 44%. A recent work conducted on *P. lividus* with copper (Volpi Ghirardini and Arizzi Novelli, 2001), reported a median CV value of 26% (n = 36), which is within the range reported in precision checks performed by the US EPA using *Arbacia punctulata.*

The second purpose of this work was to provide the combination between total eggs and sperm: egg ratio leading to the maximum repeatability and intralaboratory reproducibility. Because fertilization is a result of sperm- egg encounter, it seems intuitive that eggs concentration should be important for fertilization success. Indeed, statistical analysis performed on raw data confirmed what mentioned above in the 50% of the experiments ($p = 0.005$ and $p = 0.002$), whereas, in the remaining 50% of the cases the fertilization success was not influenced by eggs concentration ($p = 0.085$ and $p = 0.827$). Lillie (1915) found no significant effect of eggs concentration on fertilization with *Arbacia*, and Levitan *et al*. (1991) noticed

TABLE I
Coefficients of variations obtained for different combinations of sperm: egg ratio and total eggs (n: ranging from 3 to 9 for each combination)

	Sperm: egg ratio		
Total eggs	20000:1	15000:1	10000:1
2000	20.58	40.88	26.54
1000	33.33	36.92	15.44
300	11.61	31.99	28.42

a slight impact on fertilization of *Strongylocentrotus franciscanus* only when the concentration was over 100 eggs/μl.

On the other hand, Muchmore and Epel (1973) showed that the concentration of sperm can be very important for the sensitivity of eggs fertilization when gametes are exposed to chlorine. With a low sperm density, a given concentration of chlorinated sewage limited fertilization to only 1% of the eggs, but at a 10-fold higher sperm density, the fertilization was 100% for the same sewage concentration. Consequently, according to the authors, poor fertilization was clearly due to the effects on sperm and not on eggs. Future experiments will be necessary to better understand the interaction between fertilization success and eggs concentration.

Linear regression analysis performed on each experiment, considering both the number of total eggs and the sperm: egg ratios, lead to conclude that the best number of eggs to perform the test was 1000. In fact, the best fitting straight lines obtained for any sperm: egg ratio combined with 1000 eggs gave R^2values ranging from 0.77 to 0.92 with a standard deviation of 0.04 ($n = 8$) (that resulted the lowest observed).

Furthermore, fixing the number of total eggs at 1000, it was considered the sperm fertilization capability varying its concentration. There was a decreasing in fertilization success as sperm: egg ratio decreased (Figure 1), as demonstrated in preliminary experiments conducted with *P. lividus* (Giuliani *et al.,* 2004). The same behaviour is described for *Arbacia* (Morrison *et al*., 1989) and *Strongylocentrotus* (Dinnel *et al.*, 1987). The decrease in fertilization rate lead to reduce the EC50 values and to increase the sensitivity of the test to toxicants (Chapman, 1995). For this reason it is difficult to determine the best quantity of sperm to be used in the test, because it depends on the sensitivity demanded for any compound.

Results coming from the comparison between the performance of the test in plastic (polystyrene) or glass tubes showed a higher success in fertilization rate if

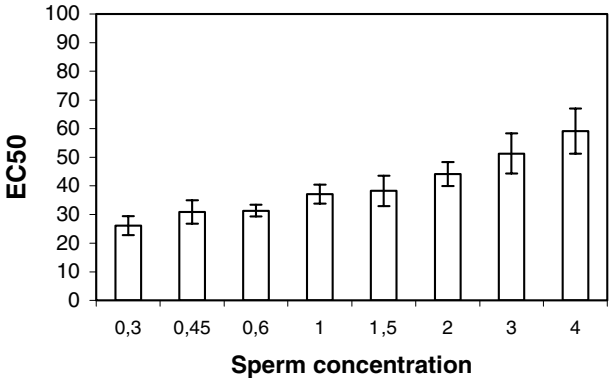

Figure 1. Pattern of EC50 values obtained with increasing sperm concentrations. Standard errors are shown for each mean EC50 value (n ranging from 3 to 9). Sperm concentration is reported in 10^7 units and the corresponding EC50 is reported in μg/l.

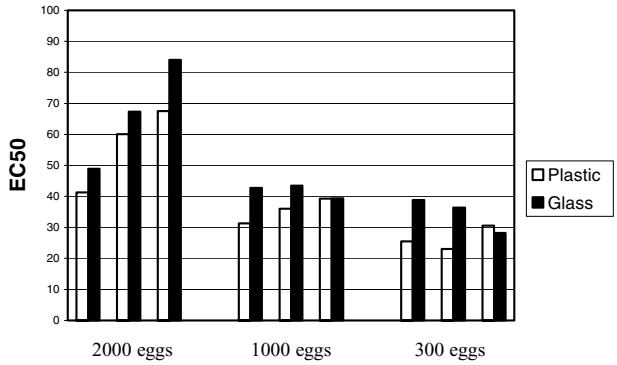

Figure 2. Median EC50 values obtained after the exposure of different sea urchin gametes concentrations (2000, 1000 and 300 eggs with sperm:eggs ratios of 1:20000, 1:15000, 1:10000, respectively) to copper in different test tubes: borosilicate glass test tubes (black) and polystyrene test tubes (white). The EC50 values are reported in μg/l.

borosilicate glass tubes were employed (Figure 2). These data did not agree with those obtained by Giuliani *et al.* (2004) with *P. lividus*, showing no differences between the type of test vessels. On the other hand, the results obtained in this research are in accordance with the experiments performed by Dinnel *et al*. (1987) showing a fertilization success in polystyrene tubes lower than the one obtained in borosilicate glass tubes. Indeed, plastic tubes are generally less expensive but they may be potentially toxic due to the leaching of chemicals from newly manufactured plastics.

4. Conclusion

The EC50 values obtained using different sperm: egg ratios and different quantities of total gametes are similar to those obtained by different authors with other ecotoxicological tests. The CVs observed in this work with the sperm cell test are within the range of other commonly applied tests and the precision obtained in this study is comparable with the most of other toxicity experiments. Nevertheless, it remains uncertain whether the precision of such tests is acceptable.

It is difficult to find a combination of factors (total eggs, sperm: egg ratio) which determines good levels of precision and repeatability. Although the non-influence of egg concentration on fertilization success has been demonstrated in previous studies, our case of study did not totally match with this conclusion. For this reason, other similar experiments will be necessary to determine exactly the cited relationship. Indeed, the best number of total eggs necessary to perform the test is 1000. Maintaining this condition, the experiments provide a high intralaboratory reproducibility and precision for any sperm: egg ratio adopted.

Fertilization success depends on sperm concentration, and the decrease in the number of this gamete lead to a decrease in fertilization rate and to an increase in test sensitivity.

Another factor which influences the fertilization success is the material of the test tubes used to perform the experiment. Such kind of materials, like plastic, are toxic and really disturb the normal activity of sperm. The result is an evident decrease in fertilization rate, which demonstrates the high sensitivity and discriminating capability associated with this bioassay.

Further research will be focused on the variations of sperm concentration fixing the number of total eggs at 1000, in order to determine the best combination between these factors, which lead to maximum precision and intralaboratory reproducibility. Then, it would be possible to begin interlaboratory experiments in order to achieve the necessary knowledge to standardize the methodology.

References

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.

ASTM: 2004, 'Standard guide for conducting static acute toxicity tests with echinoid embryos', E 1563–98, *American Society for Testing and Materials*, Philadelphia.

Bay, S., Burgess, R. and Nacci, D.: 1993, 'Status and applications of Echinoid (Phylum Echinodermata) toxicity test methods', in: W.G. Landis, J.S. Hughes and M.A. Lewis (eds), *Environmental Toxicology and Risk Assessment*, ASTM SRP 1179, Philadelphia, pp. 281–302.

Chapman, G.A., Denton, D.L. and Lazorchak, J.M.: 1995, 'Short-term methods for estimating the chronic toxicity of effluents and receiving water to West Coast marine and estuarine organisms', U.S. EPA, 600/R-95/136, Cincinnati, Ohio.

Chia, F.-S. and Bickell, L.R.: 1983, 'Echinodermata', in: K.G. Adiyodi and R.G Adiyodi (eds), *Reproductive Biology of Invertebrates, Volume II: Spermatogenesis and Sperm Function,* John Wiley and Sons, New York, pp. 545–620.

Decreto legislativo n° 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.: 1991, 'Proposed standard EXXX guide for conducting static acute toxicity tests with echinoid embryos', in: *Annual book of ASTM standards*, Draft 1, pp. 1–45.

Dinnel, P.A., Stober, Q.J., Crumley, S.C. and Nakatani, R.E.: 1982, 'Development of a sperm cell toxicity test for marine waters', in: J.G. Pearson, R.B. Foster and W.E. Bishop (eds.), *Aquatic toxicology and hazard assessment*, Fifth Conference, ASTM STP 766, Philadelphia, pp. 82–98.

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., Link, J.M., Stober, Q.J., Letourneau, M.W. and Roberts, W.E.: 1989, 'Comparative sensitivity of sea urchin bioassay to metals and pesticides', *Arch. Environ. Contam. Toxicol.* **18**, 748–755.

Environment Canada: 1991, 'Fertilization assay with echinoids (sea urchin and sand dollars)', *Environmental Protection Series,* EPS 1/RM, Ottawa, Canada.

Environment Canada: 1992, 'Biological test method: fertilization assay using Echinoids (sea urchins and sand dollars)', *Environmental Protection Series*, EPS 1/RM/27, Ottawa, Canada.

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* (L.) al variare di alcune condizioni sperimentali', *Biol. Mar. Medit.* **11(2)**, 330–333.

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.

Klemm, D.J., Morrison, G., Nurberg-King, T., Peltier, W. and Heber, M.: 1994, 'Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms', U.S. EPA, 600-4-91-003, Cincinnati, Ohio.

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 $Cu(NO_3)_2.3H_2O$ come tossico di riferimento su animali stabulati e non stabulati', *Biol. Mar. Medit*. **10** (2): 1101–1104.

Lera, S. and Pellegrini, D.: 2004, 'Evaluation of the fertilization capability of *Paracentrotus lividus* sea urchin storaged gametes by the exposure to different aqueous matrices', *Environ. Monit. Assess.* in press.

Levitan, D.R., Sewell, M.A. and Chia, F.-S.: 1991, 'Kinetics of fertilization in the sea urchin *Strongylocentrotus franciscanus*: interaction of gamete dilution, age, and contact time', *Biol. Bull.***181**, 371–378.

Lillie, F.R.: 1915, 'Studies of fertilization. VII. Analysis of variations in the fertilization power of sperm suspensions of *Arbacia'*, *Biol. Bull.* **28**, 229–251.

Morrison, G., Torello, E., Comoleo, R., Walsh, R., Kuhn, A., Burgess, R., Tagliabue, M. and Greene, W.: 1989, 'Intralaboratory precision of saltwater short-term chronic toxicity tests', *J. Water Poll. Control Fed.* **61**, 1707–1710.

Muchmore, D. and Epel, D.: 1973, 'The effects of chlorination of wastewater on fertilization in some marine invertebrates', *Mar. Biol.* **19**, 93–95.

Nacci, D., Jackim, E. and Walsh, R.: 1986, 'Comparative evaluation of three rapid marine toxicity tests: sea urchin early embryo growth test, sea urchin sperm cell toxicity test and Microtox', *Environ. Toxicol. Chem.* **5**, 521–525.

Nacci, D., Comoleo, P., Petrocelli, E. and Jackim, E.: 1991, 'Earl-standard operating procedure conducting the sea urchin *Arbacia punctulata* fertilization test', U.S. EPA, Environmental Research Laboratory, Narragansett, RI, pp. 125–131.

Pennington, J.T.: 1985, 'The ecology of fertilization of echinoid eggs: the consequences of sperm dilution, adylt aggregation, and synchronous spawning', *Biol. Bull.* **169**, 417–430.

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

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