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Research Article

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Analysis of Variability in Embryological Response of Two Sea Urchin Species to Spatial and Temporal features-Can these Factors Influence Responses in Standardized Ecotoxicological Assays?

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Abstract

The use of Echinoderms as bioindicator organisms, in particular in emryotoxicity test with *Paracentrotus lividus* and *Arbacia lixula*, has provided the scientific community with a remarkable number of ecotoxicological studies. In this experiment, the responses of these two species were analysed considering the spatial and temporal variability of three populations distributed in a radius of ca 10 km. The species tested in this experiment demonstrated an overall substantial different response towards metals ($p<0.001$), *Arbacia lixula* being the most sensitive species to both the three different sites and different periods of the year when adults were collected. There was a significant difference among populations for both species. The site affected most by the metal toxicity was Fortullino, which is the less human impacted area. In general, as a consequence of the passed reproductive season, embryos developed from gametes collected in May were the most affected in this study, confirming previous observations collected by our research group. No statistical difference has been recorded between the T1 (January) sampling corresponding to the beginning of reproductive season and the T5 (November) sampling-related embryos, which had the summer and the beginning of autumn as recovery period for their gamete production.

Keywords

Embryological testing; Ecotoxicological Assays; *Arbacia lixula*; *Paracentrotus lividus*

Introduction

Assessment of the environmental impacts of potentially toxic contaminants and prevention of marine pollution will continue to be an area of both public and scientific concern in the foreseeable future [1]. In the last decade the scientific community has switched from expressing pollution in terms of chemical concentration in environmental matrices, to considering the effective estimation of adverse effect on biota; a decision underpinned by data gathered from biomarker analysis and bioassays [2-5]. Among all the environmental contaminants, heavy metals are a group of major concern because of their proven toxicity for different marine and terrestrial species, in addition to representing a threat to human health [6-9].

The use of Echinoderms as bioindicator organisms has provided the scientific community with a remarkable number of Eco toxicological studies, in which the evaluation of toxicity to these organisms has been largely evaluated in their dual life stage as pelagic and benthic species [10-13]. In *Paracentrotus lividus* for example, after the fertilization of oocytes the planktotrophic larva lingers in the water column for a period of 3 to 4 weeks before metamorphosis it converts in a benthonic juvenile state [14]. Most echinoids, including *P. lividus* (Lamarck 1816) and *Arbacia lixula* are free spawners, they release a vast number of gametes into the water column eventually giving rise to planktotrophic larvae known as echinoplutei [15]. The expected pattern is that echinoderms have little genetic structure and high gene flow [16-18]. Nevertheless, there are examples showing that such expectations may be unfounded, because a variety of additional factors (biological, physical, ecological, etc.) might contribute to the shaping of the population structure of marine invertebrates through space and time [19-21].

P. lividus is distributed on horizontal or gently sloping seabed throughout the Mediterranean Sea and in the northeastern Atlantic [22,23]. It is a key species in the benthic ecology, driving algal community dynamics by eliminating erect algae and seagrasses in addition to inducing the formation of coralline barrens [24,25]. Although *P. lividus* and *A. lixula* co-occur on hard substrata in shallow subtidal habitats [26], their ecological niche is slightly different. *A. lixula* is more common on vertical substrata [22,23] showing a strong feeding preference for encrusting corallines. It is characterized by a considerable trophic plasticity, ranging from an omnivore strategy to strict carnivore [27,28]. The genetic structure of populations in these two species revealed that the Mediterranean represents a crucial environment with regard to genetic flow. In particular, as shown by Duran [29], *P. lividus* is distributed in two panmictic populations; one in the western Mediterranean and the other one in the Atlantic. These two populations have a high genetic flow within the corresponding environment, but present significant genetic differentiation, likely due to the physical barrier of the Strait of Gibraltar. *A. lixula* populations present three main haplogroups (named A, B, C). Urchins in the Eastern Atlantic, Alboran Sea and Mediterranean share A and B haplogroups, with a marked predominance of haplogroup A; in Brazilian populations haplogroup B is still present but haplogroup C is the predominant. No differentiation was found among Mediterranean sub-basins or among Eastern Atlantic sub-regions [30]. This particular aspect is currently under investigation by our research group.

Sea urchins are recognised as excellent model system for eco-embryo- and geno-toxicological studies [31-35] and many studies have demonstrated the sensitivity of *P. lividus* embryos to assess the toxicity of heavy metal [12,36-43] nevertheless, based upon our knowledge, few studies have been carried out about the sensitivity of *A. lixula* to heavy metals [44]. In response to these findings, our research group has focused on the analysis of the variable nature of *P. lividus* and *A. lixula* sensitivity towards three heavy metals of potential environmental concern: Cadmium, Zinc and Copper.

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Embryo development tests were conducted over a full year (from January 2014 to November 2014) to assess the possible role of the factor "Time" on variation of biological responses obtained. Furthermore, the contribution of the spatial variability was analysed, to weigh the importance of populations' detachment in selecting different genetic pools involved with pollutants resistance.

Materials and Methods

Animal sampling

P .lividus and *A. lixula* adults were collected from January 2014 to November 2014 from the following three intertidal rocky sites along the coast of Livorno: Fortullino (Italy) [43°25.618'N, 10° 23.804'E], Chioma [43°26.582'N 10°22.894'E] and Antignano [43°29.314'N, 10°19.621'E] respectively and immediately transported within an insulated box to the laboratory (Figure 1).

Gametes collection

Gametes were collected by injecting 1 ml of 0.5M KCl solution (Carlo Erba, Milan) into the coelom, through the peristome; sperm obtained from males within the same treatment was collected dry using a Pasteur pipette, pooled and stored in at 4°C until its use. Sperm concentration was determined with a haemocytometer (Thoma chamber) under an Olympus inverted microscope (Milan, Italy) using a 40 x objective. Oocytes obtained from at least three females per treatment were pooled into 1L beaker filled with 0.22 mm filtered seawater (FSW) (36 ± 1 psu salinity, pH=8.0 ± 0.2). The final concentration of 1000 eggs mL^{-1} was prepared by counting subsamples of a known volume with inverted microscope at 4 x objectives. Fertilization was granted by diluting sperm and eggs in 1L FSW beaker at 15000:1 sperm: egg ratio [45] and few minutes after fertilization, an aliquot of embryos was observed to verify the presence of the fertilization membrane.

Embryotoxicity experiments

Biological responses of *P. lividus* and *A. lixula* were evaluated with embryo toxicity tests to Copper, Cadmium and Zinc according to the protocol detailed in Gaion et al., [43]. To appraise the toxicity of heavy metals, a set of 5 tests was conducted from January to November 2014 (T1=January, T2=March, T3=May, T4=September, T5=November), collecting animals from their natural environment. Each chemical species was tested with six different concentrations: 30-40-50-60-70-80 µg L^{-1} for Zn, 1000-1200-1400-1600-1800-2000 µg L^{-1} for Cd and 20-30-40-50-60-70 µg L^{-1} for Cu respectively. Each solution was prepared using atomic absorption standard solutions [1g L^{-1}] (Carlo Erba, Milan). The initial standard solution was diluted 1:1000, for Zn and Cu, and 1:10 for Cd with double distilled water (Milli-Q-system, Millipore), and the resulting solutions were further diluted with FSW to reach the established nominal concentration. The achievement of desired concentrations was assessed analytically with an Agilent ICP OES 720 Series. Embryos were kept at 20 ± 1°C in a controlled temperature chamber, a constant salinity of 36 ± 1 psu and 9 h daylight. Tested concentrations were chosen according to existing literature data [36-38,44-48]. Six replicates were prepared for each concentration and the final volume within each well plate was 10 mL. After microfiltration (0.22 µm), water collected 1.5 nautical miles offshore from an uncontaminated coastal area was used for dilution and as negative control. Larval growth was allowed until the specimens in the control group reached P4 stage for more than 90%. Sea urchin plutei P4 stage is the larval stage which presents 4 arms and commonly occurs after 48h incubation at 20°C for both species (Figure 2) [49,50], consequently no different exposition time were set for *P. lividus* and *A. lixula*. The acceptability of the results was fixed at a percentage of normal plutei>80 % in negative control tests [36,43,45,51]. Normal and abnormal P4 were identified according to Pagano et al., [52]: fully developed and normal shaped P4 larvae were considered as "normal", whereas retarded gastrulae, pre-gastrulae, prism stages and malformed plutei (showing defects in the skeleton and/or digestive apparatus) were considered abnormal.

The relative percentage of abnormal P4 was normalized for each treatment in each concentration with respect to negative control according to Abbott's formula [53]. Normalized data were used to calculate EC_{50} values (Median effective concentration inducing 50% of abnormal P4 stages) values using Trimmed Spearman-Karber [54].

Statistical analysis

To verify the main differences among factors (Species, Site, and Time) and their interactions, three way analyses of variances (ANOVA) with Tukey post hoc test was performed. Assumptions of ANOVA had been checked and normality confirmed by the results of Shapiro-Wilk test. Statistical significance was fixed at values $p<0.05$. Statistical analyses were performed with IBM SPSS statistical software.

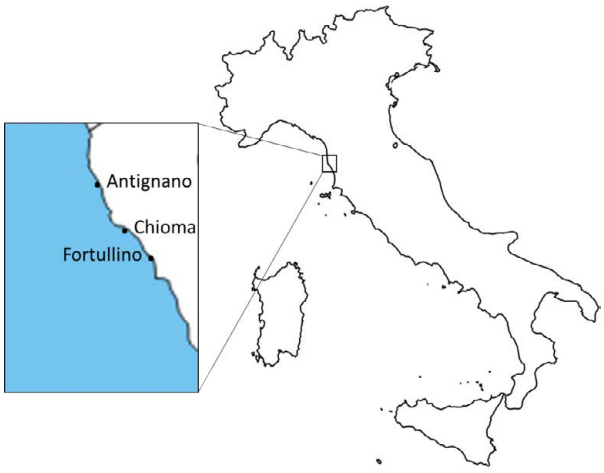

Figure 1: Location of the three populations analyzed in the present study.

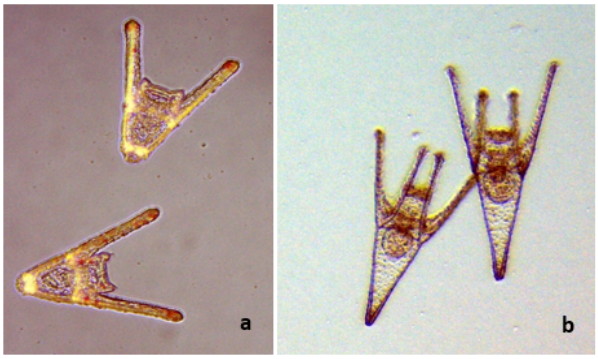

Figure 2: Four arms pluteus stage (P4) for *Arbacia lixula* (a) and *Paracentrotus lividus* (b).

Results

The good quality of gametes and exposure solutions was tested as reported in the Material and Methods section, with percentages of normal shaped plutei in control treatments >86% and concentrations measured in the range 94%<[Metal]<109%. EC_{50} values obtained in this experiment are reported in Table 1.

Cd

Among the three metals, Cd disrupted the embryological development with the highest variability among sites and times ($p<0.05$). The two species, demonstrated a different sensitivity to the solutions tested with *A. lixula* gametes being more affected by this metal ($p<0.001$, Figure 3). Populations of the three sampling sites registered different responses to the exposures, in particular the toxicity registered was Fortullino>Chioma>Antignano ($p<0.002$). On the contrary, toxicity registered in the populations of *P. lividus* in Chioma and Antignano was not different ($p=0.63$), with only the population in Fortullino presenting a higher sensitivity towards Cd ($p<0.001$).

Temporal variability affected the two species with a similar pattern; except similarities in the toxicity among the year, both *P. lividus* and *A. lixula* gametes collected in May (T3) were more sensitive if compared with the ones collected at the beginning and at the end of the year. In particular with regard to *P. lividus*, a difference was registered with T1, T2 and T5 ($p<0.001$); with regard to *A. lixula* the difference was between May (T3) organisms and T1, T4 and T5 ($p<0.001$).

Cu

As reported for Cd, *A. lixula* embryos were more affected by Cu exposure if compared with embryos of *P. lividus* (< 0.05). Among sites, the only significant difference was between Fortullino and Antignano for *P. lividus* populations, and between Fortullino and the other sites for *A. lixula* populations (Figure 4).

The plutei obtained after the fertilization of gametes belonging to specimens collected in May presented the lower EC_{50} values, with a statistical difference with the second part of the year for *P. lividus* (T4, T5) and a significant lower value compared to the first two and the last samplings for *A. lixula* (T1, T2, T5).

Zn

Zn was the metal that confirmed the lower toxicity in this experiment for *P. lividus* ($p<0.05$), but it affected plutei of *A. lixula* with similar toxicity of Cu ($p=0.63$). Sea urchins from Antignano population showed a higher resistance to Zn if compared with the other two sites only for *P. lividus* ($p<0.002$) whereas *A. lixula* showed no difference among all the three sites ($p>0.66$, Figure 5).

The temporal trend registered for *P. lividus* can be seen in EC_{50} values obtained for Zn, with results from the first and the last sampling (T1,T5) statistically different from the central part of the year ($p<0.009$ and $p<0.01$ respectively). The difference between the plutei obtained with gametes from the first sampling (T1) and the sampling in May (T3) resulted also for *A. lixula* ($p=0.01$).

Discussion

The two species tested in this experiment provided an overall substantial different response towards metals ($p<0.001$), *A. lixula* was the most sensitive species to both the three different sites and different periods of the year when adults were collected. Very few studies have been published on the analysis of potential dissimilarities between these sea urchins when used in toxicological assays, and no statistical difference has been registered between the two species [44-56]. In particular, Cesar et al., [44] tested the adverse effect of sediment elutriate in three different species of echinoderms, including *P. lividus* and *A. lixula*, using zinc sulfate as reference toxicant for the positive control. The number of normally developed plutei after exposure to toxicant was similar for all the species analysed. The same reference toxicant was used in Carballeira [55,56] and other EC_{50} values from previous studies were reported. Although the order

Table 1: EC_{50} values obtained after exposure of sea urchin embryos to different solutions of metals, values are expressed as μg L^{-1} (ppb). SD = Standard deviation.

	Paracentrotus lividus						*Arbacia lixula*					
	Antignano		Chioma		Fortullino		Antignano		Chioma		Fortullino	
	EC_{50} ppb	SD	EC_{50} ppb	SD	EC_{50} ppb	SD	EC_{50} ppb	SD	EC_{50} ppb	SD	EC_{50} ppb	SD
	Cd											
T1	2021.52	243.67	1612.34	262.12	1514.18	285.42	1688.45	176.35	1429.23	190.49	1164.36	254.56
T2	2212.56	160.63	1542.36	191.38	1589.38	216.38	1512.64	152.27	1512.32	186.85	901.24	40.39
T3	1500.52	160.32	1415.78	123.13	1412.69	182.86	1312.25	58.65	1385.08	154.35	887.45	39.47
T4	1765.28	202.17	1694.23	208.51	1238.45	84.89	1625.36	102.39	1418.67	83.15	1365.22	67.26
T5	1615.28	82.26	1827.34	81.37	1444.45	72.22	1532.16	211.68	1366.67	68.60	1545.85	77.29
	Cu											
T1	53.69	2.41	43.12	2.14	44.89	4.61	46.26	3.90	44.98	3.59	35.99	2.39
T2	53.02	2.71	44.06	3.10	40.73	2.34	42.42	4.14	44.16	4.95	37.02	2.38
T3	47.18	8.23	37.64	2.14	42.64	2.12	37.42	2.98	34.12	3.65	29.16	3.60
T4	52.91	2.24	56.17	3.52	49.97	3.93	45.25	4.19	36.25	3.27	33.15	1.66
T5	52.64	2.32	55.36	1.91	48.45	1.06	47.33	3.25	40.12	0.06	39.02	1.95
	Zn											
T1	101.84	20.42	88.16	5.81	91.03	9.47	41.14	3.12	45.63	2.65	46.18	2.95
T2	87.14	10.40	90.72	7.58	78.18	6.63	44.38	9.27	46.32	3.95	40.18	4.27
T3	82.15	8.76	79.79	6.18	72.15	6.37	32.15	2.84	33.15	3.12	28.25	2.84
T4	99.35	6.01	82.14	4.59	88.74	5.06	42.12	3.12	39.88	3.53	45.89	2.29
T5	92.15	4.64	90.16	4.56	91.14	4.62	42.99	5.44	40.15	2.21	47.28	2.75

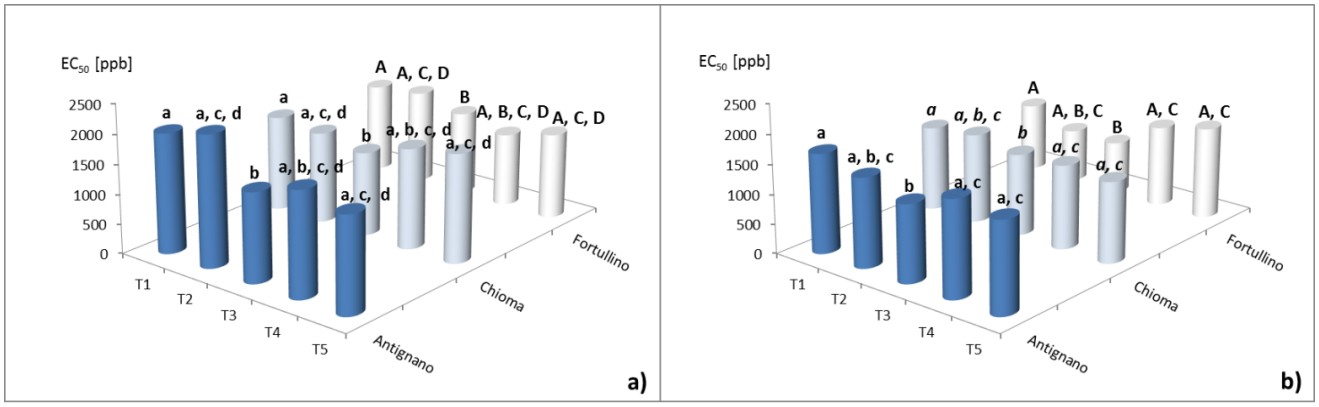

Figure 3: EC_{50} values referring to *P. lividus* (a) and *A. lixula* (b) embryos exposed to Cd solutions belonging to the three populations in analysis (Antignano, Chioma, Fortullino). T1=January, T2=March, T3=May, T4=September, T5=November. Significant differences among levels of the factor "Time" is expressed by different letters, and among levels of the factor "Site" by the use of normal, italic and capital fonts (see details in the text). Statistical significance $p<0.05$.

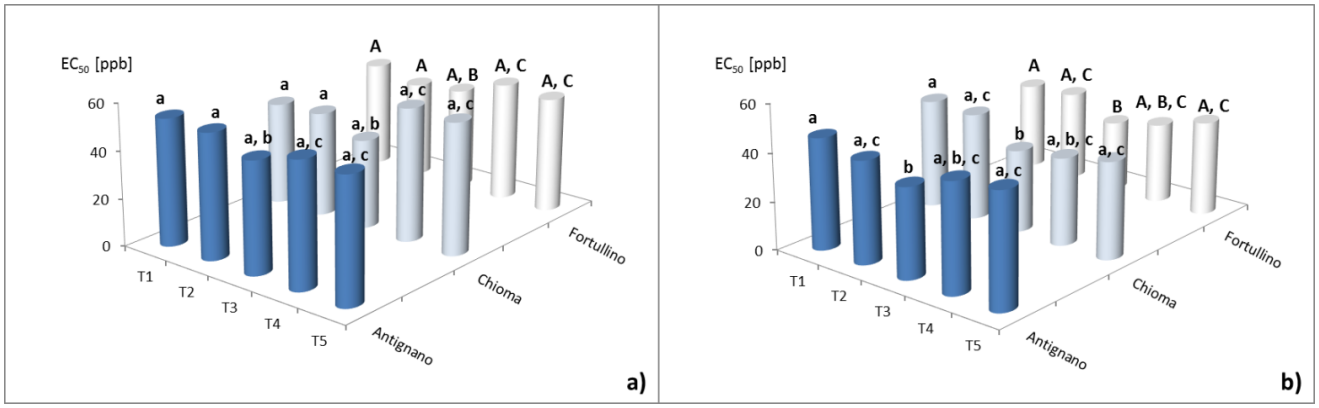

Figure 4: EC_{50} values referring to *P. lividus* (a) and *A. lixula* (b) embryos exposed to Cu solutions belonging to the three populations in analysis (Antignano, Chioma, Fortullino). T1=January, T2=March, T3=May, T4=September, T5=November. Significant differences among levels of the factor "Time" is expressed by different letters, and among levels of the factor "Site" by the use of normal, italic and capital fonts (see details in the text). Statistical significance $p<0.05$.

of magnitude of the results is similar to the values obtained in the present study, in the reported literature no statistical difference has been registered regarding the two species. To explain this discrepancy three hypotheses could be addressed. The first is related to the method used: no sperm/egg ratio has been set in the reported papers and a wider scale of concentrations was chosen for the EC_{50} calculation, resulting in a less accurate value in comparison with the present study. The second hypothesis could refer to a different chemical standard used in the experiment; or, finally, the genetic difference linked with spatial variability of populations within the Mediterranean basin select different metabolic pathways and, consequently, different sensitivities. Along with the first hypothesis, the latter seems to be the most probable explanation, considering the different responses registered in this study from three populations distributed within a radius of ca 10 km.

Fortullino, the site where embryos showed the highest sensitivity, is an area characterized by low population density and does not present in close proximity any productive activities, beach facilities, restaurant business or sewage discharges and for this reason it has been classified as "low human impact zone". The two other areas examined in this paper are more exposed to human interactions, in particular: the site "Chioma" is located near a small canal port built at the mouth of the river Chioma, the "Antignano" site is located within a short distance from a small marina (<1 km) and 7 km south from the Port of Livorno, one of the largest Italian and Mediterranean seaports. Furthermore, Fortullino is an area where, for over ten years, specimens of *P. lividus* have been collected for setting up biological assays, and whose sensitivity to the reference toxicant (Copper) has been recorded in a laboratory control chart [57]. This environmental quality of these sites has been investigated by the Regional Agency for Environmental Protection of Tuscany, and the results can be found in the report on Monitoring of Coastal Waters in Tuscany. In particular: in sediments from Antignano, the concentration of Hg, As, Cr, Cd, Ni benzo [b] fluoranthene, benzo [k] fluoranthene, exceeds to the chemical limits set in the DM 56/2009 (Italian Legislation), and in addition the concentration of Hg in water of Antignano exceeds the limits listed in Table 1/A of DM 56/2009, resulting in Hg concentrations in *Mytilus galloprovincialis* present in this area from 2010 to 2014 above the regulatory limits reported in Table 3/A DM 56/2009 [58]. Although the pluteus represents the planktonic larval stage of the sea urchin, the latter considerations underpin the correlation between the sensitivity of plutei to heavy metals or other contaminants with health of adult organisms which those plutei originate from. *P. lividus* (adult) is a species that lives in contact with the substrate and generally feeds with macroalgae, thus directly exposed to contaminants presents in the substrate.

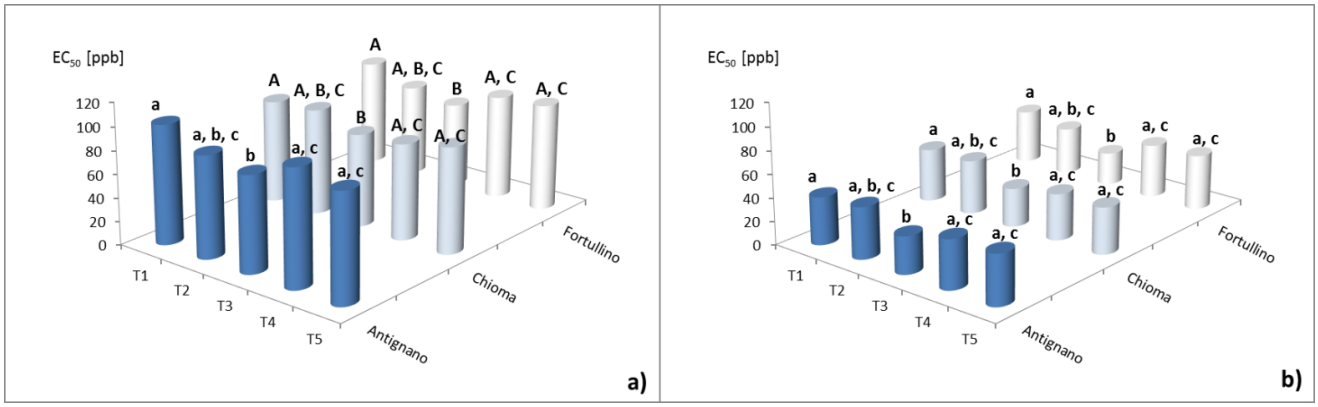

Figure 5: EC_{50} values referring to *P. lividus* (a) and *A. lixula* (b) embryos exposed to Zn solutions belonging to the three populations in analysis (Antignano, Chioma, Fortullino). T1=January, T2= March, T3=May, T4=September, T5=November. Significant differences among levels of the factor "Time" is expressed by different letters, and among levels of the factor "Site" by the use of normal, italic and capital fonts (see details in the text). Statistical significance $p<0.05$.

The quality of the gametes produced by *P. lividus* will therefore be affected by the presence or absence of contaminants in the area in which adults live and feed.

Interestingly, the significant difference between populations in this experiment for both species (Figures 3-5) and the outcome of Fortullino as the site most affected by metal toxicity (the less human impacted area), indicate that these populations have developed no form of biological resistance to trace metals. In all probability, the normal embryological development of their offspring can be remarkably affected by these pollutants. In general, as a consequence of the passed reproductive season, which in the Mediterranean Sea generally starts from October to June, the embryos developed from gametes collected in May were the most affected in this study, confirming previous observations collected by our research group (unpublished data). For all the metal tested, no statistical difference has been recorded between the T1 sampling corresponding to the beginning of reproductive season and the T5 sampling-related embryos, which had the summer and the beginning of autumn as recovery period for their gamete production (Figures 3-5).

With regard to literature data evaluating the toxicity of the same metals employed in this study, data are inconsistent and vary according to the metal analysed, in particular, in Cesar et al., [44,58,59] EC_{50} values relative to Cd ranged from 2.06 to 2.18 mg L^{-1} and from 1.80 to 2.06 mg L^{-1} for *P. lividus* and *A. lixula* respectively, whereas for Zn effective concentrations were 0.05 mg L^{-1} for *P. lividus* and 0.04 -0.05 mg L^{-1} for *A. lixula,* data similar to results presented in our trial. In the same works, similar EC_{50} were obtained when toxicity was assessed using *Sphaerechinus granularis*. Novelli [48], testing the effects of heavy metals on the embryo-development of *P. lividus* after 72 h, obtained EC_{50} values of 0.049 (0.045-0.503) mg L^{-1} for Zn, 0.062 (0.053-0.071) mg L^{-1} for Cu and 0.23 (0.1-0.27) mg L^{-1} for Cd. In general, with regard to Cu and Cd toxicity to *P. lividus*, EC_{50} values reported in literature agreed with data published in the present work, ranging from 0.02-0.11 mg L^{-1} and 0.5 to 11.24 mg L^{-1} respectively; whereas EC_{50} values obtained for Zn were lower than those obtained in our trials (0.02 to 0.58 mg L^{-1}) [36-38,43,46,47,51,57,60,61]. The variability of EC_{50} values in literature referred to Echinodermata underpins the conclusions demonstrated in the present paper, identifying temporal and spatial features as significant factors to be considered in Eco toxicological testing.

This assumption can be further confirmed analysing data reported for Cd toxicity to crustacean amphipods: *C. orientale* collected from the estuary of Magra river (La Spezia, Italy) showed EC_{50} data ranged from 1.21 mg L^{-1} in summer to 5.39 mg L^{-1} in winter; whereas for the same species sampled from a river 90 km distant (Serchio river, Pisa, Italy) EC_{50} values ranged from 1, 34 mg L^{-1} in summer to 7.39 mg L^{-1} in winter [45].

Other invertebrates have demonstrated to be more affected than sea urchins by the contaminants investigated in the present study. Indeed, EC_{50} values for Cu obtained with oyster *C. gigas* varied from 0.125 to 0.329 mg L^{-1} [62,63]; in another experiment, Nadella et al., [64] employed the model organism *Mytilus trossulus* to assess the toxicity for Cd, obtaining an EC_{50} value of 0.502 mg L^{-1}. The latter has been tested on *Crassostrea rhizophorae* and *Mytilus edulis* embryos, resulting in EC_{50} values ranging between 0. 211 and 0.316 mg L^{-1} and 1.200 mg L^{-1} respectively [65,66].

This study provides stimulating insights for consideration when performing embryo toxicity tests with *P. lividus* and *A. lixula* in both research and monitoring contexts. The findings of this study demonstrate that a spatial variability of population (ca 10 km radius) and the collection time of spawning animals can affect the outcome of the assay dramatically. Further analyses are required to assess the variability of a larger distribution of populations, considering the genetic variability that can be linked to the different responses obtained. The embryo toxicity test with echinoderms was again proven to be a very sensitive tool in environmental biology, but such sensitivity could be detrimental if spatial, temporal and genetic variability is not considered when interpret its results.

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