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PhD Thesis

Toxicological effects of metals on embryo development in sea urchin: applicative perspectives for ecotoxicological assessment criteria in harbor areas

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1. Introduction

1.1 General introduction

Ports play an important role in human society, being areas where economic activity related to the transportation, exchange and production of goods is concentrated, but they can heavily influence the marine environment (Onorati *et al.* 2013). In fact, industrial and urban activities such as shipping, loading and bunkering operations, shipyards, accidental spills and wastewater emissions are responsible for typically elevated concentrations of pollutants in seawater. Additionally, in harbor areas, modifications to the original morphology and the building of infrastructures create areas of low hydrodynamic energy, where contaminants in seawater are associated and subsequently accumulate with sediments (Montero *et al.* 2013; Bocchetti *et al.* 2008). Many literature studies have demonstrated over several decades that chemicals in sediments are responsible for toxicity (Williams *et al.* 1986; Chapman 1990, 2002; Carr *et al.* 1998; Chapman *et al.* 2002; Ho *et al.* 2002) and adverse ecological effects (Swartz *et al.* 1994; Bailey *et al.* 1995; Hartwell *et al*. 1997; Hatakeyama and Yokoyama, 1997). In this context dredging operations, which are necessary to maintain accessibility and navigational depth, disrupt the natural equilibrium between sediment and water, leading to remobilization of chemicals from the sediment with an increase in toxicity for marine organisms. Moreover, during excavation and discharge operations sediments are oxygenated and dispersed, and so, because of the change in redox potential, the contaminants may change their chemical speciation, and can be adsorbed on to silt particles and then enter the trophic food chain (Eggleton and Thomas 2004). For these reasons a proper assessment and management of dredged sediments is required, and various international conventions provide useful guidelines on this issue, including the protocols to the London Convention (IMO 1996, 2007), the Barcelona Convention (UNEP-MAP 1995a, b), OSPAR and the Helsinki Convention. These different conventions are in agreement on three basic principles:

- the precautionary principle: not all materials can be disposed of into the sea, but only after their chemical and biological characterization.
- the responsibility principle: those responsible for environmental contamination must bear the costs of reducing the pollution.
- coastal area integrated management: all aspects must be considered for every act in coastal areas, including environmental and socio-economic issues.

However, these guidelines are not mandatory, and European legislation does not deal specifically with dredged materials. In fact, these are usually regarded as waste, with the exception of sediments

relocated inside surface waters, which can be excluded from the scope of the Waste Framework Directive (2008/98/EC) if it is proven that these sediments are not hazardous. Therefore, the issue of contaminated dredged material has been regulated in each European country, with legislation often based on different approaches (Alvarez-Guerra *et al.* 2007; Manz *et al.* 2007; Onorati *et al.* 2013). In Italy offshore dumping of dredged material is regulated by Article 109 of Legislative Decree no. 152/2006, at present incomplete for lack of technical annexes. Other fragmented indications as to dredging material management can be found in Article 21 of Law no.179/2002 (coastal areas), Article 48 of Law no. 27/2012 and the Ministry Decree of 7 November 2008 (contaminated sites of national interest). Because of this lack of updated and comprehensive legislation on the handling of marine sediment, the National Institute of Environmental Protection and Research (ISPRA) has developed a handbook containing technical guidelines about the management of dredged materials (ICRAM-APAT 2007). The assessment criteria consist in a holistic and multidisciplinary approach integrating multiple lines of evidence to assess and manage the pollution of marine sediments. More specifically, sediment quality was defined after the integration of physical, chemical and ecotoxicological data. This started from the definition of the Sediment Quality Triad by Chapman *et al.* (1987), Chapman (1995), Chapman and Smith (2012), which involves three separate components, each of which can comprise one or more measurement endpoints: sediment chemistry analyses which measure contamination, laboratory toxicity tests which measure effects under standardized conditions (experimentation), and assessments of resident community alteration (generally the benthic infauna) which measure field conditions (observation) (Chapman 1996). Based on this approach, there is evidence of the unsuitability of only the chemical characterization for predicting the toxicity of complex matrixes such as dredged sediments (McCauley *et al.* 2000; Crane 2003). This "chemical" approach can be conservative in terms of protecting human health and the environment, but the real risk may be overestimated, resulting in long and costly management decisions (Bradham *et al.* 2006). For this reason, chemical analyses are integrated with toxicity bioassays, which have been applied worldwide to give quantifiable information about the potential for biological damage (toxic hazards) caused by bioavailable multi-factorial contamination, thus providing ecologically relevant responses not restricted by a predetermined list of contaminants (MacDonald *et al.* 1997; Wells 1999; Volpi Ghirardini *et al.* 2005). Bioassays are conducted either in controlled conditions in the laboratory or under field conditions (*in situ* bioassays) and consist of the exposure of test organisms to environmental matrices (sediment, water), in order to measure the toxicity quantifying the occurrence of toxic effects considering an ecologically-relevant quantitative response (Martinez-Haro *et al.* 2015). This is the main

difference between bioassays and toxicological studies, which aim at quantifying the toxicity of individual chemicals or mixtures of known composition by exposing whole living organisms under standardized conditions over a certain period of time (Cairns and Pratt 1989; Rand *et al.* 1995), the so called dose–response experiments.

The bioassay presents numerous advantages such as:

- Response to the bioavailable fraction of the toxicants, thus providing more ecologically relevant information
- Possible detection of the effects of non-conventional pollutants not measured by chemical analysis
- A rapid and cost-effective screening tool to detect polluted sites where thorough analytical chemistry may subsequently be performed.

However, the bioassay is not without its disadvantages. In fact, the results may not enable the identification of the individual causative agents of observed toxicity. Moreover, the data, although consisting of measured responses with implications for the biological fitness of the individual (e.g., mortality, growth, reproduction, feeding rates; see e.g., Rand *et al.* 1995), may not be extrapolated at ecosystem level but may be valid only for the test species. To minimize this limitation, a multispecies battery of bioassays is needed, including representatives of the main taxonomical groups of sea resources to be protected and possibly from very distant phylogenic groups (Beiras *et al.* 2003a). For this reason the guidelines of ICRAM-APAT (2007) on the management of dredged materials provide for the implementation of a battery comprising at least three test species of different taxonomic groups and selected from different trophic levels. These guidelines are based on a "pass to fail" approach, in which the ecotoxicological classification is determined by the worst result obtained in the battery of applied bioassays. On the basis of these data combined with chemical data, the sediment sample is assigned to one of three possible classes of quality. These guidelines have recently been revised using a multidisciplinary weight of evidence (WOE) approach, which has been validated in several environmental conditions (Piva *et al.* 2011; Benedetti *et al*. 2012, 2014; Regoli *et al.* 2014; Bebianno *et al.* 2015). The WOE approach considers various Lines of Evidence (LOEs), both as individual modules and as an integrated final evaluation, and it appears to be a powerful tool to support a more complex environmental risk assessment that addresses an overall decision-making process (Piva *et al.* 2011; Benedetti *et al*. 2012, Chiaretti *et al.* 2014). Among the possible bioassays, the sea urchin embryo test (SET) uses one of the most sensitive species, routinely used for the evaluation of dredged material, and which may easily influence the result of the entire battery. The protocol, based on standardized

methods, (ASTM 1995; USEPA 1995; Carr 1998; Environment Canada 2011) typically consists in the exposure of sea urchin eggs to elutriate of sediment and in the morphological observation of 100 randomly chosen embryos at the pluteus stage, which is reached after 48 hours at 20°C or 72 hours at 18°C (Arizzi Novelli *et al.* 2006). Alteration of embryo morphology generally consists in a delay in the developmental schedule and an impairment of the correct differentiation of germ layers: ectoderm, mesoderm and endoderm, and/or their derivatives (Timourian 1968; Livingston and Wilt 1989; Hardin *et al*. 1992; Russo *et al*. 2003; Bonaventura *et al.* 2005; Kiyomoto *et al.* 2010; Matranga *et al.* 2010, 2011; Pinsino *et al.* 2011b). However, embryos are conventionally classified in two major categories of biological quality, normal and abnormal, and the percentage of abnormal embryos in exposed groups is compared to the control frequency. In fact, although alteration of embryo development has been extensively described, these effects have rarely been considered to develop a standardized scale of toxicity assigning a different weight to various embryonic malformations depending on their severity. Only Carballeira *et al.* (2012) proposed calculating the percentage of abnormal larvae at 48h of exposure, classifying embryos in 4 classes with increasing toxicity on the basis of their observed skeletal malformation. Another general limit of the current approach is that developmental analyses are carried out only at 48h without distinguishing between different morphotypes occurring at stages before pluteus, thus preventing us from understanding if embryos exhibit malformation, block or delay of embryogenesis. Thus the evaluation and quantification of the different adverse effects on embryo morphology and development could greatly increase the sensitivity of the assay and its capability of discriminating from slight to severe levels of embryotoxicity.

The aim of this PhD study is to enhance the capability of the sea urchin embryo toxicity test to discriminate the ecotoxicity of dredging sediments, developing a sensitive, objective, and automatically readable endpoint that may influence the result of the entire battery of bioassays. In particular, after the investigation of the reversibility of teratogenic effects and the subsequent definition of a sensitive and realistic toxicity scale, a new integrative index of toxicity was developed. Different values were associated with each morphotype in relation to germ layer specific malformations and developmental stage. The overall results improved the capability of the *Paracentrotus lividus* embryo toxicity test to discriminate different samples, moving from the simple observation of general effects to the classification of their severity; this approach should allow for a better categorization of the teratogenic potential of marine sediments and their quality classification through a more sensitive and realistic index of toxicity.

1.2 Sea urchin *Paracentrotus lividus*: general characteristics

Paracentrotus lividus (Lamarck, 1816) belongs to Echinodermata phylum (class Echinoidea, Diademantoida order). Echinoderms are deuterostome with a well-developed coelom lined by peritoneum and containing coelomate fluid, which plays an important role in the circulatory system. Echinoids have a body protected by a dermal skeleton of calcareous plates, covered in spines and lined with skin, torn at the tip of each spine. The body is spherical with pentamerous symmetry. Each sector is composed of two zones, called radial and interradial respectively. Only the radial areas feature particular organelles, called tube feet, which have a locomotive and tactile function. Primary and secondary tubercles in which the spines are implanted are present in both ambulacral and interambulacral areas.

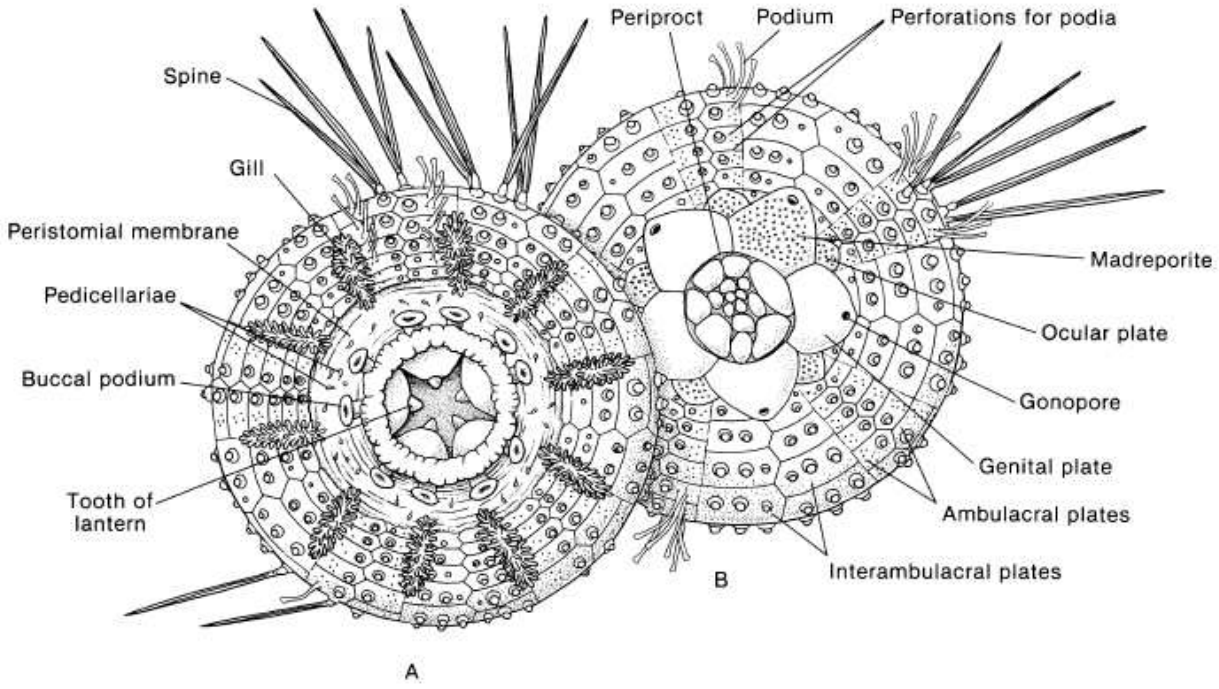


Fig. 1 – Anatomy of regular sea urchin. A: Oral view B:Aboral view. Figure from Sartori (2013).

The mouth and the anus are located on two opposite poles of the body. The oral area always faces downwards, in contact with the substrate. In the center of this area is a zone called the peristome covered with peristomial membrane and coated with small plates. The mouth is located at the center of the peristome and is formed of an ossicle system called Aristotele's lantern, a powerful masticatory organ. The intestine is long and flows from the mouth to the opposite pole of the organism (aboral

area). Specifically the anus is located in the midst of a round shaped area called the periproct and covered by many platelets. Surrounding the periproct there are 5 genital plates, where the gonopore is situated, and 5 ambulacral plates. The ambulacral plates are smaller than the previous one and located beside the genital plates (Fig. 1). Locomotion is enabled by the aquifer system. This is composed of a ring located around the mouth from which radial channels depart that cover the entire ambulacral area and branch into the tube feet. Water is actively pumped into the aquifer system, which also has a respiratory role. In fact, there is no specialized respiratory system, but only 5 pairs of coelomic expansion called branches, and the acquifer system contributes to increasing the exchange surface with the tube feet. Branches penetrate the aboral connective tissues and one enters each of the 5 gonads. A single gonoduct emerges from each gonad, which is covered with peritoneum and is located in the interambulacral area. A non-centralized nervous system is present, with a ring around the mouth in correspondence to the acquifer system (Sartori 2013).

Paracentrotus lividus is an echinoid with spines that, albeit not very long, are sharp and strong and evenly located throughout the body. This species is a rather large sea urchin. The diameter (horizontal test diameter without spines) of larger individuals can reach 7.5 cm (Lozano *et al.* 1995). In spite of its popular name ('purple sea urchin'), its color is highly variable: black-purple, purple, red-brown, dark brown, yellow-brown, light brown or olive green. Color is not related to depth or size and depends on spinocroms contained in spines in various proportions (Tortonese 1965; Gamble 1967). The analysis of sequence variation in a fragment of the mitochondrial gene cytochrome c oxidase suggests that *P. lividus* has two randomly mating populations in the Western Mediterranean and in the Eastern Atlantic, with panmixis within these two geographical areas (Duran *et al.* 2004). In these zones the areal distribution of *P. lividus* is from Scotland to Southern Morocco and the Canary Islands, including the Azores. It is particularly common in regions where Winter water temperatures range from 10 to 15 C°, and Summer temperatures from 18 to 25 C°, such as the Western Mediterranean, Portugal and the Bay of Biscay. Its northern and southern limits more or less correspond to 8 C° Winter and 28 C° Summer isotherms respectively. The typical habitat of this species is represented by subtidal rocky bottom from the mean low water mark down to 10–20 m, and intertidal rock pools (Gamble 1965; Tortonese 1965; Régis and Arfi 1978; Harmelin *et al.* 1980; Crook *et al.* 2000) with a salinity range between 15-20‰ and 39-40‰ (Pastor 1971; Allain 1975; Le Gall *et al.* 1989). Analysis of gut contents in field conditions indicates that *P. lividus* is basically herbivorous with several preferences that are in strong relationship with the habitat and the size of the organism (Kitching and Ebling 1961; Ebling *et al.* 1966; Neill and Larkum 1966; Traer 1980; Verlaque and Nedelec 1983; Verlaque 1984; Privitera *et al.*

2008). For this reason it is often defined as an opportunistic generalist (Boudouresque and Verlaque 2013). However, generally a number of species of algae are clearly 'preferred', such as *Rissoella verruculosa* (Rhodobionta, Plantae), *Cymodocea nodosa* (seagrass: Magnoliophyta, Plantae), *Cystoseira amentacea*, *Padina pavonica* and *Undaria pinnatifida* (photosynthetic stramenopiles). Other algae are strongly 'avoided', such as *Asparagopsis armata*, *Gelidium spinosum*, *Anadyomene stellata*, *Caulerpa prolifera*, *C. taxifolia* and *Flabellia petiolate*. In the Mediterranean Sea leaves of the seagrass *Posidonia oceanica* can constitute up to 40% of the gut contents of individuals located hundreds of metres from a meadow, especially in the Spring and Summer period (Maggiore *et al.* 1987; Bouderesque and Verlaque 2013). In this way, *P. lividus* plays a major role in determining the organization of benthic communities, with subtidal algal assemblages affected by both its feeding preferences and abundance (Barnes *et al.* 2002). The density of *P. lividus* shows a general and conspicuous negative correlation with erect algae coverage and biomass due to grazing activity with a subsequent shift to the macroalgal bed to extreme conditions in which only encrusting corallines are seen to grow. This particular ground is called barren (Sala and Zabala 1996; Bulleri *et al.* 1999; Bonaviri *et al.* 2011, 2012; Bertocci *et al.* 2012). Human impact can noticeably modify the ecological role of *P. lividus* by reducing its abundance through harvesting. In fact, its gonads have been appreciated as a seafood, especially in France and Spain (to a lesser extent in Italy and Greece) and *P. lividus* has been intensely harvested for export, especially in recent years (Régis 1987; Bertocci *et al.* 2012; Boudouresque and Verlaque 2013; Gianguzza *et al.* 2013). But human impact can also cause a dramatic increase in *P. lividus* density, directly through pollution or indirectly through overfishing of its predators (Sala *et al.* 1998a; Boudouresque and Verlaque; 2013). In fact, high densities of *P. lividus* and the consequent occurrence of barren ground are conditions typically linked with areas characterized by high fishery pressure and subsequently low density of target fish species such as *Diplodus sargus* and *Diplodus vulgaris*, the principal predators of the adult sea urchin and responsible for almost total predation in Mediterranean marine protected areas (Sala and Zabala 1996). However, there are also marine protected areas with low fish predation and where sea urchin fishing is not allowed. An example is Ustica Island, where a great increase in sea urchin density was observed (Gianguzza *et al.* 2006), with a subsequent increase in grazing activity and barren ground formation.

1.3 Reproduction and embryo development

In *P. lividus* the sexes are separate and there is no sexual dimorphism (Gianguzza *et al.* 2009) although hermaphroditism has been observed since the beginning of the last century (Drzewina and Bohn 1924; Neefs 1937; Byrne *et al.* 1990), and in this very particular case the sex ratio seems to change throughout the year and from one year to the next (Cellario and Fenaux 1990). Generally sexual maturity occurs at 20mm diameter and 5 months, but may occur later *in situ*. Overall, the gonad index is higher in 40–70mm than in 20–40mm size-classes (Martinez *et al.* 2003; Sanchez-Espana *et al.* 2004). In both sexes, the structure of the gonadal wall consists of 2 sacs of tissues (outer and inner). Each consists of several characteristic layers (Fig. 2B). Throughout the gonad, the genital coelomic sinus (GCS) separates the outer sac from the inner sac. The outer sac, which includes a visceral peritoneum (VP), consists mainly of a connective tissue layer (CTL). The inner sac is a genital haemal sinus (GHS) that supports the germinal epithelium on its luminal face. The principal functions of the inner sac are gametogenesis, very limited nutrient storage in the GHS, and extensive nutrient storage in the nutritive phagocytes. During the annual gametogenic cycle, gonads of both sexes of the sea urchin pass through a predictable series of structural changes briefly synthesizable as the accumulation of reserve material (growing phase), the effective spawning period (or maturation phase) and the reabsorption of relict gametes (spent/regeneration phase)), as is well documented in *P.lividus* (Byrne *et al.* 1990; Lozano *et al.* 1995; Spirlet *et al.* 1998; Martinez *et al.* 2003) and also in other sea urchin species such as *Strongylocentrotus droebachiensis* (Walker *et al.* 2005) and *Pseudocentrotus depressus* (Unuma *et al.* 2010). These changes are classified by the activities of two major populations of cells that compose the germinal epithelium: germinal cells and somatic cells called nutritive phagocytes (NPs) that are present in both sexes (Holland and Holland 1969; Kobayashi and Konaka 1971) and show an inversely proportional trend (Walker *et al.* 2005, 2013). In *P. lividus* there can be a single spawning period, as described for populations from the north west Mediterranean coast (Sala *et al.* 1998b; Hereu *et al.* 2004). In other cases, two spawning peaks are observed in early Spring and Autumn in Ireland and in some Mediterranean populations, with conspicuous differences between neighboring localities (Lozano *et al.* 1995; Fernandez 1998; Sanchez-Espana *et al.* 2004; Ouréns *et al.* 2011). Moreover, the reproductive cycle of *P. lividus* is influenced by many environmental factors such as temperature (Byrne *et al.* 1990; Lozano *et al.* 1995), photoperiod (Byrne *et al.* 1990; Lozano *et al*. 1995; Sphigel *et al.* 2004), hydrodynamic conditions (Guettaf *et al*. 2000; Giunguzza *et al.* 2013), food availability and quality (Régis 1979; Lozano *et al.* 1995; Guettaf *et al*. 2000; Sánchez-España *et al.*

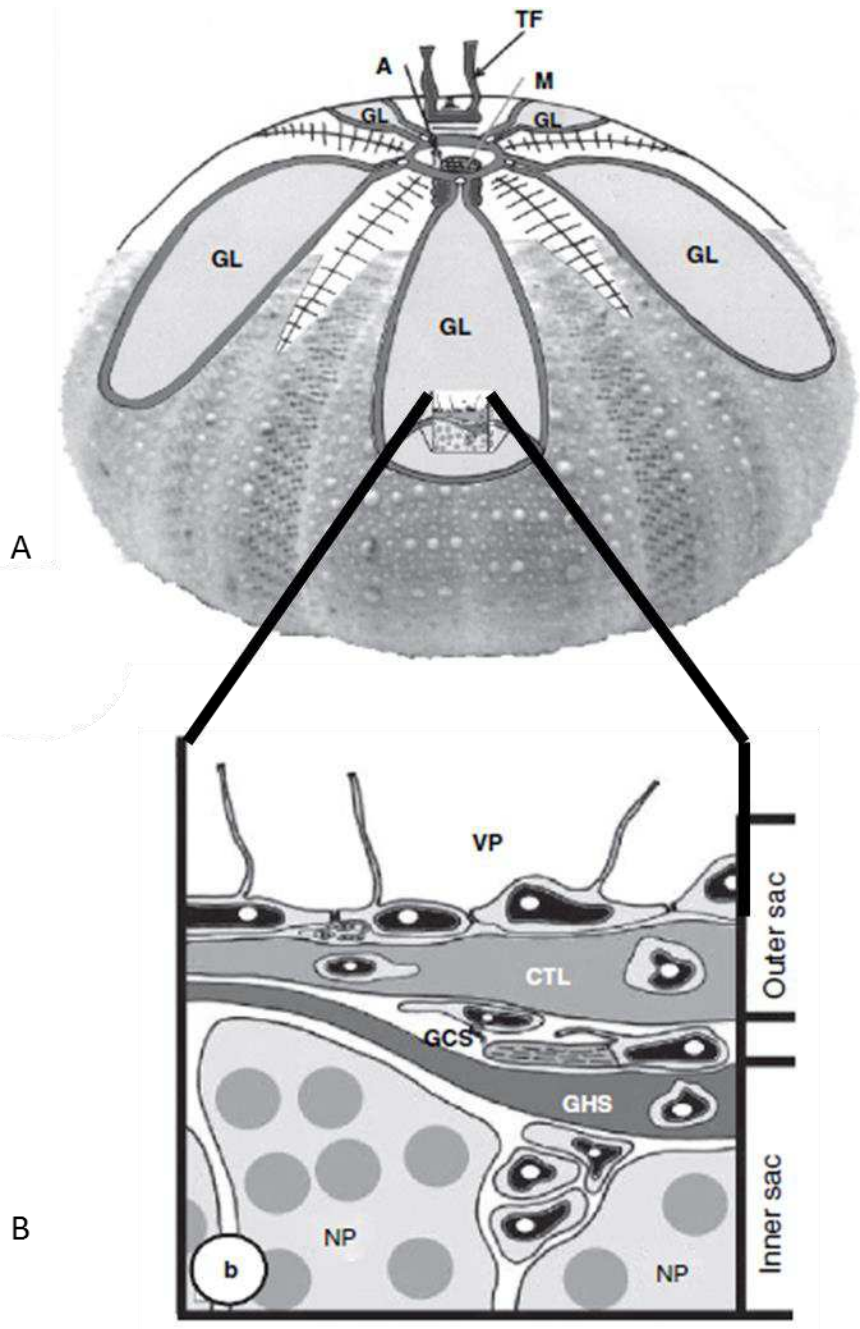


Fig. 2 – A: Diagrammatic representation of the sea urchin reproductive system in aboral side view; B: Diagrammatic representation of the tissues in the sea urchin gonad wall. A – anus; CTL – connective tissue layer; GL – gonad lumen; M – madreporite; NP – nutritive phagocytes; GCS – genital coelomic sinus; GHS – genital haemal sinus; TF – tube feet; VP – visceral peritoneum. Figure modified from Walker *et al*. (2007).

2004; Gianguzza *et al.* 2013), phytoplankton bloom (Ouréns *et al.* 2011) and it is possible to have an all-year round spawning period (Guettaf *et al.* 2000). Along the Italian coasts there occurs a single spawning period, which generally runs from October to June (Arizzi Novelli *et al.* 2002) with a maximum during the early Spring with temperatures around 13.5-18°C and a short photoperiod, in accordance with other literature studies (Byrne *et al.* 1990; Lozano *et al.* 1995; Shpigel *et al.* 2004). In this period the female gonad contains the mature eggs, which are isolecithal and possess relatively low quantities of yolk. The eggs complete their meiotic division inside the ovary and are released into sea water as haploid, metabolically inert mature egg cells (Spinelli and Albanese 1990). The sea urchin spermatozoon is a metabolically inert, elongated cell with a tail and a head occupying most of the volume and containing the acrosomal vacuole with several enzymes and other proteins. As long as they are stored in the gonads, the sperms are immobile and do not respire at a significant rate. As reported by Johnson *et al.* (1983) this is probably due to the low pH of the seminal fluid. During the reproductive season spawning occurs by muscle rhythmic contraction which cause the gamete release (Okada *et al.* 1984; Okada and Iwata 1985) in response to the carbohydrate portion of a glycoprotein produced in the intestine and stored in the aboral haemal tissues. Interconnecting nerves synchronize the activities of the gonads during spawning (Takahashi *et al.* 1990, 1991). Males spawn first and spermatozoa are activated, beginning to swim upon contact with seawater, which gives rise to an increase in the intracellular pH value. Fully mature eggs are then released into a cloud of actively swimming spermatozoa. The egg activation involves a series of signal transduction steps after sperm binds to a receptor protein on the egg surface raising the fertilization membrane and giving rise to an electrical response which imposes a fast block to polyspermy (Whitaker and Steinhardt 1985; Giudice 1986; Mah *et al.* 2005). Thus the fertilization is external and embryo development occurs in the plankton. Sea urchin exhibits a radial, holoblastic cleavage well described by Spinelli and Albanese (1990), Matranga and Bonaventura (2002) and Pruliere *et al.* (2011). The second cleavage plane falls again along the animal-vegetal (A/V) axis (meridian) and at 90° from the first, as illustrated in Fig. 3B, which report drawings of the embryo at each stage of development. The A/V axis is observable in *P. lividus* due to the presence of a subequatorial cortical pigmented band visible under blue light. The third cleavage is equatorial, perpendicular to the first two cleavage planes, and gives rise to an embryo with 8 equally sized cells (Fig. 3C). During the fourth cleavage, animal blastomeres divide equally to produce 8 mesomeres, and the vegetal blastomeres divide unequally to produce large macromeres and small micromeres located at the vegetal pole of the embryo (Fig 3 D). From this stage on the pattern of cleavage becomes more complex and the cell divisions are no longer synchronous. The 8 mesomeres in

the animal pole will form 2 tiers of 8 cells each ('an1' and 'an2'), which are the precursors of ectoderm cells. At the vegetal pole the 8 macromeres will generate, by meridian cleavage, a tier of 8 cells which will subsequently divide equatorially forming 2 tiers of 8 cells each, called 'veg1' and 'veg2' (Fig. 3E). Micromeres will generate after 2 divisions 8 cells which remain quiescent for a long time. Further divisions, still enclosed by the fertilization envelope, bring the embryo to the morula stage (Fig 3F). Six hours after fertilization the sea urchin embryo enters the early blastula stage with an empty central cavity called a blastocoel. The formation of the cavity may be due to the adhesion of the cells to the hyaline layer surrounding the embryo and other osmotic factors. The cells start developing cilia on the outer surface of the embryo to form a swimming blastula (Fig 3G). Approximately 4-6 h later midblastula, composed of 600 cells, hatch out of the fertilization envelope. At the animal pole the cilia are longer but do not beat. This "apical tuft" provides directionality to swimming, as embryos almost always move with the apical tuft region forward (visible in Fig. 3H). At this stage cell fates are fully specified and cells have begun to express particular sets of territory-specific genes (Davidson *et al*. 1998). In particular the clonal descendants of these 8-cell mesomeres rise to oral and aboral ectoderm (Cameron *et al.* 1987), which both contribute to ciliated band formation (Cameron *et al.* 1993). Some *veg1* progeny is constituted by endoderm which will contribute to the hindgut and midgut (Ransick and Davidson 1998), and the remainder will contribute to aboral ectoderm. *Veg2* domain will originate the other part of the gut (endoderm) and all mesodermal elements except for the coelomic pouch constituents, that derive in part from the small micromeres, and the skeletogenic mesenchyme. From swimming blastula morphogenic movements which will rise the internal structures of the embryo begin. In the late blastula stage the embryo becomes thickened at the vegetal pole, forming the vegetal plate. Then 32 cell descendants from the micromeres and located in the center of the vegetal plate region protruding in the basal end enter the blastocoel and start wandering with amoeboid movements. They are now differentiated in primary mesenchyme cells (PMCs) and this is the mesenchyme blastula stage (Fig. 3H). Soon after, gastrulation occurs with the invagination of the descendants of 'veg2' blastomeres, which start to form the archenteron (Fig 3I). Vegetal cells will meanwhile continue to ingress in order to form the archenteron led by filopodia extending from the secondary mesenchyme cells (SMCs), which make contact at the opposite wall of the blastocoel (Fig. 3I-L). Then the PMCs form 2 columns of cells on the 2 sides of the embryo forming 2 triradiate spicules the formation and orientation of which are defined by spatial and temporal signals depending on PMCs (Zito *et al.* 2003; Duloquin *et al.* 2007; Rottinger *et al.* 2008). The embryo starts to flatten and the area of contact of SMCs in the animal pole starts to invaginate forming the mouth (stomodeum) and the prism stage is

reached (Fig. 3M). In this phase PMCs transmit an inhibitory signal to SMCs preventing their differentiation into skeletogenic mesenchyme, thus promoting the production of a variety of differentiated mesodermal cells, suggesting that SMCs function as multipotent stem cells (Kiyomoto *et al.* 2007; Zito and Matranga 2009). The 2 triradiate spicules secerned by PMCs continue to elongate, evolving into the skeletal rods composed of 2 oral and 2 post-oral rods, two transversal rods and 2 body rods. Skeletal rods consist mainly of $CaCO_3$ (calcite) with some $MgCO_3$ (in 1:10 ratio) and organic matrix, deposited within linear synchitial aggregates formed by fusion of the PMC pseudopodia (Dubois and Chen 1989; Wilt 2002, 2005). Approximately 48 hours after fertilization and as a result of these processes, the embryo forms the four-armed pluteus larvae (Fig 3O). Embryos at this stage of development are feeding larvae, with fully differentiated skeleton, gut and contractile and nervous cells, swimming in sea water by movements of the cilia, located in the arms and coordinated by a kind of nervous system (Garner *et al.* 2015). The duration of the planktonic life of larvae of *P. lividus* (Fig. 3O-P) has been estimated to be 23–29 days *in situ* (Lozano *et al.* 1995). After this period embryos fixed at the substrate undergo a metamorphosis that will generate the adult sea urchin (Fig 3Q). In the Mediterranean Sea, benthic settlement may occur once or twice throughout the year and generally a peak is observed in late Spring (May–June), apparently regardless of substrate characteristics (Lozano *et al.* 1995; Hereu *et al.* 2004; Tomas *et al.* 2004).

1.4 *Paracentrotus lividus* as a bioindicator of environmental stressors

The adult of *P. lividus* seems to be relatively insensitive to organic pollution, and in fact such compounds clearly enhance its development (Tortonese 1965; Zavodnik 1987). Numerous populations of sea urchin are documented in polluted areas such as the Bay of Brest (Brittany-France) near the mouth of urban waste discharge, at Rabat (Morocco) in the outflow of Marseilles (France) and in Berre Lagoon, near Marseilles. At these polluted sites the density of *P. lividus* increases progressively near discharge points (Kempf 1962; Harmelin *et al.* 1980, 1981; Delmas and Regis 1985; Bayed *et al.* 2005). *P. lividus* withstands high levels of trace metal pollution, and even accumulates them (Warnau *et al.* 1998; Soualili *et al.* 2008), and is also an efficient accumulator of polychlorinated biphenyls (PCBs) (Coteur *et al*. 2003; Danis *et al*. 2005). In spite of the low sensitivity towards contaminants, the bioaccumulation capacity and the response of immune cells, the adults of *P. lividus* have been used as a bioindicator in several marine biomonitoring programs (Warnau *et al*. 1998; Soualili *et al*. 2008; Pinsino *et al.* 2008). In contrast, *P. lividus* appear to be very sensitive to a large range of contaminants during their early planktonic life stages, the most critical for their survival and in which they are

constantly exposed to pollutants deriving from different kinds of human activities (Bellas *et al.* 2008; Rosen *et al.* 2008). Considering the high sensitivity at low concentrations of contaminants and its discriminatory capability, the sea urchin embryos is considered an excellent tool to assess the toxicity of many chemical compounds and environmental stressors. Indeed, the transparency of the embryos/larvae has made the sea urchin a well-recognized developmental biology model the use of which in eco-, embryo- and genotoxicological studies has recently been appreciated (Geraci *et al.* 2004; Bonaventura *et al.* 2005; Bellas *et al*. 2008; Byrne *et al.* 2010; Kiyomoto *et al.* 2010; Romano *et al.* 2010; Aluigi *et al*. 2012). Toxic effects on *P. lividus* embryo development are well documented for a large variety of stress-inducing agents such as heat shock (Giudice *et al.* 1999), metals (Arizzi Novelli *et al.* 2003; Russo *et al.* 2003; Roccheri and Matranga 2009; Pinsino *et al.* 2010, 2011a, 2014; Moureaux *et al*. 2011), natural toxins (Romano *et al.* 2010; Varrella *et al*. 2014), pharmaceuticals (Graillet and Girard 1994; Aguirre-Martínez *et al.* 2015), biocides (Pesando *et al*. 2003; Marín *et al*. 2007), X-ray and UV radiation (Bonaventura *et al.* 2005, 2006; Matranga *et al*. 2010; Russo *et al.* 2014). In these laboratory experiments, sea urchin embryos are usually incubated with the stressor during the early stage of development in order to study the response of the organism. The classic evaluation of the effects consists in the analysis of morphological abnormalities, that are easy to observe with an optical microscope, especially from fertilization to the pluteus stage. However, several studies have analyzed the teratogenic effects on embryos and larvae also with an evaluation of gene expression and protein levels (Matranga *et al.* 2010; Pinsino *et al.* 2011a; Russo *et al.* 2014). In fact, many defense strategies and pathways have evolved to protect these embryos against adverse environmental factors, permit optimal development and guarantee species survival (Goldstone *et al.* 2006; Hamdoun and Epel 2007) and thus different cellular pathways can be investigated to find biomarkers of induced specific stressful conditions. In this context levels of Heat Shock Proteins (HSPs) are one of the most diffuse biomarkers of environmental stressors (Nesatyy and Suter 2008; Pinsino *et al.* 2011a). Stress proteins have been highly conserved during evolution and are widespread across the entire spectrum of organisms from bacteria to humans. Appropriate stress responses are essential for survival and repair of stress induced damage as well as during normal cellular homeostasis (Diller 2006).

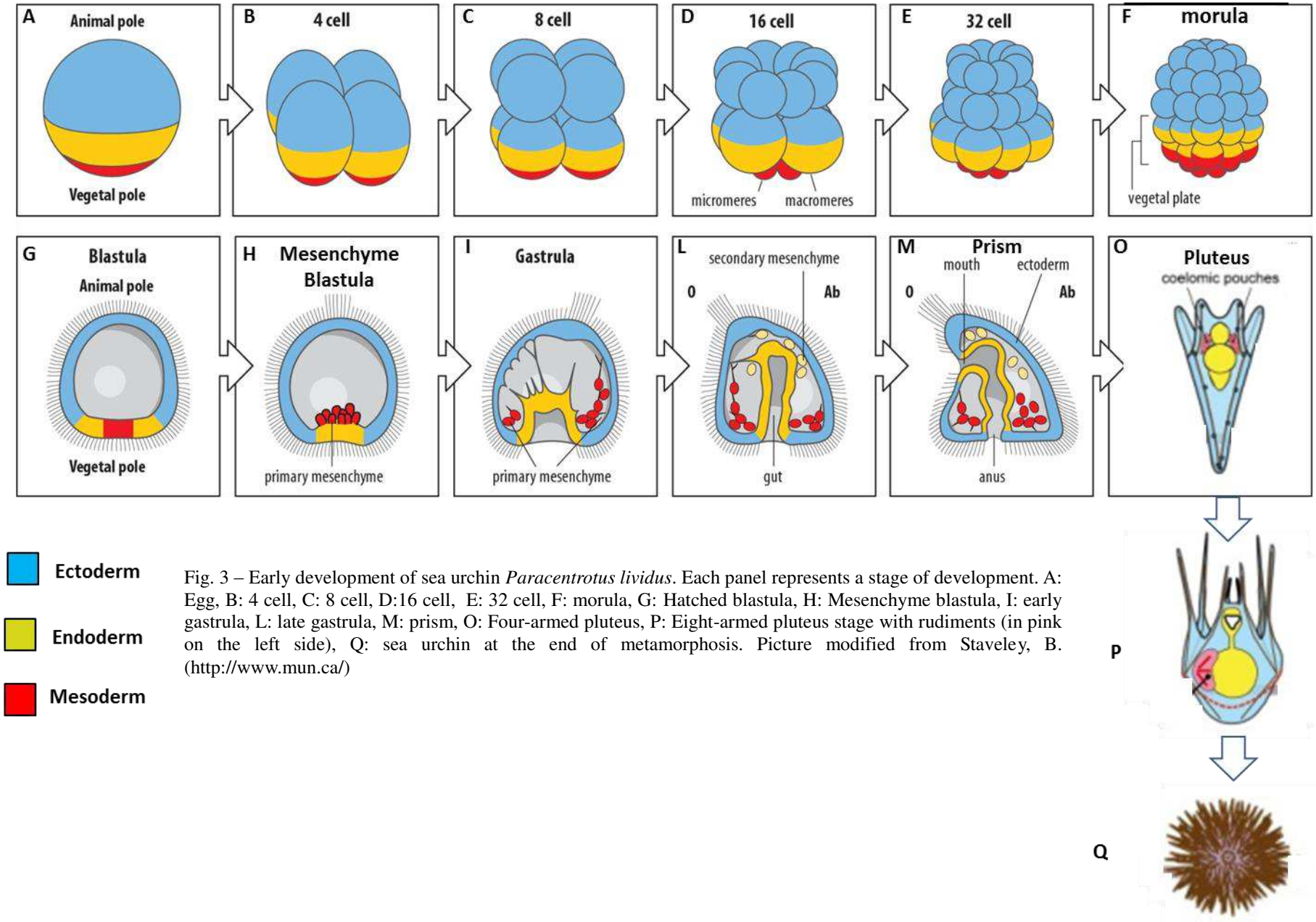


Fig. 3 – Early development of sea urchin *Paracentrotus lividus*. Each panel represents a stage of development. A: Egg, B: 4 cell, C: 8 cell, D:16 cell, E: 32 cell, F: morula, G: Hatched blastula, H: Mesenchyme blastula, I: early gastrula, L: late gastrula, M: prism, O: Four-armed pluteus, P: Eight-armed pluteus stage with rudiments (in pink on the left side), Q: sea urchin at the end of metamorphosis. Picture modified from Staveley, B. (http://www.mun.ca/)

Stress proteins belong to multigene families that include 2 different forms of proteins: constitutive forms (HSCs) and stress-inducible (HSPs) forms, which serve as molecular chaperones. Particularly the HSP70 family participates in protein synthesis and maturation; folding, assembly, and disassembly of protein complexes; proteolysis; and intracellular trafficking. HSP70 affects the activity of key regulatory proteins, cell proliferation, stress resistance and apoptosis (Gupta *et al.* 2010; Palotai *et al.* 2008). Thus, stress response is clearly recognized as a ubiquitous biomarker of environmental stressors (Pinsino *et al.* 2011a). The stress response is regulated by the heat shock transcription factor (HSF) (Wu 1995) which is present in the cytoplasm and in the nucleus in a monomeric form. Under stress conditions, HSF forms a trimer, which causes conformational changes that permit HSF to bind to the DNA-responsive element, the heat shock element (HSE), in the promoters of stress-inducible genes. Activation of HSF can increase or have no effects on transcription of HSP genes (Swindell *et al.* 2007; Tulapurkar *et al.* 2009). In the nuclei of *P. lividus* embryos, HSF binds HSE under physiological growth conditions (18–20°C), and this binding activity increases upon heat shock (31°C) (Sconzo *et al.* 1997). Furthermore, metal treatment results in synthesis of HSPs and the induction of metallothionein (MT) genes (Liu *et al.* 2009; Natoli *et al.* 2009; Forti *et al*. 2010). This induction is mediated by the cis-acting DNA element, the metal-responsive element (MRE). MTs are small metal-binding proteins that are involved in metal ion homeostasis and detoxification. In *P. lividus*, eight MT genes have been identified, and all of these genes are expressed at high levels during embryo development and larval stages (Pinsino *et al.* 2011a; Ragusa *et al.* 2013).

1.4.1 Sea Urchin Embryo Test (SET) with Paracentrotus lividus in ecotoxicological studies

Sea urchin embryos are, as reported above, often used in toxicological studies with the aim of quantifying the toxicity of well-known stressful conditions by exposing whole living organisms under standardized conditions over a certain period of time, the so called dose–response experiments. In environmental studies, the sea urchin embryo test (SET) consists of the exposure of fertilized eggs in controlled conditions to environmental matrices (water, sediment) whose toxicity it is intended to assess with an ecologically-relevant quantitative response (Martinez-Haro *et al.* 2015). Normally the test is performed in controlled conditions in the laboratory, although several authors have tried to carry out the test also under field conditions (Beiras *et al.* 2001; Salamanca *et al.* 2009), which can provide a more realistic exposure scenario than that conducted under laboratory-controlled conditions, by integrating major natural fluctuating environmental variables (Allan *et al.* 2006; Crane *et al.* 2007; Wharfe *et al.* 2007). The classic endpoint is represented by the percentage of normally developed

embryos at the 4-armed pluteus stage, which is reached 48 hours after fertilization. The observed biological effect in each sample is generally the result of the bioavailability of a complex mixture of pollutants that may be present in a sample of water/sediment, but is also dependent on physicochemical parameters of the environment (Keddy *et al.* 1995).

Thus SET is a rapid, sensitive and cost-effective biological tool for the evaluation of marine sediment and seawater quality worldwide. For these reasons it has been used for monitoring or risk assessment programs for several decades (Kobayashi 1971, 1991; Dinnel *et al.* 1988; Pagano *et al.* 1988; His *et al.* 1999; Beiras *et al.* 2003a; Kobayashi and Okamura 2004; Volpi Ghirardini *et al.* 2005; Cesar *et al.* 2009; Khosrovyan *et al.* 2013). As suggested by Dinnel *et al.* (1988) the sea urchin embryo test with *P. lividus* presents several advantages, such as:

- A very common and easily harvested species in Mediterranean Sea
- Easy to maintain in laboratory conditions and to induce it to spawn
- A long spawning period compared with other sea urchin species, with the possibility to obtain gametes all year (Guettaf *et al.* 2000).
- High number of gametes and thus the possibility to have a good replication of the experiment
- High sensitivity to a wide range of environmental pollutants
- Developmental Biology and Ecology of *P. lividus* are well-known
- The test is fast and cheap.

However, the test is not without its disadvantages, including the lack of a real methodological standardization with a clear methodology, a stricter quality control to guarantee optimum laboratory handling and biological material, and the identification of confounding factors that interfere with the measured response (OSPAR 2007; Saco-Alvarez *et al.* 2010). In fact, most studies apply a standardized protocol for embryo toxicity testing based on the method reported by EPA (ASTM 1995; USEPA 1995; Carr 1998), although slight differences may occur depending on the exposed sea urchin species and laboratory conditions.

Nowadays the SET is included within a holistic and multidisciplinary approach integrating multiple lines of evidence to assess and manage the pollution of marine waters and sediments. In this context, the use of a multispecies battery of bioassays is recommended, including representatives of the main taxonomical groups of sea resources to be protected and possibly from very distant phylogenic groups (Beiras *et al.* 2003a). Compared with many alternative test species, *P. lividus* is very sensitive to most environmental pollutants with effect concentrations comparable to those present in water column and pore-water. This is also confirmed for organic compounds and trace metals (Arizzi Novelli *et al.* 2003;

Beiras *et al*. 2003b; Bellas *et al.* 2005; Manzo *et al*. 2014), demonstrating the potential ability of SET to detect important contaminants in the ecosystem in coastal and lagoon environments. For this reason the SET has a central role in the battery of bioassays, which appear to be especially useful to investigate the causes of ecological impairment, allowing for a better understanding of the cause–effect-relationships and to work as early warning, rapid evaluation and cost effectiveness systems of ecosystem disturbance (Martinez-Haro *et al.* 2015).

1.5 Objectives and general description of the thesis

The goal of this PhD study is to enhance the capability of the sea urchin embryo toxicity test to discriminate the ecotoxicity of dredged sediments, developing a more sensitive approach to a better discrimination of the different levels of toxicity. To achieve this objective, the first part of the study was aimed at characterizing the main developmental anomalies induced by exposing *P. lividus* embryos to trace metal solutions. In these experiments, after the definitions of metal toxicity ranges, it was investigated whether specific trace metals induce specific malformations and whether such induced damage may be reversible, a still unclear but important issue for an ecological assessment. This part of the study was considered functional to the identification of a severity scale of developmental anomalies, based on the understanding of temporal evolution and reversibility of malformations during the embryo development. In particular the characterization, quantification and reversibility of developmental anomalies induced by trace metal solutions were investigated by incubating an aliquot of embryos for 24 h and then continuing the development in filtered control seawater up to 72 h. Analyses of developmental defects were performed at 24, 48 and 72 h on continuously-exposed embryos and on washed embryos. All trace metals to which embryos were exposed showed a similar trend with maximum toxic effects at 48 h, corresponding to the pluteus stage in control embryos. However, most of the embryos, especially those exposed at low and medium concentrations of metals, presented reversible anomalies, reaching the correct developmental stage at 72 h. Interestingly, specific malformations, time of occurrence and severity of effects depend on the metal considered. The results of morphological observation of metal-induced effects clearly indicated that measuring only the percentage of normally developed plutei at 48 h after fertilization is too simplistic an endopoint. Dose-response curves obtained with larval length criteria have lower slopes and the sensitivity of this endopoint is confirmed only at a low percentage of effects. Accordingly, these preliminary results highlight the urgent need to develop a different endpoint based on specific malformations at each stage of development during the early embryogenesis of the sea urchin, with particular attention to pre-

pluteus stages and to a possible reversibility of effects. The data obtained demonstrate that germ layer impairments may have different levels of severity, causing a different inability to properly differentiate ectoderm, mesoderm and endoderm territories and to recover the correct development of the embryo after 24 hours. Consequently, to obtain a more realistic and precise estimation of toxicity, it appears necessary to classify developmental anomalies into different categories on the basis of the severity of germ layer impairments. Following these criteria, a new integrative toxicity index (ITI) was developed in which the toxicity is quantified by counting the frequency of delayed and/or abnormal embryo morphologies and by quantitatively ranking the severity of effects from 0 (none) to 10 (high). Lower toxicity values were given to embryos with delay in development and absence of malformations, whereas higher scores were attributed to embryos showing contemporarily delayed and moderately impaired left/right or dorso/ventral axis symmetry as well as germ layer territories, using different categories based on severity of effects. The highest scores were attributed to delayed embryos with severe developmental anomalies, which cause the loss of the normal body organization and the appearance of embryos with aberrant morphology. Different scales and scores were tested by comparing with expert judgement the calculated indices with morphologic observations.

The toxicity index was developed applying the toxicity scale to embryos exposed to trace metal solution and elutriates of marine sediments. Furthermore, the toxicity scale was validated to estimate the embryotoxicity of sediment elutriates from Trapani harbor, comparing the elaboration of the results obtained with the conventional criteria. For these samples, morphological observations were integrated with chemical analyses of sediments and with different approaches aimed at investigating the occurrence of differing biological damage, such as the production of heat shock proteins (HSP60 and HSP70) and genotoxic damage (comet assay). The results obtained with the classical approach show that the majority of the elutriates tested exhibit a high toxicity, but also a significant decrease in toxicity after depuration. A large number of elutriates exhibiting the maximum toxicity with conventional criteria showed lower values of toxicity when elaborated with the new index (between 5 and 9): such a discrepancy is mostly attributed to the presence of less severe developmental anomalies, which also supports the high reversibility during the embryogenesis up to 72 hours. The new index of toxicity always showed as a higher discriminatory capability than conventional criteria. These biological effects observed on sea urchin development were not completely explained by chemical analyses of pollutants. Concentrations of some trace metals (Zn, V) and NH^{4+} appeared to be positively and moderately correlated with index values at 24 hours, although only NH^{4+} exhibited relatively high levels which might be responsible for the different ecotoxicological classification obtained with the 2 approaches.

Levels of HSP60 and HSP70 in exposed embryos did not exhibit evident trends and these values did not appear to be correlated with developmental endopoints and chemical parameters; the only positive correlation was found between embryos at gastrula with endoderm and mesoderm defects and HSP70 level at 24 hours. The comet assay protocol presented some technical problems in the lysis phases and a reliable procedure has still to be standardized before making any potential associations with embryo toxicity endopoints.

In conclusion, samples characterized by extremely low levels of embryotoxicity are not affected by the approach chosen for evaluating ecotoxicological results. On the other hand, the possibility to discriminate various intensities of adverse effects, and to provide different scores according to developmental stage and severity of malformations, appears particularly relevant for moderately and highly toxic sediments. Considering these samples, the new index indicates that the toxicity was often overestimated by the standard method. With the newly developed approach, the sensitivity of the *P. lividus* embryo toxicity assay is enhanced, supporting its usefulness in practical applications such as sediment classification and the choice of more appropriate management options in harbor areas.

2. Characterization of metal induced embryo malformations in *P. lividus*

2.1 Introduction

Urban and industrial activities in coastal areas introduce significant amounts of pollutants. Trace metals represent one of the most dangerous contaminants for marine organisms. The term "trace metals" identifies a large group of metallic elements which are present in living organisms in concentrations lower than 1 µg/g of dry weight (Templeton 2003). These elements persist in the environment, move up the food chain and could cause toxic effects if they are concentrated in larger amounts than physiological conditions (Ringwood 1992; Watzin and Roscigno 1997; Manzo *et al*. 2010). Trace metals are usually divided into two subclasses; the first includes Fe, Mg, Mn, Co, Zn and Cu, which are "essential" for the correct functioning of biochemical processes. Cd, Hg, Cr, Pb etc. belong to the second subclass, which is made up of metals without any established biological function and includes the more important contaminants in the aquatic environment (Viarengo 1985). A fraction of trace metals is directly dissolved in the water (Puls *et al.* 1997), but the greater part also tends to be adsorbed upon suspended particulate matter (SPM) and subsequently accumulated in the sediments. Sediments can accumulate metals at concentrations 10,000 times higher than in the overlying water column (Förstner 1979), constituting an important source of contamination and risk for living organisms, especially during the remobilization caused by dredging operations (Fichet *et al*. 1998; Geffard *et al*. 2007). Many biomonitoring studies which intend to assess the ecotoxicological quality of contaminated sediments routinely use larval bioassays with marine invertebrate embryos. In this context, the sea urchin embryo test is one of the most sensitive and widespread bioassays. Several studies demonstrate negative effects of trace metal on sea urchin embryos exposed to polluted elutriate of marine sediments (Beiras *et al*. 2003) and also waters (Kobayshi and Okamura 2004, 2005). However, most authors performed dose–response experiments incubating, under standard conditions, sea urchin embryos with known compositions of individual metals or mixtures in order to quantify the induced teratogenic effects (Warnau *et al*. 1996; Fernandez and Beiras 2001; Radenac *et al.* 2001; Arizzi Novelli *et al.* 2002; Phillips *et al.* 2003; Xu *et al*. 2011). Most of these studies were carried out using the standard method, classifying the embryos only at the pluteus stage into normal/abnormal and estimating metal toxicity as Median Effect Concentration (EC50). Slight differences in observed effects may depend on the genetics of the sea urchin population, the protocol used and the laboratory conditions. Generally, these articles dedicate only a part to a synthetic description of induced malformations which, according

to several authors, seem to involve mostly the correct development of skeletal rods and cause the appearance of undeveloped embryos. Although other studies have investigated several cellular pathways affected by metal detoxification (Geraci *et al.* 2004; Filosto *et al.* 2008; Pinsino *et al.* 2011a; Ragusa *et al*. 2013; Migliaccio *et al*. 2014), an explanation and hypothesis of a mechanism of cellular toxicity on the sea urchin embryo has rarely been discussed. Moreover, the specific morphological effects of trace metal have rarely been considered, and no studies have assessed the possible reversibility of these effects during the early life stage, yielding no understanding of whether embryos exhibit, block or delay embryogenesis before the pluteus stage.

The aim of this part of the study was to characterize and quantify the main metal-induced anomalies in *P. lividus* embryo development. In particular, after the definitions of metal toxicity ranges, it was investigated whether specific trace metals induce specific malformations and if this induced damage is reversible or not. To achieve this objective, experiments were carried out allowing to discriminate between developmental block and developmental delay, an unclear point in many literature studies. This study is part of a broader context and is functional to the identification of a severity scale of development anomalies, based on their temporal evolution and/or reversibility during embryo development.

2.2 Materials and methods

Collection and maintenance of P. lividus: Adults of sea urchin *P. lividus* were collected in a subtidal zone far from sources of disturbance caused by human activity and located off the Southern coast of Livorno (43°25'37.10"N, 10°23'48.27"E), NW Mediterranean Sea, Italy. Immediately after collection the animals were placed in polystyrene containers partially filled with seawater from the sampling site, in order to minimize the stress due to transport, in accordance with the indications of Volpi Ghirardini and Arizzi Novelli (2001); Falugi and Angelini (2002). The animals were acclimatized for up to one week in flowing seawater from the sampling site maintained at a temperature of 15 ° C ± 1, a salinity of 38 ‰ and natural photoperiod.

Embryo bioassay: The sea urchin embryo toxicity test was performed starting from standard procedure USEPA (1994, 1995, 2000); ASTM (2004) and literature data: Arizzi Novelli (2003); Volpi Ghirardini *et al.* (2005); Carballeira *et al.* (2012). Three male and three females were induced to spawn by injecting 1 ml of 0.5 M KCl through the perioral membrane. After fertilization, 1 ml of fertilized egg suspension (1000 embryos per ml) was added to 9 ml aliquots of test solution and incubated in a dark

room at 20°C for 48 h using sterile capped polystyrene six-well microplates. At the end of the experiment, the samples were preserved in 1ml of concentrated buffered formalin and 100 embryos were counted.

Definition of metal toxicity ranges: Embryos were exposed to different toxicants in order to identify the most suitable substances and concentration ranges. Metal solutions were prepared using a concentrated standard solution for atomic absorption spectroscopy (1 mg/L): cadmium nitrate, copper nitrate, mercury nitrate, lead nitrate and zinc nitrate in nitric acid 0.5 M/L (Sigma Aldrich). The bioassay was conducted on 4 different dates, exposing embryo suspension at 5 increasing concentrations of each toxicant solution (3 replicates per concentration) (Fig. 4) prepared by dissolving concentrated solutions of metals in 35‰ and 0.45 µm filtered seawater (FSW). FSW was collected at the same site and date as the adult sea urchins and was also used for negative control. After 48 h of incubation at 20°C, the percentage of normally developed plutei and the typology of malformation observed were checked. Embryos are classified as normal if they satisfy the following morphological criteria: (1) correct schedule in reaching the developmental endpoint (gastrula at 24 h and pluteus at 48 h), (2) left/right and dorso/ventral embryonic axis symmetry, (3) differentiation of oral/aboral ectoderm and endoderm territories, (4) skeleton development and patterning (for a review see Henry 1998; Pinsino *et al.* 2010). Embryos were classified as normal plutei, malformed plutei or undeveloped embryos (the stages before pluteus). The acceptability of the test results was fixed as a percentage of normal plutei ≥ 70% in control tests (Volpi Ghirardini *et al*. 2005) and only if the EC50 using the reference toxicant (Cu) fell within previously defined acceptability ranges for both tests (34.598 – 68.344 µg/l).

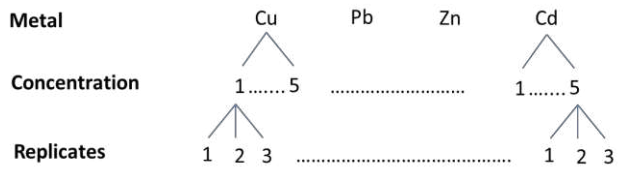


Fig. 4 – Experimental design

Characterization of malformation typologies and reversibility of developmental anomalies: In this part of the study cadmium nitrate, copper nitrate, lead nitrate and zinc nitrate in nitric acid 0.5 M/L (Sigma Aldrich) were used. The bioassay was performed at 20°C exposing embryo suspension, as described above, at 4 increasing concentrations of each toxicant solution (Table 1) selected on the basis of the first part of the study. Different aliquots of the same pool of embryos were incubated with metal for 24

h and then allowed to continue developing in filtered control seawater for up to 72 h. Analysis of developmental defects was performed at 24 h, 48 h (standard time of incubation) and 72 h after fertilization. The embryo was classified using the standard method described above. At each morphological observation, the following were also checked: 1) stage of development 2) occurrence of malformation. Embryos were considered as being well developed that had correctly reached the late gastrula stage (24 h after fertilization) and the pluteus stage (48 and 72 h after fertilization). In addition, at morphological analysis larval length was also checked at 0, 1, 18, 24, 48 and 72 h on embryos exposed to each metal. Larval length was measured as the distance between the apex and post-oral arm, which represents the maximum dimension. Minimum sample size for each replicate was fixed at n=30 embryos, as recommended by Saco-Álvarez *et al.* (2010).

Data analysis: The percentages of abnormal larvae and size inhibition were considered to evaluate the percentage of effect. Toxicity data are expressed as EC50 and EC10 values. The EC values with 95% confidence limits were calculated by the Probit method. Responses to each were corrected for effects in control tests by applying Abbott's formula (Volpi Girardini and Arizzi Novelli 2001).

2.3 Results and discussion

Definition of metal toxicity ranges: the embryo test showed a negative control of 85 ± 3% of normally developed pluteus (n=4). Regarding positive controls, copper was used as reference toxicant and showed a mean EC50 ± SD of 52.275 ± 4.755 µg/l (n=4), a value within the acceptability range (34.598 – 68.344 µg/l).

Toxicity data expressed as median effective concentration (EC50) are reported in Table 1. The metals tested show in order of toxicity: Cu> Zn> Pb> Cd and values are comparable with those of the literature regarding *Paracentrotus lividus* (Warnau *et al*. 1996; His *et al*. 1999; Fernandez and Beiras 2001; Radenac *et al*. 2001; Arizzi Novelli *et al.* 2003). The major part of these data are similar to those reported in other studies. For example, Cu shows values (EC50= 52.28 µg/l) close to those found by other authors such as Arizzi Novelli *et al*. (2003) (EC50 = 62 µg/l); Fernandez and Beiras (2001) (EC50 = 67 µg/l). Also for Zn, the data are comparable with Radenac *et al.* (2001) (26 and 96% of abnormalities at 50 and 250 µg/l) and with Arizzi Novelli *et al*. (2003) (EC50 = 49 µg/l). Comparable data were also obtained for Pb (EC50 = 124.38 µg/l in the present study and 68 µg/l in Arizzi Novelli *et al*. (2003). In contrast, Cd values are not in agreement with those reported by Fernandez and Beiras (2001) (1655.2 µg/l in this study and 9240 µg/l reported by the authors). The differences between these studies are probably due to different methods for testing and in the sea urchin pool of gametes.

Regarding the embryo toxicity test, further considerations of trace metals involve the observation of embryotoxic effects. All tested metals show a similar trend, with a gradual decrease in normally developed plutei at increasing concentrations and a gradual increase in undeveloped embryos. The metals show, at medium concentrations, moderate malformations at the pluteus stage (between 1 and 25 %) (Fig. 5). At high concentrations there is a gradual increase in the percentages of undeveloped embryos up to 100%. The increase in percentage of malformations at the pluteus stage is particularly evident for Zn, Pb and Cd. In particular, Zn and Pb show a great increase in malformed plutei at concentrations between 70 and 100 s µg/l. For Cd, particularly grave anomalies were observed at concentrations between 1.5 and 2 mg/l. Most of these anomalies induced by metals seem to involve an impairment of the pluteus skeleton.

On the basis of toxicity data and morphological observations obtained, 4 suitable concentrations were selected for each metal (Table 2) to use for the ensuing experiments having the aim of characterizing the malformation typologies and of assessing the reversibility of developmental defects.

METAL	EC50 (µg/l)	LITERATURE RANGE (µg/l)
Cd	1655.23 (1123.69-2038.14)	230- 9240
Cu	52.28 (47.91 – 57.34)	20-115
Pb	124.38 (114.34 – 132.36)	40-540
Zn	91.30 (76.22 – 116.38)	20-580

Table 1 - EC50 values obtained for embryo bioassay compared with EC50 data from literature. Data are expressed in µg/l with respective 95% confidence limits.

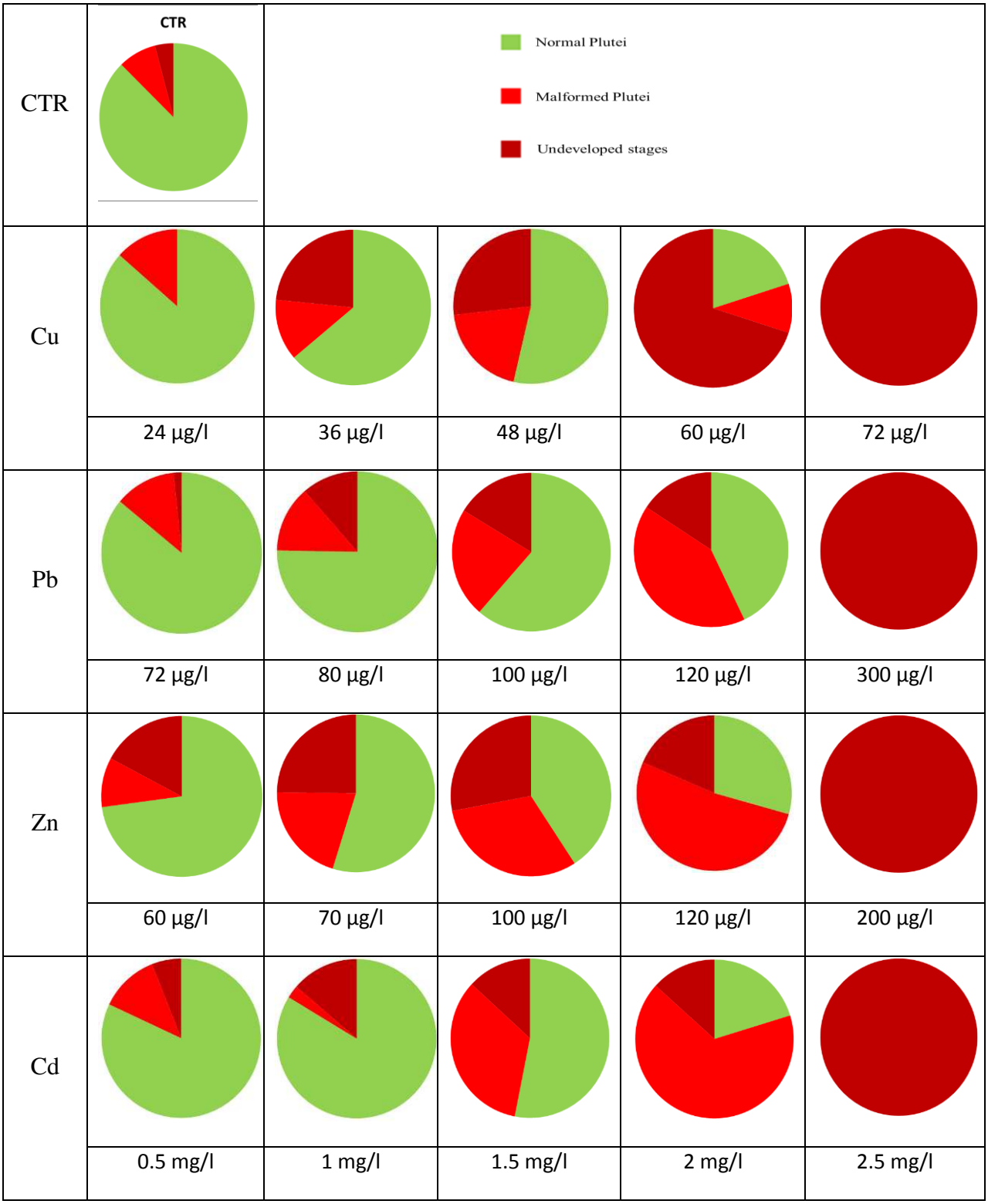


Fig. 5 – Analysis of embryo developed is reported in the pie charts. The green sectors represent the percentage of normally developed plutei and red sectors the percentage of abnormal plutei. In particular, light red indicates the embryos with undeveloped stages and dark red the embryos with malformations, as reported in the legend in the upper part of the panel. On the rows are reported the metals (the first one refers to control embryos) and under each pie chart is reported the exposure concentrations expressed in µg/l or mg/l.

Characterization and reversibly of malformations typologies: On the basis of EC50 obtained during the first experiments 4 concentrations of 4 trace metals were selected (Table 2). Bioassay results show negative control of 87 ± 2% of normal plutei at 48 h and an EC50 = 45.78 ± 4 µg/l for Cu, used for positive control. These values fall within acceptability ranges (≥70% negative control, between 34.598 and 68.344 µg/l positive control).

METAL	CONCENTRATIONS (µg/l)			
Cu	30	50	60	70
Pb	80	100	120	250
Zn	60	70	100	120
Cd	1000	1500	2000	2500

Table 2 – Nominal metal concentrations used in the experiments expressed in µg/l.

Copper: *t*oxicity results show that copper appeared to be toxic already at 24 h with 24% of abnormal embryo at 60 µg/l and 45% at 70 µg/l (Fig. 6). At high Cu concentrations a percentage of these embryos reached mesenchyme blastula stage correctly (20% at 60 µg/l) (Fig 6B) or blastula stage with severe malformations (Fig. 6C), a morphotype which increases with increasing Cu concentrations (4% at 60 and 37% at 70 µg/l). At 48 h an increase in toxicity was observed at all metal concentrations. In particular, slight skeletal malformations (between 6 and 13%) at the pluteus stage at 30 and 50 µg/l with crossed (Fig. 6E) and separated skeletal rods on the apex (Fig 6F) were observed. Embryos exposed to high Cu concentrations show a greatly increased prism stage (26% at 50 µg/l, 41% at 60 µg/l and 71% at 70 µg/l) (Fig. 6G). A percentage of embryos exhibited severe malformations and were blocked at the gastrula stage (13% at 50 µg/l, 30% at 60 µg/l and 6% at 70 µg/l). 24 hours later (72 h after fertilization) embryos appeared to reach the early pluteus stage (Fig. 6D) at 50 µg/l (32%), while at 60 and 70 µg/l fewer than 7% reached the pluteus stage and development was arrested at the prism stage. These data fit with Warnau *et al.* (1996) and Arizzi Novelli *et al.* (2003), who observed a decrease in plutei and an increased prism stage at 70 µg/l. At 72 h, a slight decrease in toxicity was observed, on average, at all metal concentrations.

Concerning the reversibility experiment, there is no significant difference at 48 h between embryos washed at 24 h and non-washed embryos (Fig. 7). A great decrease in toxic effect was observed at 72 h in washed embryos, especially at higher Cu concentrations. In fact, at 50 µg/l there is a shift from 32%

(48 h) to 70% of normal plutei (72 h). This tendency is more evident at 60 and 70 µg/l, concentrations which show 10% of normal plutei at 48 h and 63% of normal plutei at 72 h. Regarding the developmental analysis at 60 µg/l, only 15% of embryos were blocked at the pluteus stage; most of them were merely delayed, reaching the pluteus stage correctly (63%). At 70 µg/l the percentage of embryos blocked at the prism stage went from 70% (48 h) to 13% (72 h). The other delayed embryos reached the pluteus stage (67%). In non-washed embryos the difference between 48 h and 72 h was less marked, with a maximum increase in normal developed pluteus of 15%. Moreover, some of these embryos that reach the prism stage showed a delay in development of up to 60 µg/l (40% of prism stage at 48h and 20% at 72 h), while at 70 µg/l all embryos (70% of total embryos) are blocked at the prism stage.

Cu is known to render its toxic action, among other ways, through inhibiting the activity of membrane located carrier proteins such as ATPases (Li *et al.* 1996) as well as carbonic anhydrase (Zimmer *et al*. 2012), resulting in a disruption of the ionic balance. In addition to changes in whole-body Na, K, and Mg levels, large increases in whole-body Ca concentration occurred accompanied by substantial variations in unidirectional Ca uptake from the external seawater and in Ca^{2+}-ATPase activity, a key enzyme of Ca metabolism. A Ca deficiency was found by Tellis *et al*. (2014a) in sea urchin *Strongylocentrotus purpuratus* exposed to Cu already at 24 h after fertilization, with a return to normal Ca uptake rates at 48 h. One hypothesis is that these tendencies, which differentiate Cu effects from those caused by the other metals, may partially explain the greater number of observed malformations already at the gastrula stage also in *P. lividus* embryos. Moreover, the metal-induced Ca deficiency may have serious implications for normal sea urchin larval development considering that this ion is a major constituent of the spicule (Wilt 1999, 2002; Raz *et al*. 2003) and could explain the slight spicule problems observed at 48 h. Pinsino *et al.* (2011a) observed the implication of different cellular pathways in *P. lividus* embryos exposed to Cu. In particular, this article demonstrates that Cu alters the phosphorylation state of HSC70, which may result from modulation of HSC70 via a post-translational mechanism that does not involve gene transcriptional induction. Moreover, Cu appears to be involved in metal-chelating systems through an increase in two Metal Regulatory Element (MRE) complexes (C1 and C2), which bind the consensus MRE sequence and regulate the expression of Metallothionein (MT) genes. Pinsino *et al.* (2011a) also observed greater morphological effects at 48 h, and this fits with the present study. Despite this morphological observation, the first evidence of the detoxification mechanism was observed 2h after fertilization. A possible explanation for the observation of the current study is that the detoxification mechanism started during segmentation. With a continuous

exposure, embryos exhibit toxic effects at the pluteus stage, but embryos washed at 24 h can partially make up for the delay at prism stage at 72 h thanks to the effects of detoxification mechanisms and the absence of Cu in seawater.

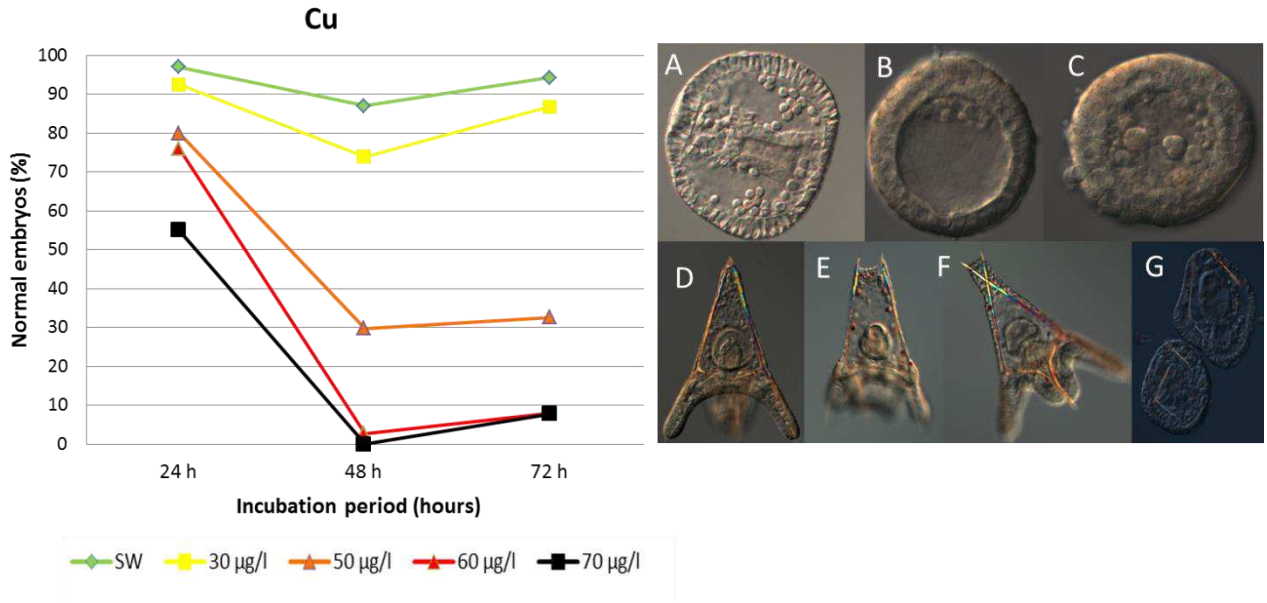


Fig. 6 –Non-washed embryos: Percentage of normal embryos (ordinate) at different times of incubation (abscissa) for each Cu concentration (colored lines). SW: control. On the right side the observed morphotypes : A: Late Gastrula stage, B: Mesenchyme blastula stage, C: malformed blastula stage, D: pluteus stage, E: separated tip, F: crossed tip, G: prism stage.

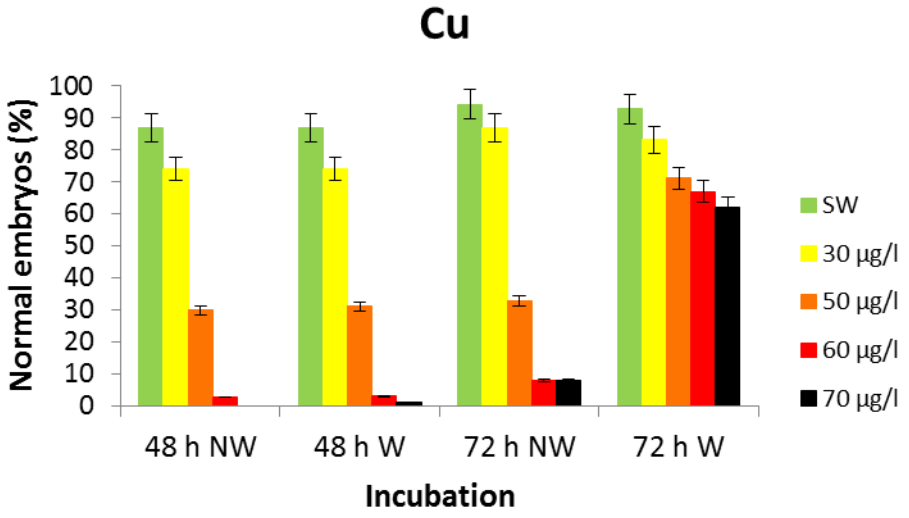


Fig. 7 – Histograms represent the results from the morphological analysis expressed as mean percentage (%) of normal embryos ± standard deviation. Data refer to non-washed embryos (NW) and to washed embryos (W) 48 hours after fertilization (first 2 groups of bars from the left) and 72 hours after fertilization (third and fourth groups of bars from the left). The colored bars represent the different exposure to Cu concentrations as reported on the right side of the figure.

Lead: Pb-exposed embryos show moderate developmental anomalies at 24 h with 65% of normal embryos at 250 µg/l and percentages higher than 75% at other Pb concentrations (Fig. 8). Malformed embryos at the blastula stage (Fig. 8C) (between 6 and 9% at each Pb concentrations) and embryos which reach the gastrula stage correctly with a slight delay (Fig. 8B) (19% at 120µg/l and 25% at 250 µg/l) were observed.

From 24 h to 48 h an increase in toxicity was observed, especially at higher concentrations (from 73% to 60% of normal embryos at 100 µg/l, from 72% to 20% of normal embryos at 120 µg/l and from 65% to 0% of normal embryos at 250 µg/l). From these data it appears clear that Pb caused skeletal malformations at the pluteus stage with crossed (13% at 80 µg/l and 11% at 100 µg/l) (Fig. 8F) and speared spicules (3 and 18%). At concentrations over 120 µg/l there was a shift from skeletal abnormalities to the prism (Fig. 8G) and late gastrula stage (Fig. 8A). Fewer effects were observed at 72 h, with a great decrease in toxic effects and with a percentage of normal embryos greater than 80% except for embryos exposed to up to 120 µg/l of Pb. In particular, the delay at gastrula and prism stage at 120 µg/l appeared reversible and all embryos reached the pluteus stage (with only 15% of skeletal anomalies). At 250 µg/l a fraction of prism embryos observed at 48 h appeared to reach the early pluteus stage at 72 h (29% correctly and 8 % with skeletal anomalies) and Gastrula embryos reached the prism stage, which at 72 h represented 54% of total embryos. At 250 and 72 h there were only 10% of normal pluteus, that is, from 8 to 9 times fewer than at lower Pb concentrations.

Concerning the reversibility experiment, there is no significant difference at 48 h between embryos washed at 24 h and non-washed embryos (Fig. 9). Also at 72 h very slight differences were observed between washed and non-washed embryos at concentrations of up to 120 µg/l, with comparable values greater than 80% of well-developed plutei. Only at 250 µg/l were there more normal plutei in washed embryos (28%) than non-washed embryos (10%). In washed embryos all the gastrula and prisms observed at 48 h reached the pluteus stage, although 72% exhibited severe skeletal malformations, with the absence of arm differentiation. In general Pb toxicity is thought to occur by replacing divalent ions such as Zn and Fe and by calcium mimicry (Ballatori 2002). Tellis *et al.* (2014b) is the only study with the aim of investigating the toxicity mechanism of Pb on sea urchin embryos. The study demonstrates Pb as having the most pronounced effects on gastrulation, as Ca uptake and accumulation. Ca^{2+}-ATPase levels were the most affected at this stage. This is the stage of development at which spicule formation accelerates, and thus can involve spicule calcification after gastrulation and may explain the skeletal anomalies observed at the pluteus stage in the current study. This may also explain why marked toxic effects were not already observed at the beginning of gastrulation. Moreover, Tellis *et al.*

(2014b) observed an intermittent recovery of unidirectional Ca uptake rates on Pb-exposed embryo, suggesting the occurrence of damage repair during continued Pb exposure. Considering this trend, it is possible to hypothesize that the great decrease in toxicity observed at 72 in non-washed embryos (especially at concentrations lower than 120 µg/l) could depend on the end of Ca uptake alteration and on the damage repair mechanism, which also appears to involve the activation of HSP70 and, moderately, the induction of MT genes (Pinsino *et al.* 2011a). Probably the embryos exposed to 250 µg/l of Pb develop too severe skeletal damage during spicule formation, enabling the washed embryos to reach the pluteus stage (differently to non-washed embryos), albeit developing evident problems in calcification of the arms.

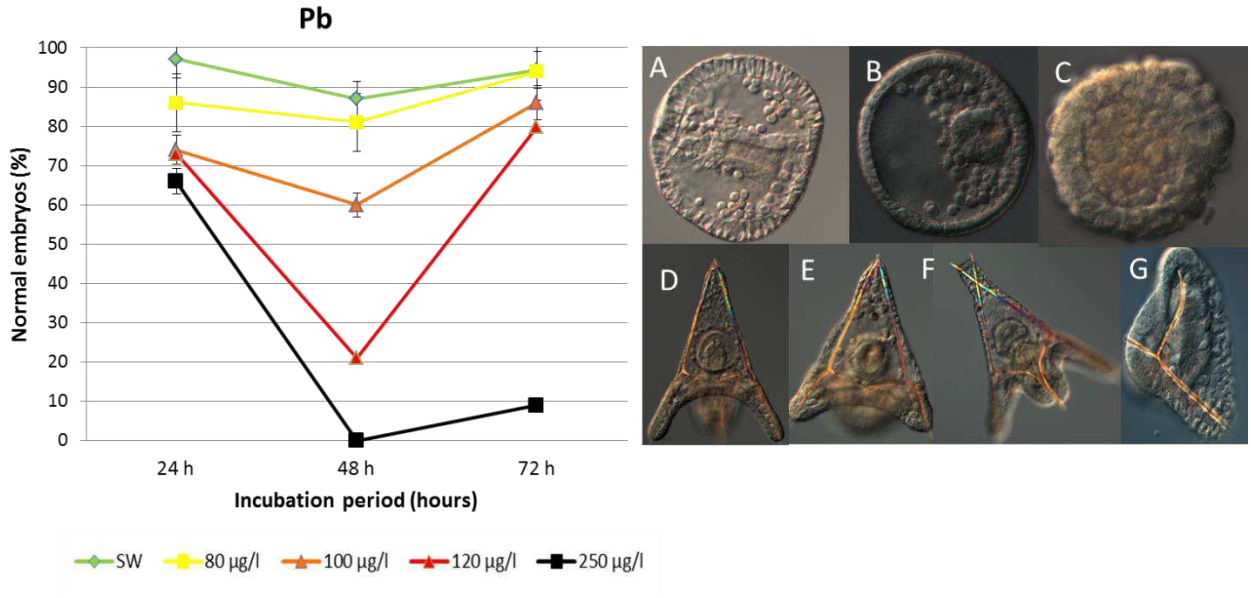


Fig. 8 – Non-washed embryos: Percentage of normal embryos (ordinate) at different times of incubation (abscissa) for each Pb concentration (colored lines). SW: control. On the right side the observed morphotypes : A: Late Gastrula stage, B: Early gastrula stage, C: malformed blastula stage, D: pluteus stage, E: early pluteus stage, F: crossed tip, G: prism stage

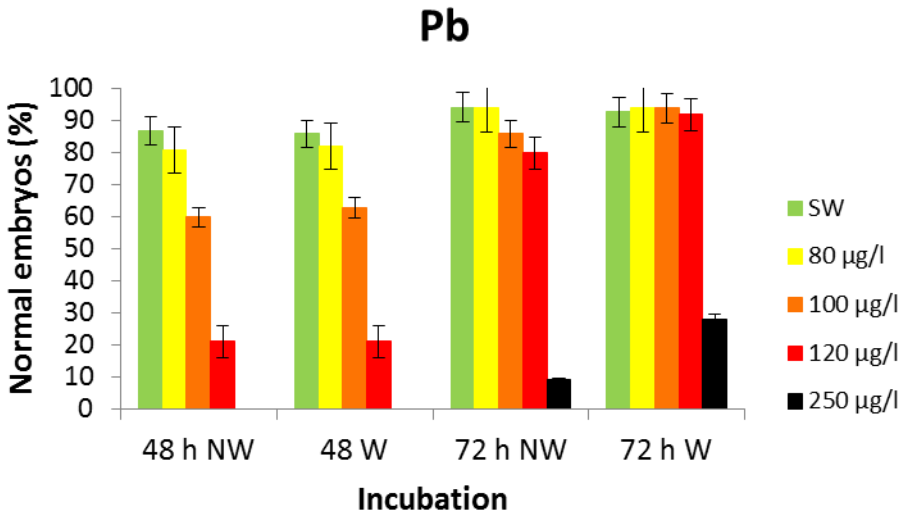


Fig. 9 – Histograms represent the results from the morphological analysis expressed as mean percentage (%) of normal embryos ± standard deviation. Data refer to non-washed embryos (NW) and to washed embryos (W) 48 hours after fertilization (first 2 groups of bars from the left) and 72 hours after fertilization (third and fourth groups of bars from the left). The colored bars represent the different exposure to Pb concentrations as reported on the right side of the figure.

Zinc: Zn caused lower effects at 24 h than 48 and 72 h, but nevertheless slight malformation and delay at the blastula stage (20%) were observed at 100 µg/l and 120 µg/l. Embryos observed at 48 h showed moderate skeletal malformations (21%) at 60 µg/l with an increase in malformed pluteus and an increased prism stage (Fig. 10D) starting from 100 µg/l (76 % at 100 µg/l and 89 % at 120 µg/l). Observing embryos 24 h later (72 h after fertilization), a lower toxicity was also observed for Zn although the repair of the toxic effect appeared lower than for Pb- and Cd-exposed embryos. In particular, up to 100 µg/l all embryos delayed at the prism stage reached the pluteus stage. Some of these embryos exhibited a correct development (55% of total embryos at 70 µg/l and 28% at 100 µg/l) although a high percentage reached the pluteus stage with severe skeletal malformations (45% of total embryos at 70 µg/l and 72% at 100 µg/l). At 120 µg/l all prism stages were not blocked but only delayed. However, only 3% reached the pluteus stage correctly, and the other embryos exhibited skeletal malformations at the pluteus stage (17%) or at the early pluteus stage (80%). Almost all of these abnormalities are represented by radialized arms that in some cases caused the deformation of the entire body shape (Fig. 10E). In fact, 42% of total embryos at 70 µg/l and 70% at 100 µg/l showed this phenotype at the pluteus stage, while 80% of total embryos exhibited this malformation at the early pluteus stage.

Regarding the reversibility experiments, also for Zn, no significant differences were observed at 48 h between washed and non-washed embryos (Fig. 11) . More normally developed plutei were observed in washed embryos than in non-washed ones at 72 h: 84% and 55% respectively at 70 µg/l, 56% and 28% respectively at 100 µg/l, 15% and 3% respectively at 120 µg/l (Fig. 11). At all metal concentrations at which washed embryos were exposed, the embryos delayed at the prism and early pluteus stage at 48 h reached the pluteus stage at 72 h. But also in this case a particularly high percentage of plutei with radialized arms was observed (37% at 100 µg/l and 84% at 120 µg/l), meaning that the reversibility of Zn-exposed embryos was lower than that of the embryos exposed to the other metals.

It has been well known for several decades that Zn is one of the most active metals affecting early stages of echinoids (Lallier 1955; Timourian 1968; Kobayashi 1990). In fact, zinc treatment animalizes (anteriorizes) the embryos and leads to embryos with no or reduced endomesodermal cells. This trend is evident from the germ layer formation and the effects are observable from the beginning of gastrulation in relationship to Zn concentrations. In this experiment, at the Zn concentrations used animalization effects were not observed at the gastrula stage, differently from studies by other authors such as Kobayashi and Okamura (2004), who observed exogastrula and apollo-like gastrula in Zn-exposed embryos of sea urchin *Anthocidaris crassispina.* On the other hand, these authors describe skeletal radialization at the pluteus stage, which is the same developmental anomaly as that observed in this experiment. This could be due partly to the inhibition of Ca^{2+}-ATPase induced by Zn, which causes the disruption of Ca homeostasis and thus skeletal anomalies from gastrulation onwards (Tellis *et al*. 2014b). This study demonstrates that Zn, differently from Pb, causes a continuous and not intermittent alteration in Ca uptake rates during all the early development of the sea urchin *Strongylocentrotus purpuratus* causing less reversible damage. This could explain the lower reversibility effects of Zn compared to those of the other metals used in the present work and the high percentages of skeletal malformations occurring also in washed embryos 72 h after fertilization.

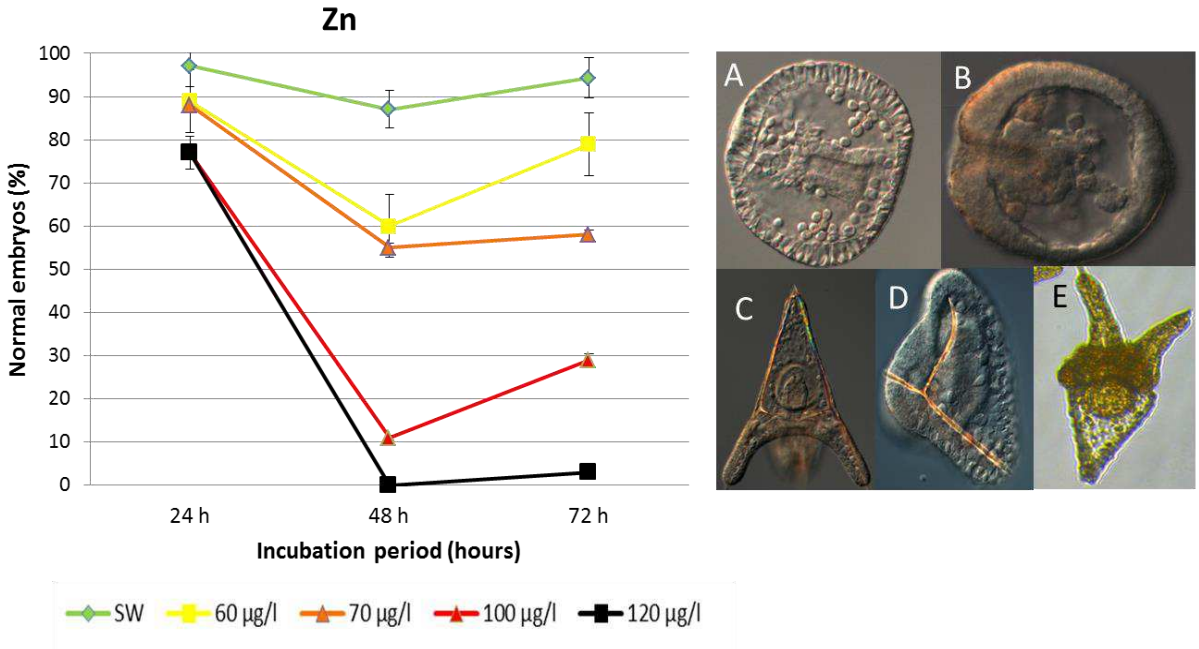


Fig. 10 – Non-washed embryos: Percentage of normal embryos (ordinate) at different times of incubation (abscissa) for each Zn concentration (colored lines). SW: control. On the right side the observed morphotypes : A: Late Gastrula stage, B: Malformed gastrula stage, C: pluteus stage, E: prism stage, F: pluteus with arms malformations and radialized shape.

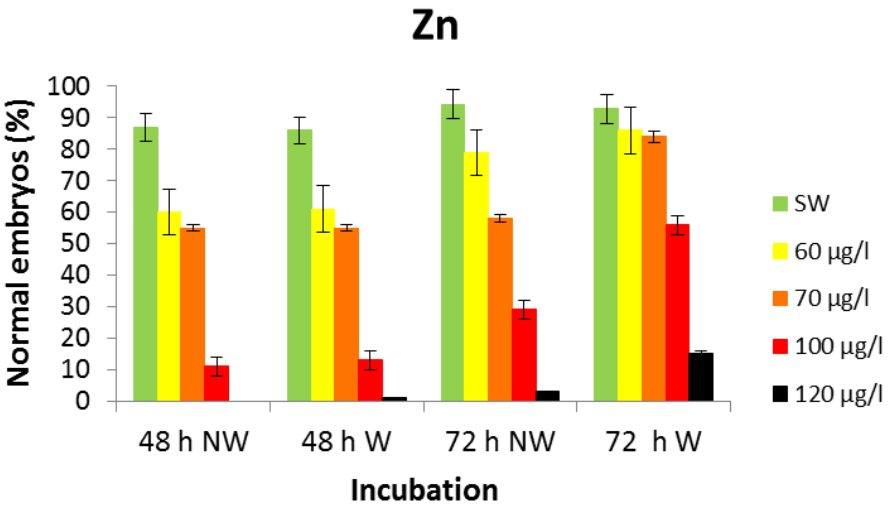


Fig. 11 – Histograms represent the results from the morphological analysis expressed as mean percentage (%) of normal embryos ± standard deviation. Data refer to non-washed embryos (NW) and to washed embryos (W) 48 hours after fertilization (first 2 groups of bars from the left) and 72 hours after fertilization (third and fourth groups of bars from the left). The colored bars represent the different exposure to Zn concentrations as reported on the right side of the figure.

Cadmium: At 24h the toxic effects were almost absent with percentages of normal gastrula higher than 85% at all Cd concentrations (Fig. 12). At 48 h a decrease in normal embryos was observed, especially at high concentrations with 60%, 23% and 0% at 1.5 mg/l, 2 mg/l and 2.5 mg/l respectively. Considering the morphological analysis, it was evident that Cd also induced skeletal malformation at

the pluteus stage, but only crossed spicules at the apex (Fig. 12E) were observed (1 and 1.5 µg/l with 18% and 25% respectively). At higher concentrations a delay in development and skeletal malformations were shown by exposed embryos. In particular, early pluteus and prism embryos with crossed skeletal rods (Fig. 12F) were observed at 2 and 2.5 µg/l with 42 and 50%, respectively. This malformation at the pluteus stage is also reported by Arizzi Novelli *et al.* (2003). At 72 h early pluteus embryos partially reach the pluteus stage, but still exhibit crossed spicules in 21% of total embryos at 2 µg/l and 55 % at 2.5 mg/l. Interestingly, embryos transferred to clean seawater at 24h show at 48 h skeletal malformation comparable with those observed in non-washed embryos, but in washed embryos the anomalies appear reversible. In fact, at 72 h washed embryos show an almost complete absence of skeletal anomalies, with a percentage of well-developed embryos over 80%, slightly lower than in embryos checked at 24 h (Fig. 13).

Experimental evidence suggests that the metal crosses the plasma membrane as a bivalent ion, Cd^{2+}, exerting an agonistic role against calcium ionic channels (Viarengo and Nicotera 1991; Rainbow *et al.* 2005). Cadmium has been associated with: blockage of oxidative phosphorylation, glutathione depletion and antioxidant enzymatic activity inhibition, production of ROS, DNA damage, and inhibition of relative repair mechanisms, and a general reduction in protein synthesis coupled to an increase in stress proteins (Kesseler and Brand 1994; Shimizu *et al.* 1997; Schröder *et al.* 1999, 2005; Ercal *et al.* 2001; Roccheri *et al.* 2004; Lin *et al.* 2007; Filosto *et al*. 2008).

Thus the mechanisms by which cadmium interacts with cellular components and molecular targets are probably various and in the case of sea urchin embryos still poorly understood. However, several studies have demonstrated that *P. lividus* embryos accumulate a certain amount of Cd, which induces apoptosis (Roccheri *et al.* 2004; Agnello *et al.* 2006, 2007; Filosto *et al.* 2008). Therefore, the occurrence of abnormalities would depend on cell loss caused by extensive apoptosis. It is known that sea urchin embryos can activate physiological mechanisms of programmed cell death at specific developmental stages (Roccheri *et al*. 2002; Sato *et al.* 2006; Vega Thurber and Epel 2007), but an increase in apoptotic processes was observed in response to the accumulation of Cd (Agnello *et al.* 2006, 2007; Filosto *et al.* 2008). Apoptosis is supposed to be a way of removing cells whose DNA has been heavily damaged by cadmium, as documented in the case of a vertebrate model system (Hamada *et al.* 1997). Thus, we can assume that also in sea urchin embryos/larvae, apoptosis can be considered as part of a defense strategy in response to cadmium. Although several studies have investigated the mechanisms by which different chemicals affect sea urchin embryonic development, detailed defense mechanisms have not yet been described (Goldstone *et al.* 2006). The molecular pathways activated in

response to cadmium have been partially described and have involved HSPs synthesis (Roccheri *et al.* 2004), DNA damage as single cell breaks (Schröder *et al*. 2005), metallothionein gene expression (Russo *et al.* 2003), and caspase-3 activation (Agnello *et al*. 2007). Considering the results of this study, it is evident that Cd effects, which are partially due to its agonistic role against calcium ionic channels and to apoptosis induction, produce evident morphological anomalies after gastrulation. One hypothesis is that the most commonly observed effect of crossed spicules is partially due to a miss-regulation of cellular remodeling caused by a supernumerary number of apoptotic cells and/or the perturbation of Ca homeostasis, which is fundamental for spicule formation. Moreover, it is possible that molecular pathways involved in the detoxification process are activated before the end of gastrulation and the effects are evident in embryos incubated with no Cd after 24h. In fact, washed embryos are restored to the normal body organization 72 h after fertilization and skeletal anomalies appear almost completely reversible, differently from non-washed embryos, which at 72 h exhibit severe malformations.

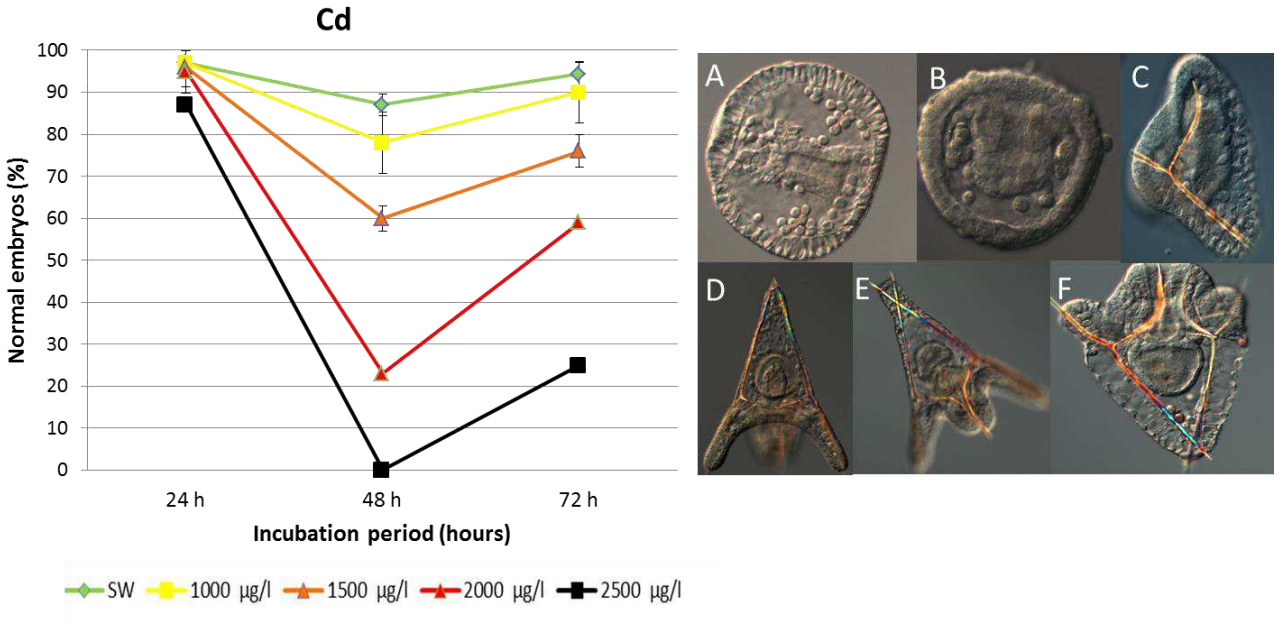


Fig. 12 – Non-washed embryos: Percentage of normal embryos (ordinate) at different times of incubation (abscissa) for each Cd concentration (colored lines). SW: control. On the right side the observed morphotypes: A: Late Gastrula stage, B: Malformed gastrula stage, C: prism stage, D: pluteus stage, E: crossed tip, F: crossed early pluteus stage.

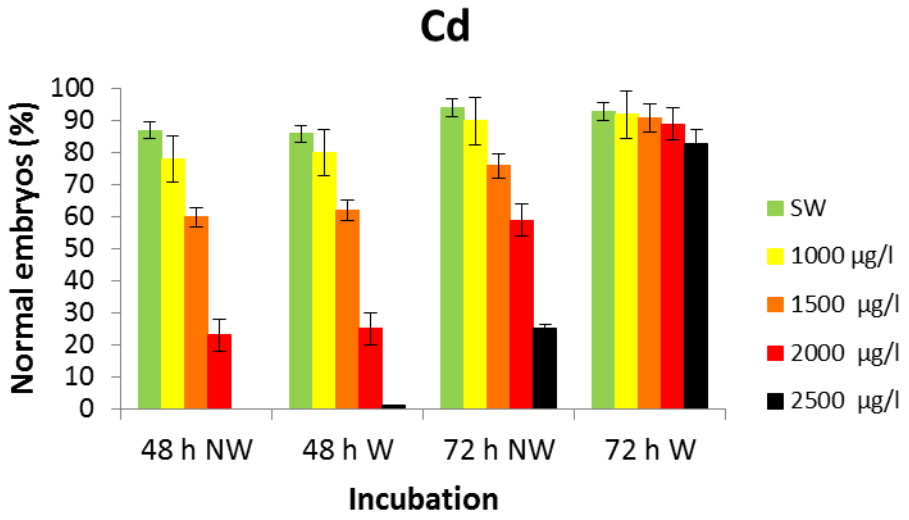


Fig. 13 – Histograms represent the results from the morphological analysis expressed as mean percentage (%) of normal embryos ± standard deviation. Data refer to non-washed embryos (NW) and to washed embryos (W) 48 hours after fertilization (first 2 groups of bars from the left) and 72 hours after fertilization (third and fourth groups of bars from the left). The colored bars represent the different exposure to Cd concentrations as reported on the right side of the figure.

Inhibition of larval length: Concentration dependence in growth inhibition was also found for all tested metals at 0,1, 18, 24, 48 and 72 h, as illustrated in Fig. 14. Results suggest that during early life stages size increase is very slight. Fertilized eggs measure a mean size ± SD of 90 µm ± 1.49 (n=30). Cleavage, Morula and Blastula stages have approximately the same diameter. Gastrula registered a dimension of 113 µm ± 3.77, and from prism to pluteus stage there is an increase of more than 300 µm. At 72 h control pluteus are 456 µm ±26 µm in length. These control sizes are comparable with sizes found by Saco-Alvarez *et al.* (2010).

Data regarding the metal inhibition of larval length are reported in Fig. 14. Before 18 h all values are very close to those of the control embryos, with size decrease between 0 and 3%, except for Cu at 70 µg/l (larval reduction of 10.4%) and Pb at 250 µg/l (larval reduction of 6.3%). At 24h, slight effects on size were observed for Cd and Pb (larval reduction between 0 and 3%) and greater inhibition was observed in embryos exposed to Cu (larval reduction of 10.6% and 12% at 60 and 70 µg/l, respectively) and Cd (6% of larval reduction at 120 µg/l). In fact, Cu-treated embryos were partially delayed at the blastula stage at 24 h, while Cd-treated embryos exhibited a larval reduction at the gastrula stage but no significant developmental delay. At 48 h, concentrations of 30 µl and 70 µg/l of Cu caused a larval length reduction of 38 and 66% respectively. For Pb, concentrations of 80 and 250 µg/l caused a size decrease of 7% and 45%. For Cd, an inhibition of 37% was observed at 60 µg/l, and this increased to 52% at 120 µg/l. Cd caused less inhibition of size than other metals at the

concentrations tested, with a length reduction of between 2% (at1 mg/l) and 11% (at 2.2 mg/l). At 72 h, larval length continued to increase although size inhibition caused by metals was very similar to that found at 48 h for all metals except Cd. In fact, this metal caused at concentrations of 2 and 2.5 mg/l a size reduction of 24 % and 44 % at 72h, as against 3% and 11% at 48 h. Concerning the effects of Cd, also at 72 h the increase in normal embryos was not followed by an increase in larval length.

Comparing EC10 estimates using percentages of normal larvae with those obtained using larval length (Table 3) some differences were observed. In particular at 48 h and 72 h, EC values obtained using larval length were lower than those calculated for normal larvae (except for Cd at 48 h). Early larval growth seems to be a more sensitive response than embryogenesis success. This observation is in accordance with Saco-Alvarez *et al.* (2010) and the conclusion reported by Fernandez and Beiras (2001). However, the straight-line slope values reported in Table 3 are significantly higher for curves obtained using percentages of normal larvae than those obtained using larval length, up to 11 times higher, as for Pb at 48 h (14.50 with normal larvae and 2.38 with larval length). In fact, considering EC50 values, unlike EC10 values, inhibition of larval grow appear to be less sensitive than embryogenesis success for all metals, a consequence of the different slopes of the curves. For example, in the case of Pb EC50, using larval length is more than 2 times greater than EC50 using normal larvae with 266.72 µg/l and 315.66 µg/l at 48 and 72 h respectively with larval length compared with 108 µg/l and 164.45 µg/l at 48 and 72 h respectively with normal larvae.

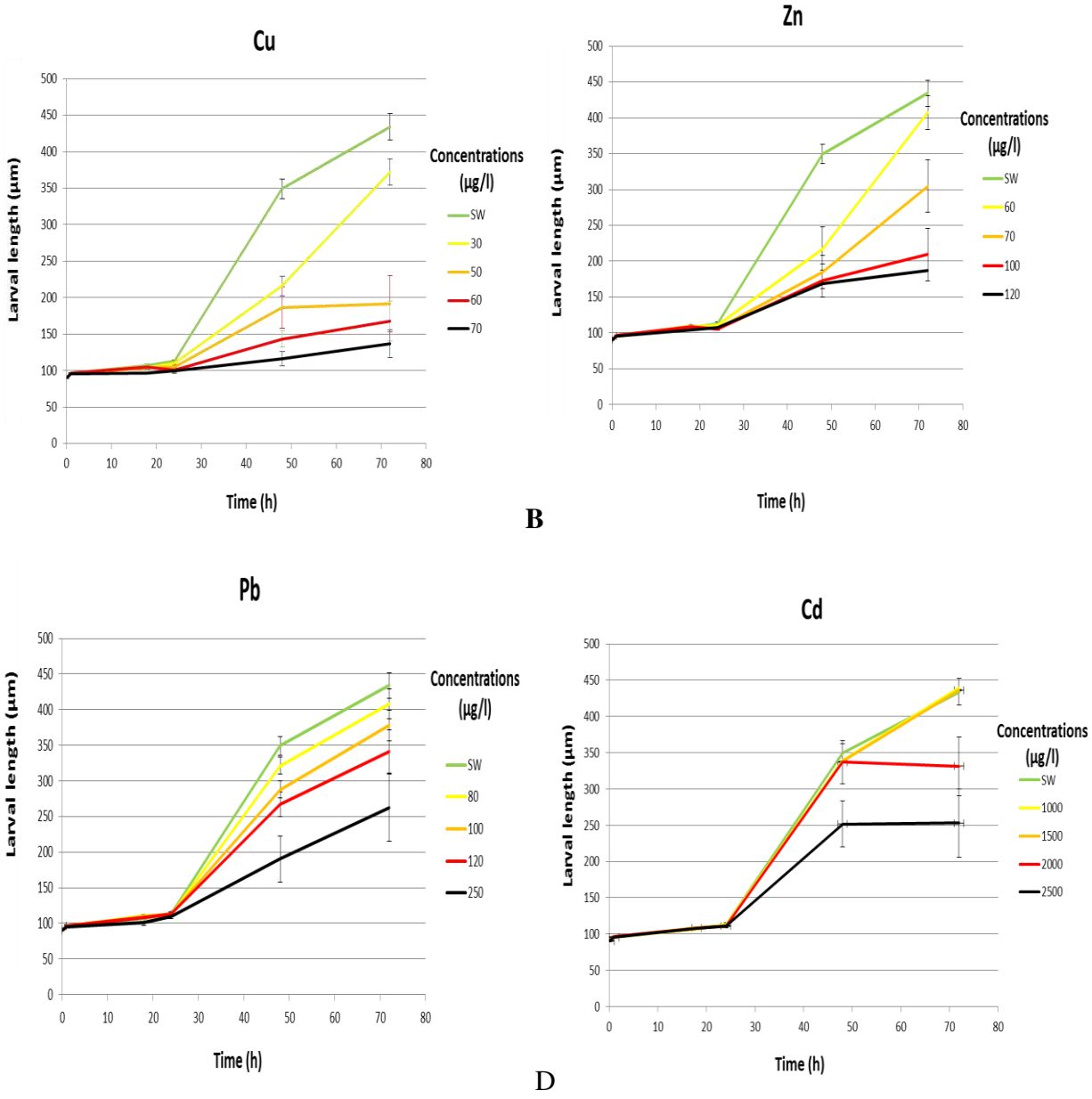


Fig. 14- Mean larval length size ± SD (n=30) (ordinate) at different incubation times (abscissa) for Cu (A), Zn (B), Pb (C) and Cd (D). Concentrations are represented by colored lines. The green line represents control embryos.

Endpoint	NORMAL EMBRYOS			LARVAL LENGTH		
Incubation time	24h	48h	72h	24h	48h	72h
Cu	**28.15** (22.42-32.21) *9.53*	**37.84** (31.75-41.25) *15.50*	**40.65** (36.38 - 43.90) *6.64*	**59.74** (43.83-114.62) *1.54*	**5.47** (0.67-11.35) *1.42*	**19.60** (14.38-23.86) *3.26*
Pb	**56.57** (26.30-79.01) *1.73*	**87.90** (75.96-94.48) *14.40*	**108.53** (93.3- 21.14) *7.10*	**0.20** (n.c) *0.19*	**77.31** (57.43-92.61) *2.38*	**88.43** (66.88-105.10) *2.32*
Cd	**68.25** (32.00 -85.10) *2.34*	**52.99** (29.85-67.82) *9.34*	**56.93** (50,13- 64,87) *7.82*	**23.68** (11,53-45.88) *0.59*	**6.42** (0-23.56) *1.06*	**54.51** (4.06-71.88) *4.90*
Cd	**2536** (2186,7-2635,5) *5.90*	**1352.70** (1195.6-1464,0) *12.15*	**1710.20** (1530.9- 1830.4) *6.87*	**5273.60** (4581,7-5347,9) *1.43*	**2527.20** (1902-5150.7) *1.60*	**1377.10** (1115.3-547.9) *6.50*

Table 3 – Toxicity results are reported for each metal (indicated in the first column on the left) as Effect Concentration 10% (EC10) using the standard criteria of the percentage of normal embryos (left column). These data are compared with the EC10 obtained using larval length criteria (right column). All toxicity data are reported in bold in the upper part of each cell and are expressed in µg/l . The lower values represent the respective 95% confidence limits. The lowest values of each cell reported in italics are the respective straight-line slopes.

2.4 Conclusions

Generally speaking, all trace metals to which the embryos were exposed show similar trends of toxicity. In particular, toxic effects were higher at 48 h than at 24h and 72 h. In fact, the pluteus stage appears to be more sensitive than embryos at the end of gastrulation. However, most embryos analyzed at 48 h, especially those exposed to low and medium concentrations of metals, appear to present reversible anomalies of embryo development. In fact, at 72 h most embryos reached the correct developmental stage. This trend is much more evident for embryos transferred to clean seawater at 24 h. At 48 h no significant differences were observed between washed and non-washed embryos, but at 72 h washed embryos showed higher values of normally developed plutei than embryos checked at 48h than non-washed embryos at 72 h. However, specific malformations, time of occurrence and magnitude of effects depend on the metal considered. For example, Cu causes greater effects already at 24 h than Pb, Zn and Cd, causing mainly developmental delay at 48 h. Differently, Pb, Zn and Cd cause less damage at 24 h and more skeletal malformations at 48 h: in Zn exposed embryos most of these anomalies are permanent, while those caused by Cd and Pb are more reversible at 72 h. Skeletal malformations induced by Pb exposure are more reversible than those caused by the other metals also for non-washed embryos. Specifically, Zn seems to cause mainly radialization of the arms and Cd the crossing of spicules. These toxic effects caused by metal exposure probably depend on mechanisms by

which each individual metal interacts with cellular components and molecular targets. These mechanisms are varied and in the case of sea urchin embryos still poorly understood. Generally, these metals render their toxic effects through disruption of Ca homeostasis (Tellis *et al.* 2014a, 2014b), likely through different proximate mechanisms and effects on skeletal development, influencing swimming ability. However, Zn is also known as an animalizing agent and Cd induces apoptosis (Agnello *et al.* 2006, 2007; Filosto *et al.* 2008). The observed partial reversibility of the teratogenic effects could be due to different molecular pathways activated in response to metals, which involved for example HSPs synthesis, DNA damage such as single cell breaks, metallothionein gene expression and caspase-3 activation. Concerning the morphological observation of metal-induced effects, it is evident that the percentage of normally developed plutei achieved 48 h after fertilization is a simplistic endopoint. In fact, it is not possible to detect the magnitude of specific effects and their reversibility. Contrarily size inhibition, an alternative endpoint used by several authors (Fernandez and Beiras 2001; Saco-Alvarez *et al.* 2010), is considered as being a more sensitive criterion than percentage of normal larvae. However, these results show that dose-response curves obtained with larval length criteria have a lower straight-line slope and hence the sensitivity of this endpoint is confirmed only at low percentages of effects. In fact, at high toxicant concentrations size inhibition does not fall to zero as a percentage of well-developed embryos. In contrast, at lower toxicant concentrations there are smaller plutei and pre-pluteus embryos together, and consequently larval size can discriminate better than the standard endpoint based on embryogenesis success.

In conclusion, this part of the Thesis demonstrated that classic endpoints are inadequate to estimate teratogenic effects on sea urchin embryos because too many different effects (with differing reversibility) are not considered, especially at the pre-pluteus stage. These embryos exhibit a wide range of teratogenic effects but very similar dimensions, a phenomenon that causes a decrease in the sensitivity of the larval length endpoint. At the same time all these larvae are considered as being abnormal with embryogenesis success endpoints. Hence the need to develop a different endpoint based on specific malformations at each stage of development during the early embryogenesis of the sea urchin.

3. New Integrative Index of Toxicity (ITI) development, application and validation

3.1 Introduction

The sea urchin embryo toxicity test is classically used to assess the noxious effects of contaminated marine waters and sediments. In Italian guidelines on the quality of dredged sediments, the standard toxicity criteria used for this assay are based on a single endpoint at 48 hours of development, corresponding to the pluteus stage; different typologies of abnormalities, including those which occur at earlier stages, are not categorized, thus preventing the evaluation of the actual teratogenic hazards. The results of characterization of developmental anomalies and investigation of the reversibility of the effects (reported in Ch. 2) demonstrate the occurrence of a large scale of malformations at different stages of development and with a various degree of reversibility depending on the dose and the typology of pollutant. Generally most of the embryos observed, especially those exposed to low and medium concentrations of metals, presented reversible anomalies, reaching the correct developmental stage at 72 hours after fertilization. These data clearly indicate that the standard criteria based on the percentage of normally developed pluteus at 48 h and the size increase criteria appear to be inadequate endopoints to obtain a realistic estimate of toxicity. In particular, there would appear to be an urgent need to develop a different endopoint based on specific malformations at each stage of development during the early embryogenesis of the sea urchin, with particular attention to the pre-pluteus stage and to the possible reversibility of toxic effects. Consequently, with the aim of enhancing the capability of the sea urchin embryo toxicity test to discriminate the ecotoxicity of marine sediments, a new toxicity scale has been developed in this study associating different values to each morphotype in relation to germ layer specific malformations and developmental stage. This information was synthesized into a new integrative index of toxicity quantifying the frequency of delayed and abnormal embryos and quantitatively ranking the severity of effects.

The integrative toxicity index (ITI) was developed considering toxic effects induced by trace metals and by elutriate samples of marine sediments on sea urchin embryo development. On the basis of the obtained results, the scale of toxicity was further optimized dividing developmental anomalies into different categories on the basis of the severity of germ layer impairments. In fact, the result of morphological observations demonstrates that germ layer impairment may have various levels of severity, causing a different inability to properly differentiate ectoderm, mesoderm and endoderm territories and to recover the correct development of the embryo after 24 hours. To obtain a more

realistic and precise estimation of toxicity, developmental anomalies were classified into different categories on the basis of the severity of germ layer impairments. The new integrative index of toxicity calculated starting from this scale of toxicity was applied and validated exposing sea urchin embryos to elutriates of sediment from Trapani harbor, chosen as a model area. The new toxicity index was compared with other approaches and with existing normative guidelines on sediment toxicity characterization. In particular, developmental endopoints and toxicity index values were integrated with chemical data and with new methodological approaches for other biological effects, such as DNA damage and HSPs levels in exposed sea urchin embryos. Trapani harbor, in the framework of a larger project, was chosen as a model case-study for the application of an integrated, multidisciplinary Weight Of Evidence (WOE) approach to improve the criteria of sediment quality characterization and resulting management options (Piva *et al.* 2011; Benedetti *et al*. 2012).

The overall results were expected to improve the capability of the *P. lividus* embryo toxicity test to discriminate different samples, moving from the simple observation of general effects to the classification of their severity; this approach should allow for a better categorization of the teratogenic potential of marine sediments and their quality classification through a more sensitive and realistic toxicity index.

3.2 Materials and methods

3.2.1 ITI development

Adults of *P. lividus* were collected and acclimatized in controlled conditions as reported in par. 2.2. Embryo toxicity tests were performed as explained in par. 2.2. The same paragraph explained the preparation of metal solutions and the exposure of embryo cultures. A new integrative index of toxicity was applied to estimate the toxicity of trace metal solutions and, subsequently, those of elutriate samples, prepared as follows.

Sediment Sampling and Elutriate Preparation

Sediments were collected in June/July 2012 during a large characterization and monitoring project in Trapani harbor. Elutriates from 24 representative sediment samples were prepared in accordance with USEPA (1991) guidelines and literature studies (Volpi Ghirardini *et al*. 2005). The sediment samples were mixed in a 1:4 (v/v) ratio of sediment to water and placed on a rotary shaker table for 1 hour, at a speed of 300 rpm, at room temperature. The dilutions were made up with 0.22 µm filtered sea water

(FSW) collected in a long-term monitored reference site located far from human activities. After mixing, the samples were centrifuged for 20 min at 3000 rpm (4°C) and the aqueous fractions (elutriate samples) were poured off and stored for 30 days at -20°C until used for the toxicity tests.

Toxicity criteria

The toxicity of metal solutions and elutriate samples was determined by calculating the percentage of normal embryos at the gastrula (24 h) and pluteus (48 h) stage, according to both standard criteria and a new integrative index of toxicity as reported by Morroni *et al.* (2016). The standard criteria evaluate the percentage of normal and abnormal embryos without considering different typologies of malformations or the phase at which they appear. Embryos are classified as normal if they satisfy the following morphological criteria: (1) correct schedule in reaching the developmental endpoint (gastrula at 24h and pluteus at 48 h), (2) left/right and dorso/ventral embryonic axis symmetry, (3) differentiation of oral/aboral ectoderm and endoderm territories, (4) skeleton development and patterning (for a review see Henry 1998; Pinsino *et al.* 2010). Embryos displaying impairment of the left/right or dorso/ventral axis symmetry, as well as oral/aboral ectoderm, mesoderm and endoderm territories, are scored as abnormal, without subcategories distinctions. The new integrative toxicity index (ITI) is based on a detailed analysis of development delays and germ layer impairments, i.e. their inability to properly differentiate ectoderm, mesoderm and endoderm territories. The toxicity is quantified by counting the frequency of delayed and/or abnormal embryo morphologies and by quantitatively ranking the severity of effects from 0 (none) to 10 (high). Anomalies are classified as delays in development with or without further morphological alterations, and assigned with an increasing weight depending on the severity and the stage at which they appear 24 or 48 h from fertilization. Lower toxicity values were given to embryos with a delay in development and the absence of malformations; higher scores were attributed to embryos showing contemporarily delayed and malformed morphotypes. Different scales and scores were tested by comparing with expert judgement the calculated indices with morphologic observations. Details of selected scores and stages of development are given in Table 4. After 24 hours, the assigned toxicity score is 0 for late gastrula (lG) corresponding to the normal stage, while 1 to 4 when embryos observed are gastrula (G), mesenchyme blastula (mBl), blastula (Bl) or morula (M) respectively: when such delayed stages also present morphological alterations, their toxicity scores range from 6 to 10 (Table 1). At 48 hours, toxicity score is 0 for pluteus (Pl), from 1 to 5.5 when observing delayed stages corresponding to early pluteus (ePl), prisma (Pr), G, mBl, Bl and M: toxicity

scores increase from 6 to 10 for the same delayed stages when abnormal morphotypes are also observed (Table 4).

The integrative toxicity index (ITI) is calculated as follows:

$$ITI = \sum^{n}_{i=10}(S_i * F_i)/100$$

Where S_i is the score associated with each abnormality and F_i is the frequency observed for that abnormality (i=10)

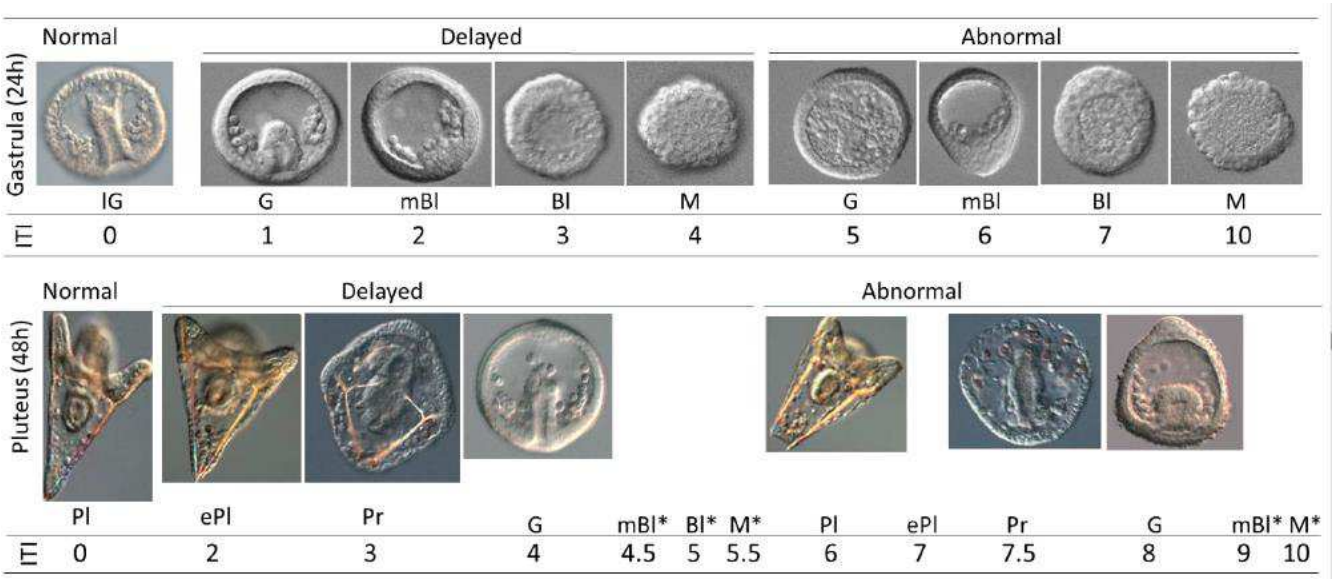


Table 4 – Classification of malformations according to the new Integrative Toxicity Index (ITI) based on detailed analysis

3.2.2 ITI application and validation

Sediment sampling

Sediments were sampled in Trapani harbor (Sicily, Italy) in June 2012. Sediment cores (from 0 to 200 cm depth) were collected in 19 square areas with sides from 50 to 200 m, at a central point fixed by geographical coordinates. Analysis was carried out on 37 samples collected at different depths in relationship to the thickness of the sediment at each station located in the dredging area (Fig.16). The elutriates were prepared as reported in 3.3.1

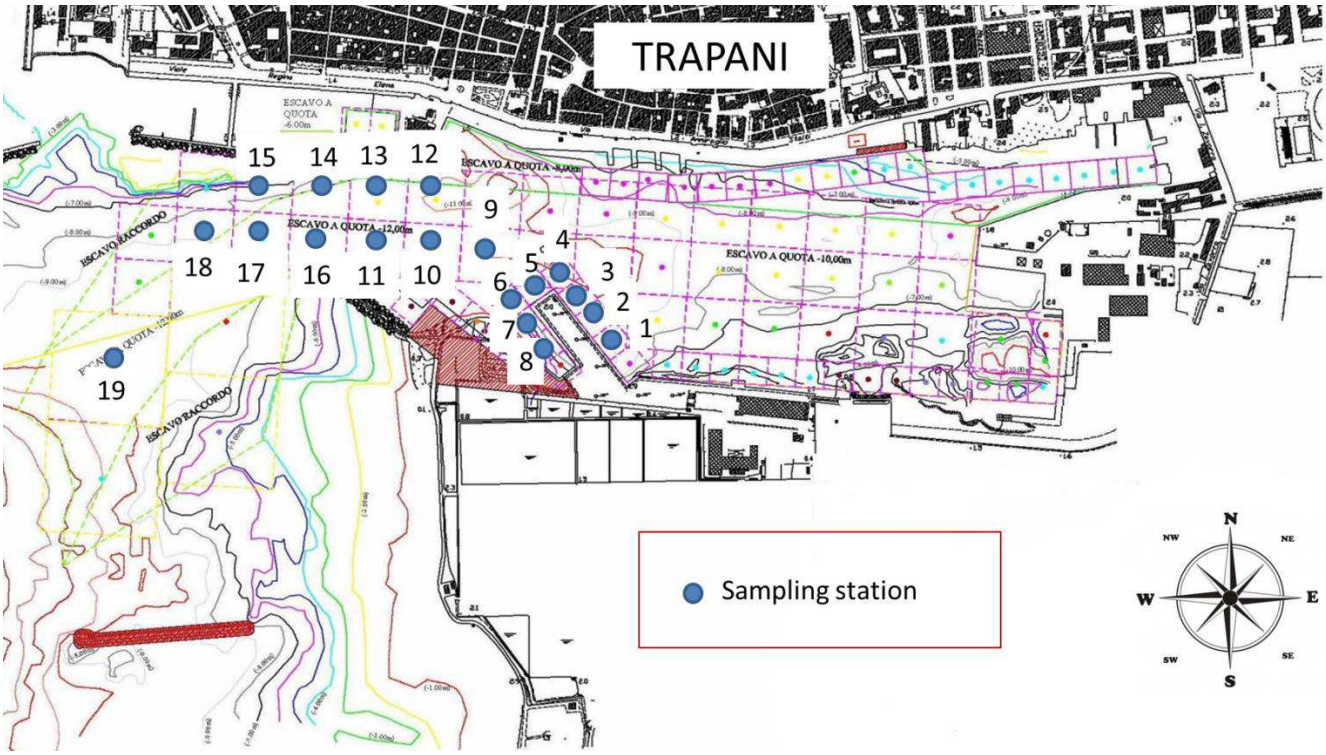


Fig. 16 - Sampling stations in Trapani harbor. Blue circles repesent sediment stations.

Sea urchin embryo toxicity test: Adult *P. lividus* were collected during the breeding season by scuba divers along the North-Western coast of Sicily (Italy). After collection, the sea urchins were transported in an insulated plastic container to the laboratory and immediately used to obtain eggs and sperms. Toxicity tests were performed in accordance with adapted U.S. Environmental Protection Agency guideline (USEPA 1995). Gametes from at least three females and three males were collected from gonads and pooled prior to fertilization as reported by Bonaventura *et al.* (2005) and Russo *et al.* (2014). The sperm was collected dry (directly from the surface of the sea urchin) using a micropipette with the end of the tip cut off, and maintained in a sealed container at 4°C. The eggs were placed in FSW, washed three times and used for bioassays within 30 min of collection. 10 µl of dry sperm were diluted in 50 ml of FSW and added to 300 ml of egg suspension diluted to 20000 eggs/ml (sperm/egg ratio 50:1). After fertilization, the embryos were maintained in 60 mm Petri dishes (10 ml per dish, corresponding to about $2x10^4$ embryos per dish) at a temperature of 18±2°C. Embryo cultures were exposed from fertilization to the pluteus stage (48 h post-fertilization) to 24 different elutriate samples prepared as described above. In accordance with the guidelines on dredged materials (APAT-ICRAM, 2007), three replicates were performed for each elutriate sample. Morphological evaluation was performed on live embryos: for this purpose, groups of 20 embryos were collected and immediately

inspected at the 24-hour and 48-hour endpoints by optical microscopy (Zeiss Axioskop 2 Plus microscope) and photographed by a digital camera.

SDS-PAGE and Western blot:

12 of 37 samples were selected to detect HSC70 and HSC60 protein levels in exposed larvae following the methodology reported by Geraci *et al.* (2004) and by Pinsino *et al.* (2011a). In particular, about 20,000 embryos were harvested 24 and 48 h after exposure and centrifuged at 1000 rpm for 5 min. After supernatant removal, pellets were either deep-frozen in liquid nitrogen and stored at -80°C.

After a maximum of 2 weeks the embryo pellets were dounce-homogenized on ice in about 250 ml of lysis buffer containing 20 mM Tris–HCl, 2 mM EDTA, 1% NP-40, 15% glycerol, and 2 mM DTT, supplemented with a cocktail of protease inhibitors such as 2 lg/ll antipain and leupeptin, 1 lg/ll aprotinin and pepstatin, 1 mM benzamidine, and 0.1 mM PMSF. Lysates were centrifuged at 10,000 rpm for 10 min, at 4°C; supernatants were collected and total protein concentrations were determined by the Bradford method. Equal amounts of proteins corresponding approximately to 30 µg were denatured at 100 °C for 5 min in Laemmli buffer and separated by SDS-PAGE. After the run (1 hour at 100 V) proteins were electrophoretically transferred to 0.45-µm nitrocellulose membranes (AmershamTM HybondTM-ECL) in a buffer containing 12.5 mM Tris, 96 mM glycine, and 20% (v/v) methanol for 30 minutes at 15 V. After blocking with 5% non-fat dried milk in 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween 20 (TBS-T), membranes were incubated overnight at room temperature with anti-bovine brain 70 kDa heat shock protein mouse monoclonal antibody diluted 1:700 and and 60 kDa heat shock protein mouse monoclonal antibody diluted 1:400. The secondary antibodies used were anti-mouse peroxidase-conjugated diluted 1:2,000 (HSC60) and anti-mouse 1:2000 (HSC70). The incubation was performed for 1 hour in a dark room at 4°C. After washing with PBS containing 0.1% of Tween 20 films were scanned and band intensities quantified by the ChemiDoc system (BIO-RAD). Results were reported as arbitrary units obtained from the volumetric analysis of bands.

Comet assay and diffusion assay

To investigate the possible occurrence of DNA damage and apoptosis in exposed sea urchin embryos a new methodology of comet assay and diffusion assay was developed. The experiments were performed at 4 cell stage, 32 cell stage, blastula stage and early gastrula stage. Pre-hatching stages required the removal of the fertilization envelope in order to perform the assays. This methodology was developed

starting from those previously described by Cervello *et al.* (1984) and Matranga *et al.* (1986). In particular, the fertilization of 1 l of egg suspension with 4000 embryos/ml was performed in para-aminobenzoic acid (PABA) 6mM in FSW. After the fertilization and the appearance of the fertilization envelope, the embryos were filtered through a 60 μm nylon mesh, in order to remove the envelope. After 3 washes in FSW, the embryos were used for incubation experiments up to the achievement of the selected stage of development. The next step necessary to perform toxicity assays was the cell dissociation. This phase was performed harvesting approximately 50,000 embryos and centrifuging at 1000 rpm for 3 minutes. The FSW present in the suspension was replaced with the dissociation medium composed of EDTA 2 mM in calcium and magnesium free seawater (CMFSW). The suspension of embryos was subsequently centrifuged at 1000 rpm for 3 minutes. The pellet was re-suspended using a Pasteur pipette in order to favor cell dissociation. The dissociated cells obtained were centrifuged for 10 min at 1000 rpm in order to remove the dissociation medium and to replace it with a 0.075 M solution of KCl in FSW. The function of this salt was to stretch the membrane by osmosis and facilitate the successive cell lysis. The incubation period was 20 minutes. After that the suspension was centrifuged again at 1000 rpm for 10 minutes and used for the comet assay (starting from Tice *et al.* 2000) placing embryos on slides adding 75 μl of low melting agarose (LMA) 0,5% in PBS per slide. The slides used in the experiments were previously laminated with normal melting agarose (NMA) 1% in PBS maintaining the slides for 5 min at 4 ° C, and then adding a third layer of LMA (85 μl per slide). After further 5 min at 4 ° C the slides were immersed in lysis solution. This was prepared fresh at each experiment from a pre-lysis solution (NaCl 2,5 M, Na2EDTA 100 mM, Tris HCl, pH 10) adding dimethyl sulfoxide (DMSO) 10% and Triton X 100 1%.

The diffusion assay was performed removing the cells from the lysis solution after 1 hour and washing them with TRIS-HCl 3 times, and then immersing them for 3 minutes in cold methanol. The samples were observed by fluorescence microscopy after staining with ethidium bromide.

The comet assay was conducted removing cells from lysis solution after 48 hours, placing the suspension in a horizontal electrophoresis cell with running buffer at pH 13 (EDTA 200 mM, NaOH 10 N in deionized water) for 10 minutes before running. The electrophoresis run was performed for 5 min at 25 V and 300 mA. At the end of the run, the nuclei were observed by fluorescence microscopy after staining with ethidium bromide.

Chemical analysis: Chemical analyses were carried out on sediment samples. After homogenization and sieving, each sediment sample was prepared for chemical analyses of pollutants. In particular, metals and organic pollutants such as Polycyclic Aromatic Hydrocarbons (PAHs), Polychlorinated Biphenyls (PCBs) and organochlorine pesticides were analyzed.

The concentration of metals (Al, As, Cd, Cr, Cu, Hg, Ni, Pb, V, Zn) in the acid extract was determined by Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) in accordance with US EPA 3052/96 (hotplate digestion technique) and US EPA 6010B/96. For PAHs, analyses were carried out using HPLC with water–acetonitrile gradient and fluorimetric detection as reported by Bocchetti *et al.* (2008). PAHs were identified by the retention time of appropriate pure standard solutions (EPA 610 Polynuclear Aromatic Hydrocarbons Mix). The determination of halogenated organic compounds, including polychlorinated biphenyls (PCBs) and organochlorine pesticides included in EPA-8081, was conducted through techniques of gas chromatography with mass detector (GC-MS).

Physico-chemical parameters (pH, temperature, dissolved oxygen and salinity) of elutriates were detected using a pH-ameter (Eutech Instruments PCD650), while chemical analyses (concentrations of sulfides and total ammonia) were performed with a spectrophotometer (Hach Lange DR3900).

Data analysis: The percentages of abnormal larvae and size inhibition were considered to evaluate the percentage of effect. Toxicity data are expressed as EC50 and EC10 values. The EC values with 95% confidence limits were calculated by the Probit method. Responses to each were corrected for effects in control tests by applying Abbott's formula (Volpi Girardini and Arizzi Novelli 2001). Pearson correlation coefficients among ITI (at 24h, 48h, 72h), HSPs levels (24h, 48h), each observed morphotype, physical and chemical parameters were estimated using the statistical software R.

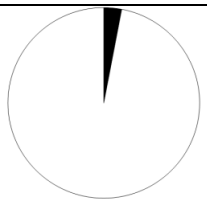
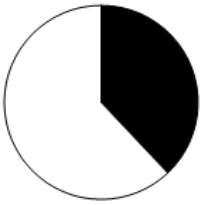
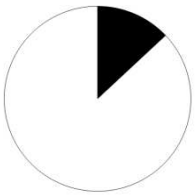
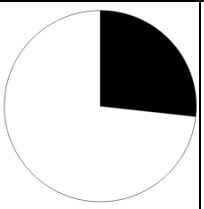
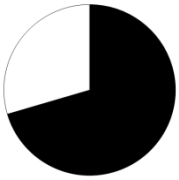
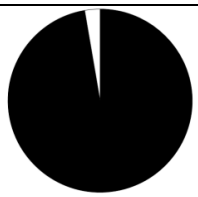
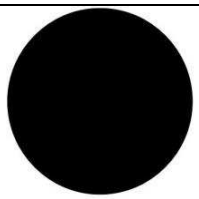
3.3 Results and discussion

3.3.1 ITI development

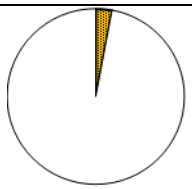
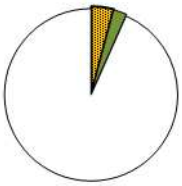
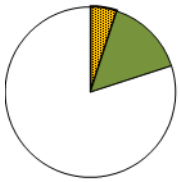
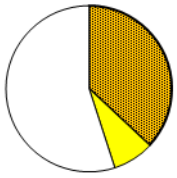
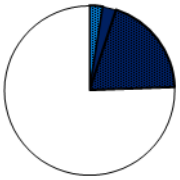
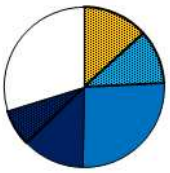
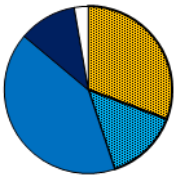
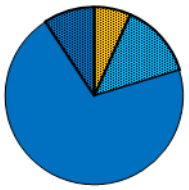
Metal exposure

The results on embryonic development calculated according to the standard criteria and to ITI are reported in Figs. 17-21 for each metal. A description of malformations and their reversibility is reported in Ch. 2. Generally speaking, it is observed that at increasing metal concentrations both the percentage of abnormal embryos and the index of toxicity increase. Developmental analysis shows this trend at 24 h and, more evidently, at 48 h of incubation (Fig. 22). However, the stage of development and the occurrence of anomalies is highly variable between different metals and different times of exposure, as is observable in Figs. 17-20. The simple observation of normal versus abnormal embryos is certainly rapid and easy to evaluate, but also less sensitive in discriminating different samples compared to ITI, which recognizes and weights the delay and degree of various abnormalities. Considering ITI, alterations are scored according to the severity of the effects, discriminating simply delayed from delayed and contemporarily abnormal morphotypes (Table 4). For this reason, metal solutions that cause similar percentages of well-developed plutei may show different index values. For example, considering the highest concentrations Cu shows 0% of normal embryos at 48h with a concentration of 70 μg/l (Fig. 17A) and an index value of 5.38 (Fig. 17B). In this case most of the embryos are delayed at the prism stage (70%), but the other embryos present developmental anomalies at the blastula, late gastrula and prism stage (Fig. 18B). Zn at 120 μg/l and incubated with embryos until 48 h also presents 100% of abnormal embryos (Fig. 19A), but an ITI value of 2.45 (Fig. 19B), which appears to be less than half of the toxicity estimated in embryos exposed to Cu. This is mainly due to the very low percentage of malformed embryos (2%), with almost all embryos reaching the prism stage correctly (90%) and early pluteus stage (8%). Another similar example is represented by Pb at 100 μg/l and Zn at 60 μg/l, which both caused a percentage of 40% of abnormal embryos exposed up to 48 h (Fig. 18A and 19A respectively): however, Pb shows an index value of 2.48 (Fig. 18B) and Zn exhibits 1.05 of toxicity (Fig. 19B). The reason is that 40% of abnormal embryos are represented by malformed embryos in the case of Pb (30% of malformed early plutei and 10% of malformed prism) (Fig. 18B) and by delayed plutei in the case of Zn, with only 16% of early plutei with developmental anomalies (Fig. 19B).

Cu

□ Normal embryos ■ Abnormal embryos	CTR	Cu 24 µg/l	Cu 48 µg/l	Cu 60 µg/l	Cu 72 µg/l
24 hours					
Abnormal embryos 24 h (%)	**3**	**9**	**20**	**24**	**45**
48 hours					
Abnormal embryos 48 h (%)	**13**	**26**	**70**	**97**	**100**

A

	CTR	Cu 24 µg/l	Cu 48 µg/l	Cu 60 µg/l	Cu 72 µg/l
24 hours					
ITI 24 h	**0.23**	**0.35**	**0.95**	**1.36**	**3.33**
48 hours					
ITI 48 h (%)	**0.79**	**1.36**	**3.50**	**4.38**	**5.38**

B

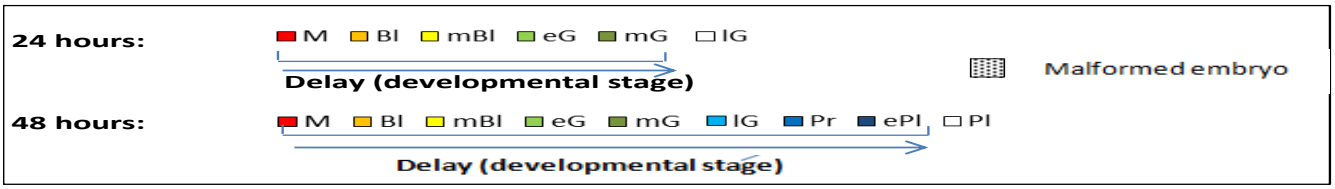
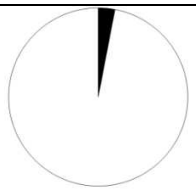
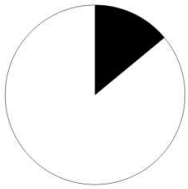
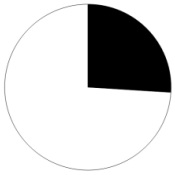
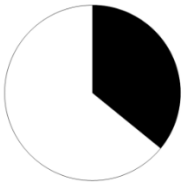
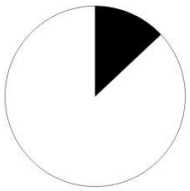
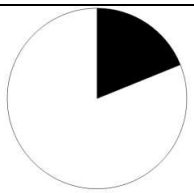
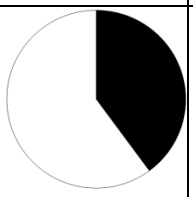
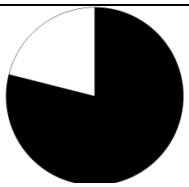
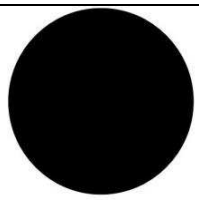
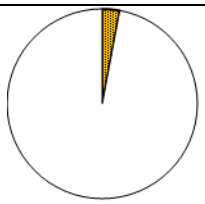
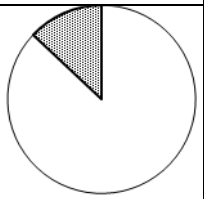
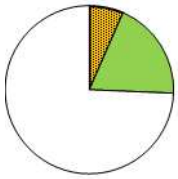
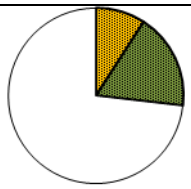
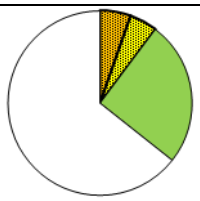
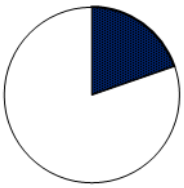
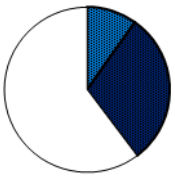
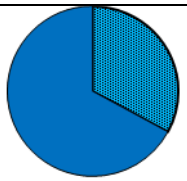


Fig. 17 – In the upper panel (A) the percentages of normal embryo are reported in white on the pie charts at 24 hours and 48 hours of incubation (on the rows) for each Cu concentration (on the columns). The percentage of normal embryos is reported in the cells under each pie chart. In the lower panel (B) the stages of development reached by embryos are reported in different colors on the pie charts (as illustrated by the legend under the panel) at 24 hours and 48 hours of incubation (on the rows) for each Cu concentration (on the columns). The ITI value is reported in the cells under each pie chart.

Pb

□ Normal embryos ■ Abnormal embryos	CTR	Pb 80 µg/l	Pb 100 µg/l	Pb 120 µg/l	Pb 250 µg/l
24 hours					
Abnormal embryos 24 h (%)	**3**	**14**	**26**	**27**	**37**
48 hours					
Abnormal embryos 48 h (%)	**13**	**26**	**40**	**79**	**100**

A

	CTR	Pb 80 µg/l	Pb 100 µg/l	Pb 120 µg/l	Pb 250 µg/l
24 hours					
ITI 24 h	**0.23**	**0.72**	**0.78**	**0.73**	**1.09**
48 hours					
ITI 48 h (%)	**0.79**	**1.20**	**2.48**	**2.05**	**4.48**

B

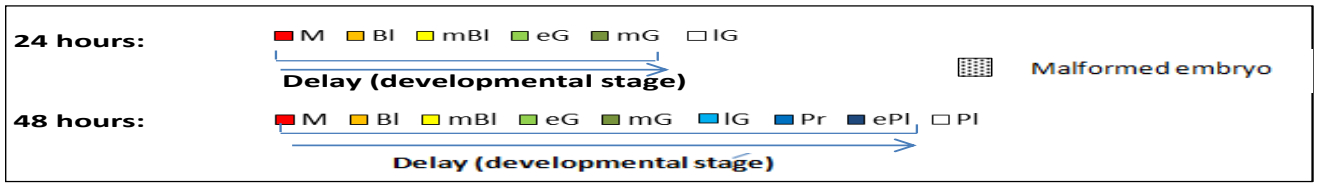
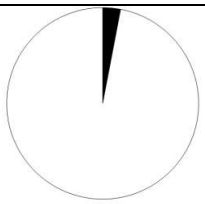
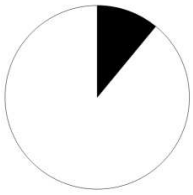
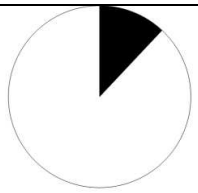
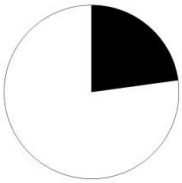
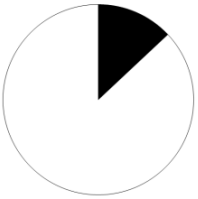
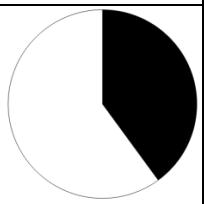
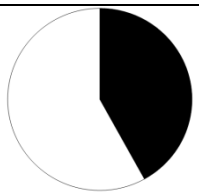
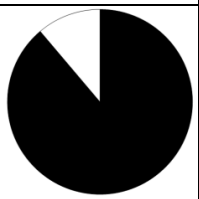
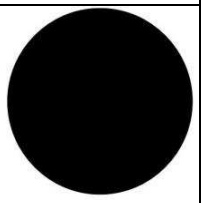
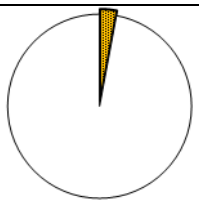
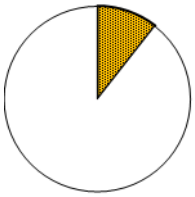
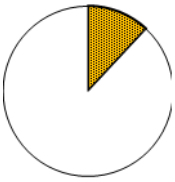
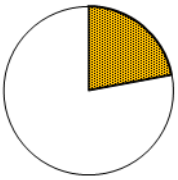
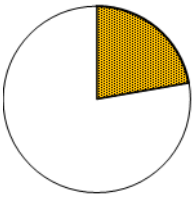
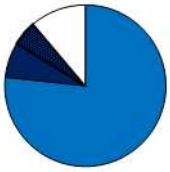
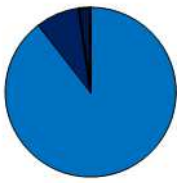


Fig. 18 – In the upper panel (A) the percentages of normal embryo are reported in white on the pie charts at 24 hours and 48 hours of incubation (on the rows) for each Pb concentration (on the columns). The percentage of normal embryos is reported in the cells under each pie chart.. In the lower panel (B) the stages of development reached by embryos are reported in different colors on the pie charts (as illustrated by the legend under the panel) at 24 hours and 48 hours of incubation (on the rows) for each Pb concentration (on the columns). The ITI value is reported in the cells under each pie chart.

Zn

□ Normal embryos ■ Abnormal embryos	CTR	Zn 60 µg/l	Zn 72 µg/l	Zn 100 µg/l	Zn 120 µg/l
24 hours					
Abnormal embryos 24 h (%)	**3**	**11**	**23**	**22**	**14**
48 hours					
Abnormal embryos 48 h (%)	**13**	**40**	**42**	**89**	**100**

	CTR	Zn 60 µg/l	Zn 72 µg/l	Zn 100 µg/l	Zn 120 µg/l
24 hours					
ITI 24 h	**0.23**	**0.84**	**1.64**	**1.78**	**1.95**
48 hours					
ITI 48 h (%)	**0.79**	**1.05**	**1.41**	**2.48**	**2.75**

B

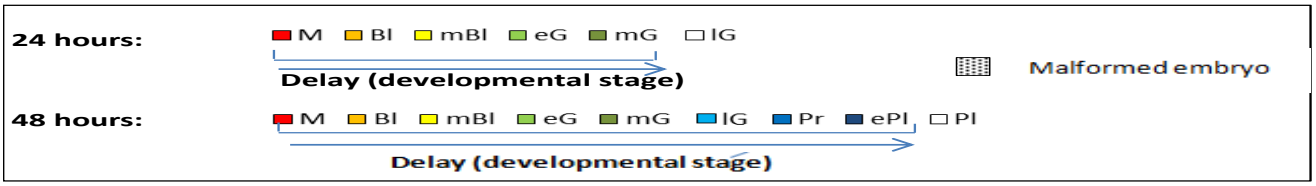
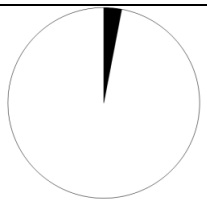
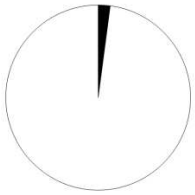
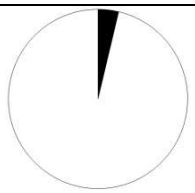
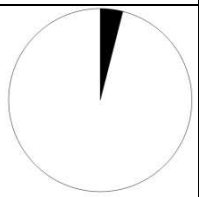
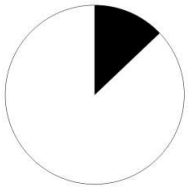
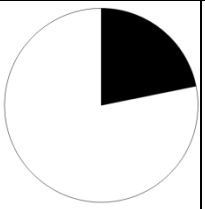
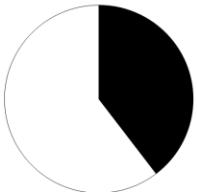
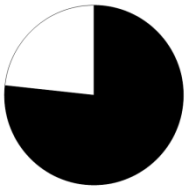
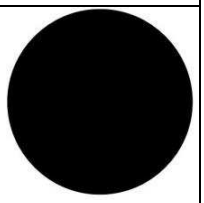
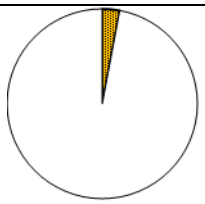
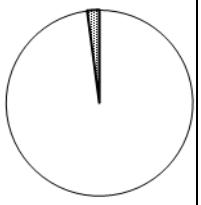
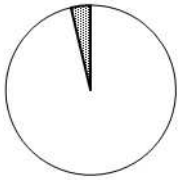
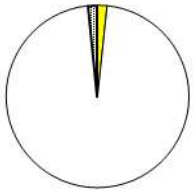
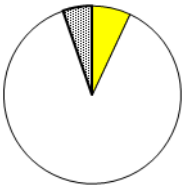
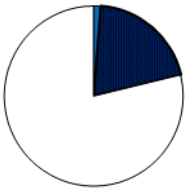
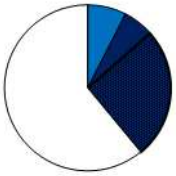
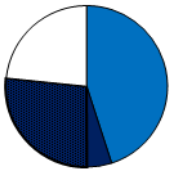
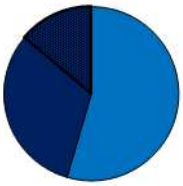


Fig. 19 – In the upper panel (A) the percentages of normal embryo are reported in white on the pie charts at 24 hours and 48 hours of incubation (on the rows) for each Zn concentration (on the columns). The percentage of normal embryos is reported in the cells under each pie chart. In the lower panel (B) the stages of development reached by embryos are reported in different colors on the pie charts (as illustrated by the legend under the panel) at 24 hours and 48 hours of incubation (on the rows) for each Zn concentration (on the columns). The ITI value is reported in the cells under each pie chart.

Cd

□ Normal embryos ■ Abnormal embryos	CTR	Cd 1 mg/l	Cd 1.5 mg/l	Cd 2 mg/l	Cd 2.5 mg/l
24 hours					
Abnormal embryos 24 h (%)	**3**	**3**	**3**	**5**	**13**
48 hours					
Abnormal embryos 48 h (%)	**13**	**22**	**40**	**77**	**100**

A

	CTR	Cd 1 mg/l	Cd 1.5 mg/l	Cd 2 mg/l	Cd 2.5 mg/l
24 hours					
ITI 24 h	**0.23**	**0.13**	**0.22**	**0.13**	**0.45**
48 hours					
ITI 48 h (%)	**0.79**	**1.24**	**1.88**	**2.79**	**3.05**

B

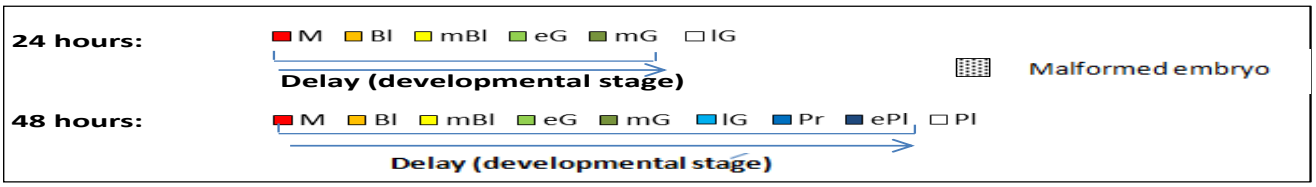


Fig. 20 – In the upper panel (A) the percentages of normal embryo are reported in white on the pie charts at 24 hours and 48 hours of incubation (on the rows) for each Cd concentration (on the columns). The percentage of normal embryos is reported in the cells under each pie chart. In the lower panel (B) the stages of development reached by embryos are reported in different colors on the pie charts (as illustrated by the legend under the panel) at 24 hours and 48 hours of incubation (on the rows) for each Cd concentration (on the columns). The ITI value is reported in the cells under each pie chart

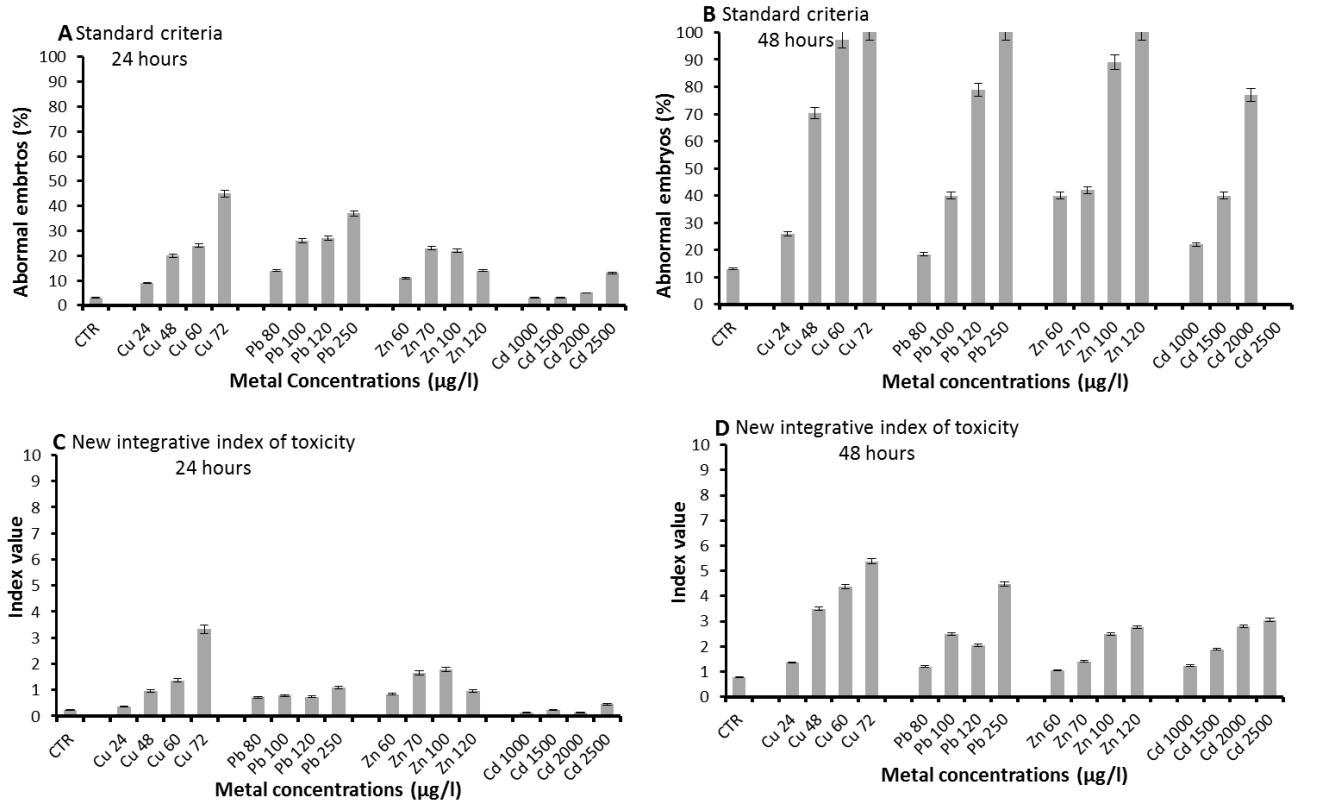


Fig. 21 – Sea urchin embryonic development evaluated according to standard criteria (A, B) and to ITI (C, D) at 24 hours (A, C) and 48 hours after fertilization (B,D). Histograms represent the results from the morphological analysis expressed as mean percentage (%) of abnormal embryos ± standard deviation (A) and values of ITI, calculated as reported in Materials and Methods (Table 4). Data refer to each metal concentrations, reported on the X axis. CTR: control.

Elutriate exposure

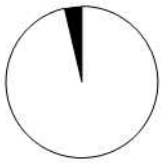
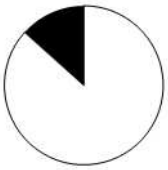
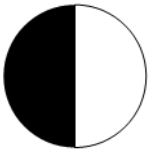
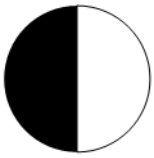
The results on embryonic development calculated according to the standard criteria and to ITI are shown on the pie charts in Fig. 22 and on the histogram in Fig. 23. Considering the evaluation of embryotoxicity obtained with the classical approach, the majority of the elutriates tested (66%, 16 out of 24 samples) showed an extremely high toxicity with a percentage of normal embryos lower than 10% at 24 h; only 17 % of samples (n=4) exhibited more than 80% of regular embryos. The extent of embryotoxicity was even higher at 48h, when 83% of elutriates (n=20) showed less than 10% of normally developed larvae (Fig. 22).

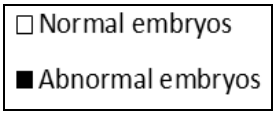
The ITI, as for metal experiments, demonstrated a major discriminatory capacity than standard criteria. With the new approach, the evaluation of embryotoxicity in sediment elutriates appeared to be more differentiated and the ITI was more homogeneously distributed between 0 and 10. This result reflects the fact that the ITI allows for a better separation of the samples according to the developmental stage

at which an effect is observed, and considering delays in development less severe than delays also showing morphological malformations. From a different perspective, the embryotoxicity of several harbor elutriates was overestimated by the classical evaluation method, since all the different effects are similarly considered as reflecting the maximum toxic level.

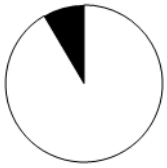
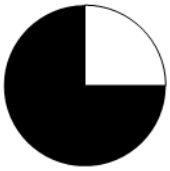
As an example, sample 2 exhibited the highest toxicity with the standard criteria, exhibiting more than 90% of abnormal larvae (Fig. 22A); however, the new ITI provided an intermediate value for this sample (Fig. 22B) because the majority of abnormal embryos were delayed but without any further morphological alteration. Several samples revealed an elevated percentage of delayed embryos at 24 hours, which, however, reached the blastula stage at 48 hours, confirming that development was delayed and not blocked. The results obtained indicated that the evaluation of embryotoxicity through the standard criteria or the ITI provided similar results for those samples characterized by a very limited or very high toxicity. On the other hand, when elaborated with the new ITI (delays in development prevailing on malformations), sediment elutriates characterized by an intermediate toxicity still appeared as highly toxic according to the classical criteria, with no distinguishing of the severity of the observed effects. These moderate effects are responsible for the different ecotoxicological classification obtained with these 2 approaches for several samples, with significant implications in the final assignment of the quality class to such sediments and in the resulting choice of more appropriate management options (Morroni *et al.* 2016).

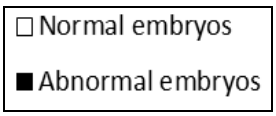
A – Standard criteria - 24 hours

					
CTR- 3%	**7** - 13%	**21** – 4%	**9** – 9%	**11**-13%	**13** – 94%
					
15 – 50%	**22** – 50%	**10** – 71%	**1** -87%	**3**-99%	**23**-100%
					
4-100%	**8**-100%	**2**-100%	**24**-100%	**14**-100%	**20**-100%
					
6-100%	**19**-74%	**5**-35%	**12**-58%	**16**-100%	**18**-100%

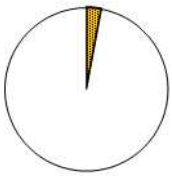
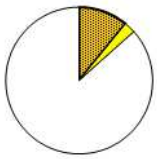
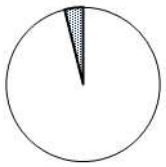
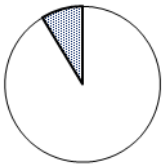
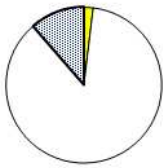
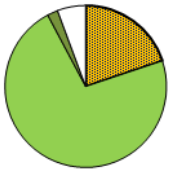


B- Standard criteria - 48 hours

					
CTR-9%	**7** -28%	**21** -58%	**9**-55%	**11**-80%	**13**-82%
					
15 -86%	**22**-87%	**10**-99%	**1**-100%	**3**-100%	**23**-100%
					
4-100%	**8**-100%	**2**-100%	**24**-100%	**14**-100%	**20**-100%
					
6-100%	**19**-100%	**5**-100%	**12**-100%	**16**-100%	**18**-100%



C – ITI - 24 hours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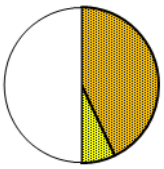	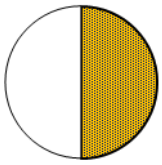	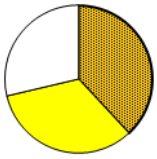	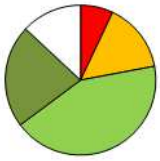	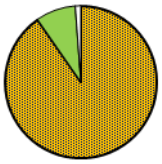	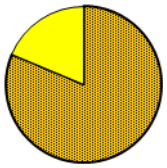
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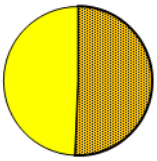
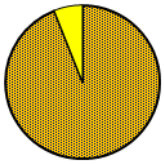
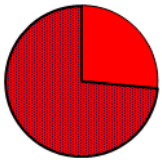
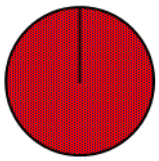
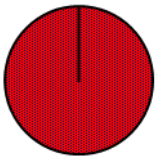
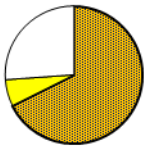
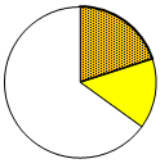
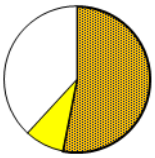
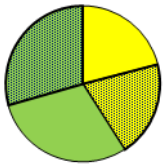
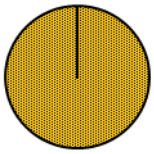
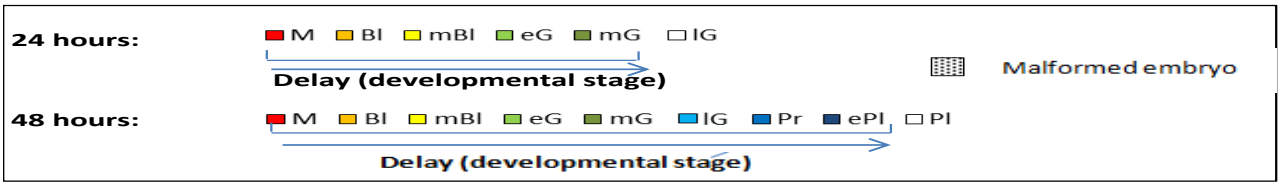
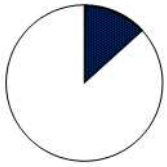
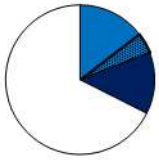
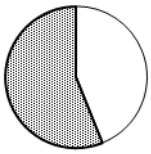
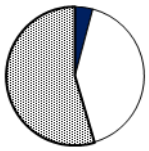
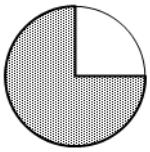
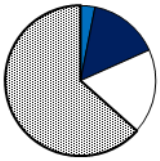
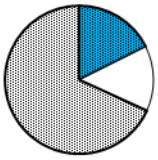
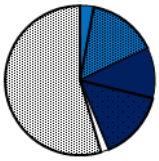
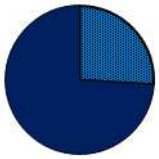
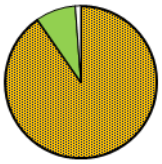
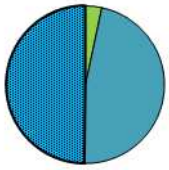
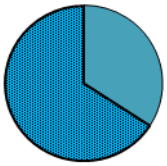
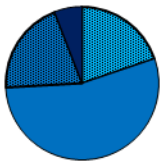
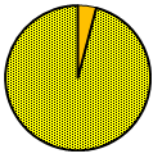
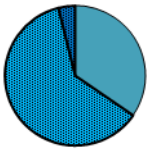
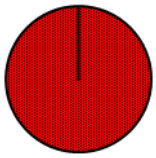
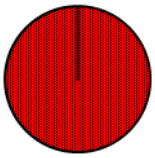
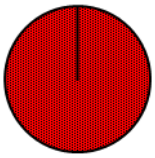
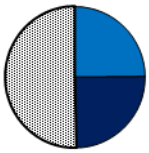
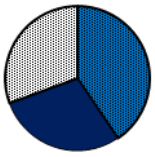
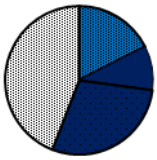
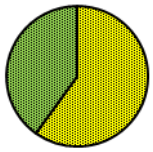
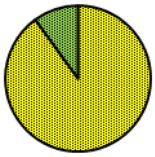
					
4-5.55	**8**-1.16	**2**-2.94	**24**-8.57	**14**-4.85	**20**-10
					
6-10	**19**-6.21	**5**-2.1	**12**-4.96	**16**-3.88	**18**-9



D – ITI - 48 hours

					
CTR-0.1	**7**-1.02	**21**-3.36	**9**-3.38	**11**-4.5	**13**-1.6
					
15-4.24	**22**-5.48	**10**-5.6	**1**-5.96	**3**-7.95	**23**-5.74
					
4-6.31	**8**-4.63	**2**-8.25	**24**-6.27	**14**-10	**20**-10
					
6-10	**19**-4.66	**5**-5.91	**12**-6.19	**16**-7.8	**18**-9

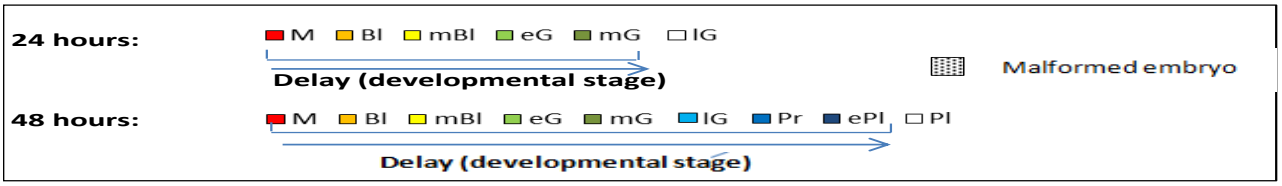
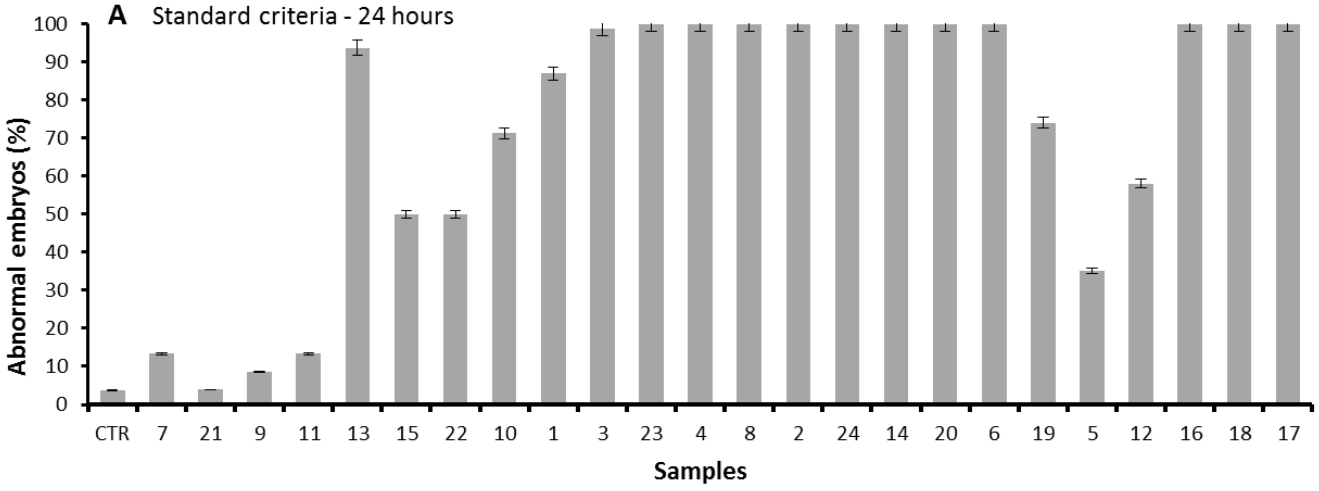
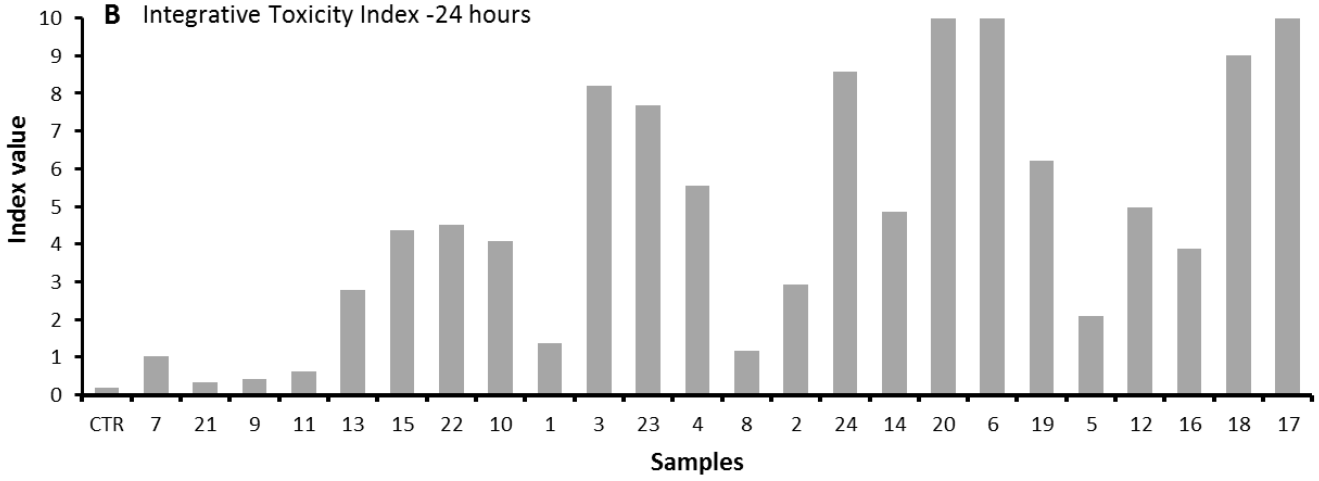


Fig. 22 –Percentages of abnormal embryo are reported in white on the pie charts at 24 hours (A) and 48 hours (B) of incubation for each elutriate sample. The sample code (in bold) and percentage of normal embryos are reported in the cells under each pie chart. In the lower panels the stages of development reached by embryos are reported in different colors on the pie charts (as illustrated by the legend under the panel) at 24 hours (C) and 48 hours (D) of incubation for each elutriate samples. The ITI value and the sample code are reported in the cells under each pie chart.


A Standard criteria - 24 hours
Abnormal embryos (%)
100
90
80
70
60
50
40
30
20
10
0
CTR 7 21 9 11 13 15 22 10 1 3 23 4 8 2 24 14 20 6 19 5 12 16 18 17
Samples


B Integrative Toxicity Index -24 hours
Index value
10
9
8
7
6
5
4
3
2
1
0
CTR 7 21 9 11 13 15 22 10 1 3 23 4 8 2 24 14 20 6 19 5 12 16 18 17
Samples

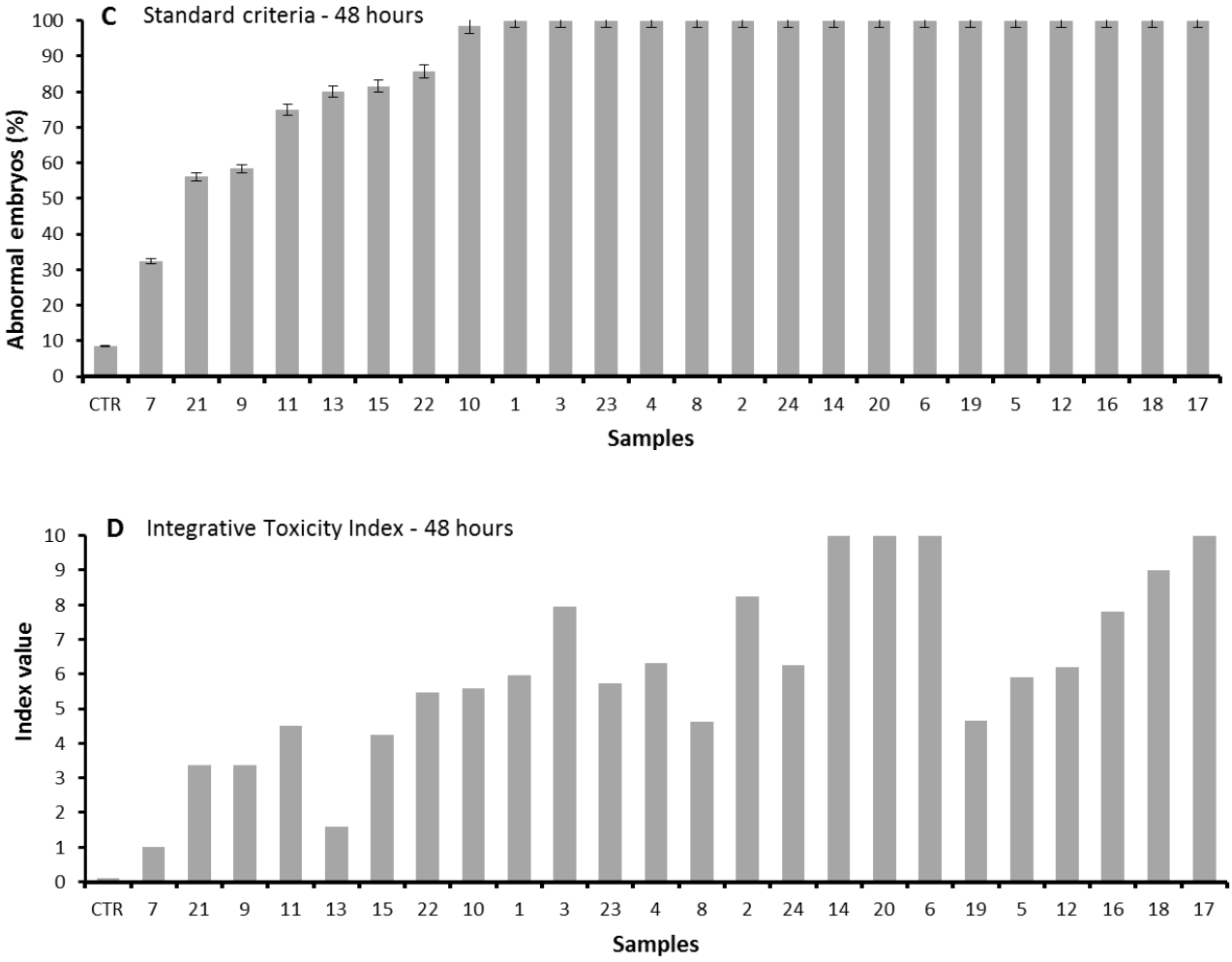


Fig. 23 – Sea urchin embryonic development evaluated according to standard criteria (A, C) and to ITI (B, D) 24 hours (A, B) and 48 hours after fertilization (C,D). Histograms represent the results from the morphological analysis expressed as mean percentage (%) of abnormal embryos ± standard deviation (A) and ITI values, calculated as reported in Materials and Methods (Table 4). Data refer to each elutriate sample, reported on the X axis. CTR: control.

3.3.2 *ITI application and validation*

Applying the ITI to embryos exposed to metal solution (3.3.1) and elutriate of marine sediments (3.3.2), a greater discriminatory capacity than that of standard criteria was demonstrated. In fact, in both cases the embryotoxicity evaluation appeared to be more differentiated with ITI values distributed between 0 and 10. The solutions which exhibited medium and high toxicity with the classical approach showed different effects on embryo development detectable and synthesized by ITI, which indicate that the toxicity was often overestimated by the standard method.

However, this approach has also demonstrated several limitations. In fact, it was observed that at each stage of development embryos can exhibit malformations with differing severity and, consequently, with differing reversibility. For this reason embryos with similar index values can show differing reversibility of toxic effects. To further improve the ITI estimation the scale of toxicity was modified considering the severity of germ layer impairments.

Solutions that caused an intermediate toxicity when elaborated with the new ITI still appeared as highly toxic according to the classical criteria, with no distinguishing of the severity of the observed effects. In fact germ layer malformations may have differing levels of severity, and consequently differing inability to properly differentiate ectoderm, mesoderm and endoderm territories and to recover the correct development of the embryo. Also in this new scale of toxicity embryos were considered normal if they satisfied the following morphological criteria: (1) correct schedule in reaching the developmental endpoint (gastrula at 24h and pluteus at 48 h), (2) left/right and dorso/ventral embryonic axis symmetry, (3) differentiation of oral/aboral ectoderm and endoderm territories, (4) skeleton development and patterning. To calculate the index score, lower toxicity values were given to embryos with delay in development and absence of malformations. Higher scores were attributed to embryos showing contemporarily delay and moderate impairment of the left/right or dorso/ventral axis symmetry, as well as germ layer territories, using different categories based on the severity of the effects. The highest scores were attributed to delayed embryos with severe developmental anomalies, which cause the loss of the normal body organization and the appearance of embryos with aberrant morphology. In particular, after 24 hours the assigned toxicity score is 0 for late gastrula (lG) corresponding to the normal stage, and 1 to 4 when the embryos observed are gastrula (G), mesenchyme blastula (mBl), blastula (Bl) or morula (M) respectively. When embryos at G also present germ layer defects, their toxicity score is fixed at 5 for ectoderm anomalies, 6 for endoderm malformations and 7 for mesoderm defects. When embryos exhibit more severe developmental

anomalies a score range from 8 to 10 is assigned to aberrant G (8), aberrant mBl (9) and aberrant Bl or M (10) (Table 5A). At 48 hours, the toxicity score is 0 for pluteus (Pl), from 1 to 5 when observing delayed stages corresponding to early pluteus (ePl), prism (Pr), G, mBl and Bl. Toxicity scores increase from 6 to 8 for ePl, Pr and G when abnormal morphotypes are also observed. Finally the highest index values are assigned to aberrant embryos delayed at Pl or Pr (9) and at Bl or M (10) (Table 5B).
Different scales and scores were tested by comparing with expert judgement the calculated indices with morphologic observations. Details of selected scores and stages of development are given in Table 5. After the development of the scale of toxicity, this was applied and validated estimating the embryotoxicity of 38 elutriate samples from Trapani harbor, chosen as a model area. The developmental analyses were conducted with standard criteria, size criteria and ITI. Developmental endopoints were compared with results obtained by physico-chemical analysis and with other methods to investigate different biologically induced damage such as HSP60 and HSP70 production and DNA damage in exposed embryos, as described in detail in Material and methods. The results are reported in the following paragraphs.

Inhibition of larval length criteria: Concentration dependence in growth inhibition was found for all elutriate samples at 48 h. Table 6 shows the effect concentrations expressed as EC10 and EC50 comparing larval length criteria with normal embryo standard criteria. Data refer to the dilution (expressed in percentage) of elutriate samples. Observing the results, the data appear to confirm the trend already observed in metal-exposure experiments. In fact, once again, the straight-line slope values reported in Table 6 are much higher for curves obtained using percentages of normal larvae than those obtained using larval length. Differently from EC10, with the analysis of EC50 values, the inhibition of larval growth appears to be a less sensitive endpoint than embryogenesis success for all metals, as a consequence of the differing slopes of the curves. These data confirm that early larval growth is not a more sensitive response than the classical criteria based on embryogenesis success. In fact, to enhance the discriminatory ability of the sea urchin embryo toxicity test it is necessary to discriminate different developmental anomalies, which can cause the appearance of morphotypes of a similar size and considered as being at the same toxicity level with the classical approach.

	Toxicity Categories										
Gastrula (24 h)	***Normal*** 	***Delayed*** 				***G with germ layers defects*** 			***Aberrant morphologies*** 		
	lG	**G**	**mBl**	**Bl**	**M**	**Ectoderm**	**Endoderm**	**Mesoderm**	**G**	**mBl**	**Bl, M**
	0	1	2	3	4	5	6	7	8	9	10

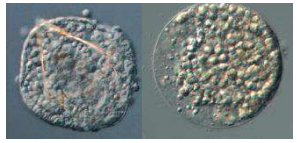
	Toxicity Categories										
Pluteus (48-72h)	***Normal*** 	***Delayed*** 					***Delayed + defects*** 			***Aberrant morpholohies*** 	
	Pl	**Pl**	**Pr**	**G**	**mBl**	**Bl**	**Pl**	**Pr**	**G**	**Pl-Pr**	**Bl-M**
	0	1	2	3	4	5	6	7	8	9	10

Table 5 – Classification of malformations according to the ITI based on detailed analysis of developmental delays (delayed embryos) and germ layer impairment (abnormal embryos)

	Normal embryos (standard criteria)			**Size inhibition**		
	Slope	EC10	EC50	slope	EC10	EC50
93 C/D	0	>400	>400	0	>400	>400
81 C/D	2.951	91.677	249.199	2.358	91.463 (70.745 146.456)	319.616 (179.547 2791.482)
78/79 E	1.280	39.252	393.359	0,700	21.357	>400
48 C/D	3.406	11.208 (20.862 35.235)	58.747 (50.467 66.304)	0.819	2.491 (0.001 9.133)	91.491 (59.997 488.234)
78 C/D	2.971	49.542 (11.352-67.789)	133.742 (104.465-345.223)	1.862	46.719 (31.830 59.050)	227.787 (146.059-680.502)
93 A	2.775	35.444 (15.076-49.304)	102.637 (83.122 144.170)	0.784	32.234 (19.321-35.402)	222.647 (183.522 244.189)
49 C/D	7.544	55.429 (35.565 66.516)	81.963 (69.413 89.915)	1.794	42.401 (27.666 53.989)	219.555 (141.498 641.339)
47 C/D	4.414	30.108 (20.854 37.364)	58.747 (50.467 66.304)	1.150	15.834 (10.458 27.644)	76.738 (55.697 80.344)
49 A	4.96	34.782 (24.252 42.645)	63.038 (54.353 70.740)	1.744	14.160 (6.580 20.819)	76.861 (62.571 105.182)
79A	2.733	9.933	29.231	1.379	14.162 (4.409 22.629)	120.191 (86.222 255.192)
73/74 E	6.585	40.276 (31.993 46.585)	63.046 (56.372 69.261)	2.455	44.592 (33.300 53.698)	148.346 (114.399 241.130)
48 A	2.730	12.686	37.380	3.390	30.486 (24.288	72.785 (64.928

					35.728)	83.353)
78 A	3.085779	10.946 (6.284 15.158)	28.483 (22.571 33.559)	1.779	7.644 (3.655 15.860)	35.823 (26.751 40.795)
73 A	Nc	Nc	nc	nc	nc	nc
76A	5.418	19.959 (15.512 23.714)	34.408 (30.077 38.339)	2.728	11.569 (9.630 21.156)	50.477 (32.767 68.328)
45 A	4.014	10.255 (7.305 12.774)	21.389 (18.287 24.310)	1.993	7.483 (5.840 13.471)	30.630 (18.287 24.310)
74 C/D	5.365	11.145 (7.899 13.432)	19.318 (16.909 21.588)	1.998	8.253 (0.755 16.258)	36.130 (19.382 88.446)
46A	4.096	11.997 (8.183 15.149)	24.656 (20.827 28.138)	1.0964	8.647 (4.533 12.836)	32.746 (28.846 35.648)
75 A	7.050	29.032 (14.777 35.499)	44.122 (36.656 48.437)	2.074	16.605 (9.632 22.602)	68.853 (58.127 86.013)
72A	2.866	9.183	25.713	1.614	5.678 (0.246 14.341)	35.324 (13.944 366.702)
47 E/F	2.857	4.892	13.742	1.663	5.190 (0.064 13.064)	30.598 (11.520 105.209)
81 A	10.328	25.855 (20.943 29.460)	34.405 (30.381 37.739)	1.596	6.992 (3.885 10.130)	44.432 (36.830 54.925)
79 C/D	4.043	13.431 (9.465 16.872)	27.866 (23.548 31.818)	2.080	17.701 (0.000 36.185)	73.105 (35.591 193)
44A	9.626	21.563 (18.287	29.298 (26.726	2.816	19.075	54.381

		23.974)	32.110)			
43 A	8.419	20.550 (17.225 23.041)	29.176 (26.569 31.822)	4.986	45.212 (38.366 50.809)	81.707 (74.779 90.468)
49 E	7.521	29.210 (19.871 34.781)	43.244 (37.131 47.363)	2.245	26.819 (18.077 33.863)	99.849 (82.055 136.162)
73 C/D	3.696	12.707 (8.037 16.507)	28.235 (23.538 32.593)	2.684	26.399 (19.310 32.299)	79.256 (68.605 95.899)
48 E/F	3.682	15.622	34.816	1.336	4.567 (0.655 9.790)	41.560 (29.673 53.488)
75 C/D	7.812	5.318 (4.430 6.010)	7.758 (7.025 8.460)	0.104	nc	nc
77 C/D	3.792	11.261 (5.403 14.716)	24.520 (20.708 30.891)	2.094	8.239 (0.000 22.552)	33.700 (3.470 148.199)
76 C/D	9.195	12.500 (9.990 14.353)	17.230 (15.177 18.925)	1.865	6.456	31.411
42 A	9.374	22.760	31.181	1.802	6.673	34.307
76/77 E	3.578	4.115	9.388	1.508	3.515 (0.034 9.134)	24.885 (10.025 62.536)
46 C/D	6.119	4.771 (3.654 5.609)	7.727 (6.832 8.543)	1.693	3.253 (0.021 8.532)	18.599 (5.992 63.057)
47A	2.215	5.511 (2.237 8.254)	20.879 (16.297 28.637)	2.589	4.808 (0.005 13.165)	28.584 (8.139 134.307)
71 A	8.396	19.020 (15.479	27.030 (24.674	3.532	27.244 (21.808	62.809 (56.453

		21.410)	29.380)		31.903)	70.620)
74 A	5.660	13.142	22.135	2.289	12.096 (9.453 14.644)	43.902 (38.359 50.913)

Table 6- – Toxicity data expressed as EC10 and EC50 obtained using percentages of normal embryos (left column) compared with those obtained using size inhibition (right column). Data are reported in µg/l with respective 95% confidence limits and straight-line slope value.

Standard criteria and ITI

The results on embryonic development calculated according to the standard criteria and the new ITI are reported in detail on the pie charts in Fig. 24. On the graphs are reported the percentage of well-developed embryos for each sample (A,B,C) and the morphological analysis with the developmental stage reached by embryos and the occurrence of malformations or aberrant morphologies reporting the ITI value for each sample (D,E,F). These data are synthesized on the histogram in Fig. 25.

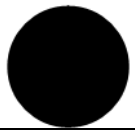
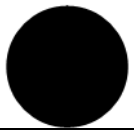
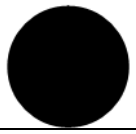
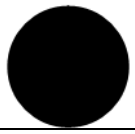
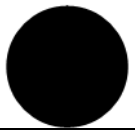
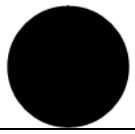
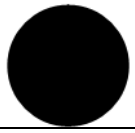
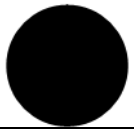
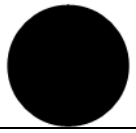
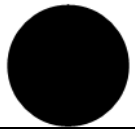
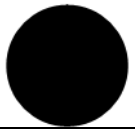
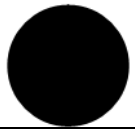
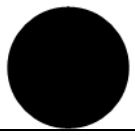
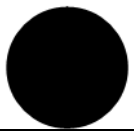
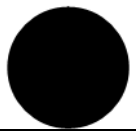
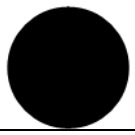
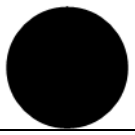
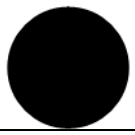
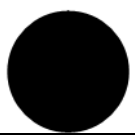
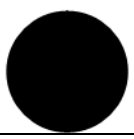
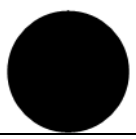
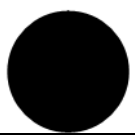
Considering the classical approach, the majority of elutriates tested (58%) showed an extremely high toxicity with a percentage of normal embryos lower than 10% already at 24 h; only 13% of samples (n=5) exhibited more than 80% of regular embryos (Fig. 24A, 25). The extent of embryotoxicity was even higher at 48h when 76% of elutriates (n=29) showed less than 10% of normally developed larvae (Fig. 24B, 25). At 72h a significant toxicity decrease was observed and the 76% of elutriates which caused less than 10% of normal embryos at 48h decreased to 42% of elutriates at 72 h.

Comparing these results with those obtained applying the ITI, it is evident that using the new approach the evaluation of embryotoxicity is more differentiated, with index values distributed between 0 and 10. This trend is much less evident in samples characterized by low and moderate toxicity, with percentages of abnormal embryos generally up to 80%. In fact, these elutriates, compared to those with similar percentages of well-developed embryos, exhibit very similar values of integrative toxicity, approximately between 0.5 and 3. Slight differences in ITI value were observed only at 24 hours for samples 6B and 8A, which showed 33% of abnormal embryos with an ITI of 1.66 for 6B and 2.55 for 8A (Fig. 24 A, D).

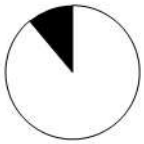
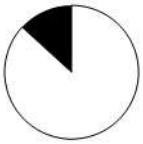
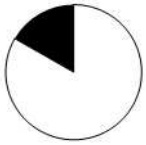
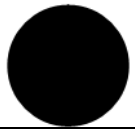
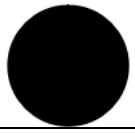
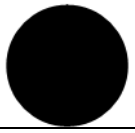
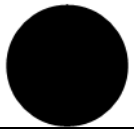
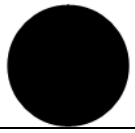
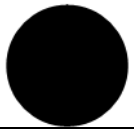
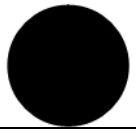
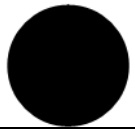
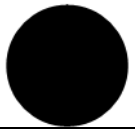
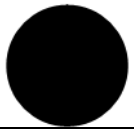
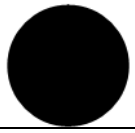
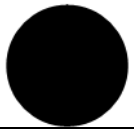
A – Standard criteria - 24h

					
CTR -6%	**19B** -9%	**18B** -19%	**15C** -15%	**7B** -59%	**15B** - 11%
					
19A -20%	**8B** -14%	**6B** -33%	**8A** -33%	**17A** -46%	**11C** -58%
					
7A -42%	**15A** -70%	**11A** -38%	**14A** -40%	**4A** -74%	**10B** -100%
					
5A -42%	**9A** -39%	**12A** -100%	**6C** -73%	**18A** -22%	**17B** -34%
					
3A -52%	**2A** -100%	**8C** -71%	**11B** -46%	**7C** -48%	**9B** -100%
					
16B-100%	**14B**-100%	**1A**-100%	**14C**-100%	**5B**-100%	**6A**-100%
					
13A-100%	**10A**-100%				

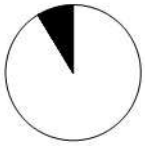
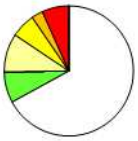
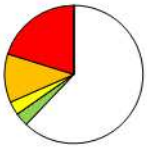
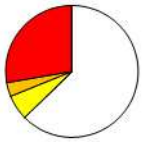
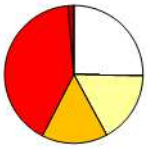
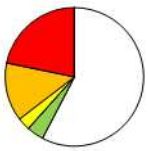
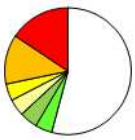
B – Standard criteria - 48h

					
CTR-12%	**19B**-15%	**18B**-22%	**15C**-29%	**7B**-36%	**15B**-42%
					
19A-53%	**8B**-77%	**6B**-75%	**8A**-87%	**17A**-90%	**11C**-92%
					
7A-96%	**15A**-96%	**11A**-97%	**14A**-99%	**4A**-100%	**10B**-100%
					
5A -100%	**9A**-100%	**12A**-100%	**6C**-100%	**18A**-100%	**17B**-100%
					
3A-100%	**2A**-100%	**8C**-100%	**11B**-100%	**7C**-100%	**9B**-100%
					
16B-100%	**14B**-100%	**1A**-100%	**14C**-100%	**5B**-100%	**6A**-100%
					
13A-100%	**10A**-100%				

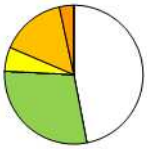
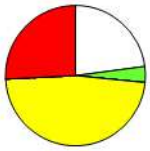
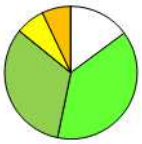
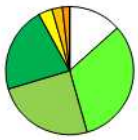
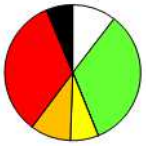
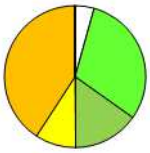
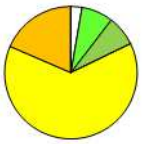
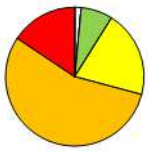
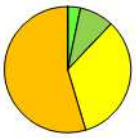
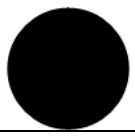
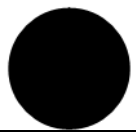
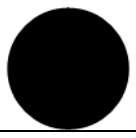
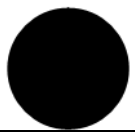
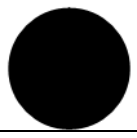
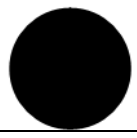
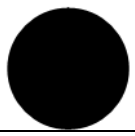
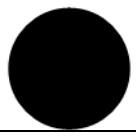
C – Standard criteria - 72h

					
CTR-5%	**19B**-11%	**18B**-20%	**15C**-13%	**7B**-30%	**15B**-17%
					
19A-28%	**8B**-26%	**6B**-19%	**8A**-25%	**17A**- 35%	**11C**-90%
					
7A-10%	**15A**-63%	**11A**-18%	**14A**-27%	**4A**-100%	**10B**-90%
					
5A-13%	**9A**-42%	**12A**-100%	**6C**-100%	**18A**-51%	**17B**-100%
					
3A-100%	**2A**-37%	**8C**-22%	**11B**-100%	**7C**-100%	**9B**-100%
					
16B-100%	**14B**-100%	**1A**-100%	**14C**-100%	**5B**-100%	**6A**-100%
					
13A-100%	**10A**-100%				

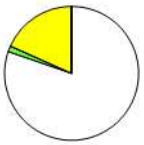
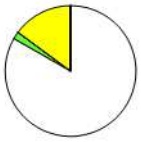
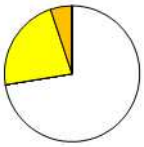
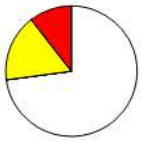
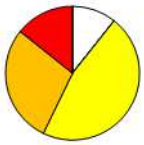
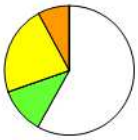
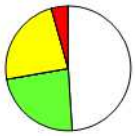
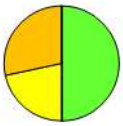
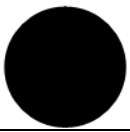
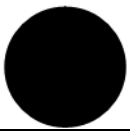
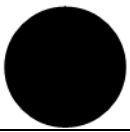
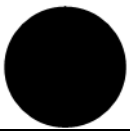
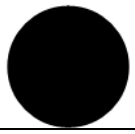
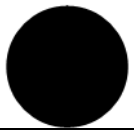
D – Index of toxicity 24 h

					
CTR-0.49	**19B**-0.87	**18B**-0.91	**15C**-0.82	**7B**-3.04	**15B**-0.71
					
19A-0.89	**8B**-0.72	**6B**-1.66	**8A**-2.55	**17A**-2.13	**11C**-3.46
					
7A-2.33	**15A**-3.58	**11A**-2.66	**14A**-2.69	**4A**-5.30	**10B**-7
					
5A-2.95	**9A**-2.43	**12A**-8.91	**6C**-5.54	**18A**-1.22	**17B**-2.57
					
3A-2.57	**2A**-7.45	**8C**-4.77	**11B**-2.74	**7C**-3.55	**9B**-9.88
					
16B-9.40	**14B**-10	**1A**-9.70	**14C**-10	**5B**-10	**6A**-10
					
13A-8	**10A**-8				

E – Index of toxicity 48 h

					
CTR - 0.99	**19B** - 1.01	**18B** - 1.03	**15C**-0.87	**7B**- 1.53	**15B**-1.64
					
19A-2.24	**8B**-5.22	**6B**-1.96	**8A**-2.01	**17A**-5.06	**11C**-5.34
					
7A-4.02	**15A**-7.74	**11A**-5	**14A**-6.63	**4A**-9.33	**10B**-9.72
					
5A - 5.56	**9A**-8.44	**12A**-7.94	**6C**-9.01	**18A**-8.37	**17B**-6.36
					
3A-9	**2A**-8.59	**8C**-5.29	**11B**-6.03	**7C**-8	**9B**-10
					
16B - 10	**14B**- 10	**1A**- 10	**14C**- 10	**5B**- 10	**6A**- 10
					
13A- 10	**10A**- 10				

F – Index of toxicity 72 h

					
CTR - 0.29	**19B** - 0.32	**18B** - 1.11	**15C** - 0.90	**7B** - 1.33	**15B**- 0.91
					
19A - 1.71	**8B** - 1.32	**6B** - 1.29	**8A** - 1.55	**17A** - 2.82	**11C** - 5
					
7A - 0.71	**15A** - 3.87	**11A** - 1.04	**14A** - 1.94	**4A** - 10	**10B** - 4.72
					
5A - 0.67	**9A** - 2.09	**12A** - 9.91	**6C** - 9.07	**18A** - 2.03	**17B** - 9
					
3A - 9	**2A** - 2.17	**8C** - 1.58	**11B** - 3.78	**7C** - 8.51	**9B** - 10
					
16B- 10	**14B**- 10	**1A**- 10	**14C**- 10	**5B**- 10	**6A-** 10
					
13A- 10	**10A**- 10				

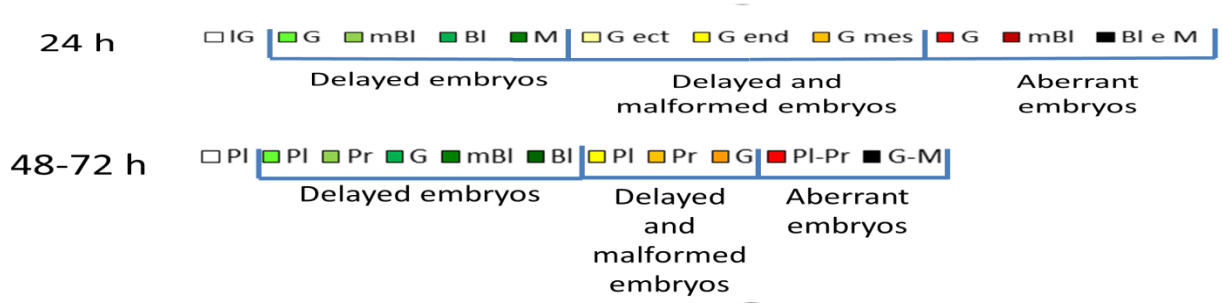


Fig. 24 –Percentages of normal embryo are reported in white on the pie charts at 24 hours (A), 48 hours (B) and 72 h (C) of incubation for each elutriate sample. The sample code (in bold) and percentage of normal embryos are reported in the cells under each pie chart. In the lower panels (D, E, F) the stages of development reached by embryos are reported in different colors on the pie charts (as illustrated by the legend) at 24 hours (D), 48 hours (E) and 72 hours (F) of incubation for each elutriate sample. The sample code and ITI value are reported in the cells under each pie chart .

In contrast, a much more evident discriminatory ability of the ITI was observed at higher toxic effects. For example, at 48 hours 6A, 8B and 17A show percentages of abnormal embryos ranging from 85 to 90% but 17A exhibits an index value more than 2 times greater than 6A and 8B (5.06 versus 1.96 and 2), as is observable from Fig. 24 B, E. This is mainly due to the 40% of aberrant embryos (34% of aberrant prisms and 6% of aberrant gastrulae) caused by 17A and partially observed in extremely lower frequencies in the other two elutriate samples (Fig. 24 E). The same phenomenon was observed considering more highly toxic samples such as 7A and 15A, both of which caused 96% of abnormal embryos but with index values of 4.02 and 7.74 respectively. Once again the higher value calculated for 15A is caused by the 73% of aberrant embryos (18% of aberrant prisms and 55% of aberrant gastrulae) present in this sample. Finally, analyzing the effects on embryos exposed to samples that give rise to 100% of malformed embryos the highest variability of index values was observed. In fact, considering, as an example, at 48 hours 8C, 5A , 12A, 9A, 3A, these samples show very differentiated ITI values with 5,29, 5,56, 7,94, 8,44, 9, respectively (Fig. 24 E). In contrast, other elutriates show the maximum toxicity value of 10, including 9B, 168, 14B, 1A, 14 C, 5B, 6, 13A, 10A. Also at 72 hours the greater discriminatory ability of ITI compared to classical criteria is evident in samples with 100% of abnormal embryos. 11B shows a low ITI (3,78) considering 100% of abnormal embryos. This is due to the absence of aberrant embryos but the presence of delayed plutei and slight malformations on all embryos. In contrast, other samples show higher toxicity values, such as 7C (8,51), 3A (9), 12A (9,91) and 10 other samples with a value of 10.

Considering the toxic effects during the incubation period both the standard criteria and the new ITI demonstrate the lowest toxic effects at 24 hours, the highest effects at 48 hours and a partial recovery at 72 hours, confirming the already observed trend in reversibility experiments using trace metals (Ch. 2). This trend is synthesized in Fig. 25. In particular, Fig. 25 C and D report the effects at 48 hours. At this time 22 samples exhibit 100% of malformed plutei (Fig. 25 C).

Considering the standard criteria, these samples show differentiated ITI index values and 9 out of the 22 samples show the maximum ITI score (10). At 72 hours a partial recovery of the correct embryo development was observed. However, 16 out of 22 samples with 100% of malformations at 48 hours continued to exhibit the maximal toxicity at 72 hours (Fig. 25 E). Interestingly, these samples were estimated as having an ITI value of 10. This is due to the total block of embryogenesis with the 100% of embryos which are at the pre-gastrula stage with an aberrant morphology. These very severe anomalies explain the impossibility of these embryos to recover the correct development, a phenomenon possible for the other embryos which exhibit a less severe developmental delay at 48 hours and not a block of embryogenesis.

Effects of elutriate exposure to protein levels of HSP60 and HSP70

In eukaryotes, the anti-apoptotic events triggered by stress-inducing agents have been shown to involve an increase in the levels of heat shock proteins (Beere 2005; Jiang *et al.* 2011). Indeed the nuclear accumulation of cytoplasmic HSP70 proteins is a ubiquitous response to heat stress and has been observed in a variety of different organisms (Chu *et al.* 2001). Previous studies on the sea urchin reported the production of the heat shock-inducible mitochondrial chaperonin HSP60 and the acknowledged cellular stress and anti-apoptotic protein HSP70 in response to different stressors such as trace metals (Geraci *et al.* 2004; Pinsino *et al.* 2011a), UV-B (Bonaventura *et al.* 2005, 2006) and X-rays (Bonaventura *et al.* 2011). In the present study, after 24 hours of incubation the exposed embryos presented levels of HSP60 and HSP70 similar to those of control embryos except for HSP70 in 8C and HSP60 in 14A, which exhibit slightly higher values (Fig. 26 B,C). Considering HSP70 production in embryos, after 48 hours of exposure they present values between 4 and 6 times higher than those of control embryos 9A, 6A, 1A. HSP60 was also found at high levels in embryos exposed for 48 hours to several samples such as 19B, 6B, 14A, with values up to 10 times higher than those found in control embryos. However, comparing these results with ITI values referring to the same samples (Fig. 26

A,E), there is no evident trend. In fact, analysis of correlation revealed the absence of a significant correlation between HSPs levels and developmental endopoints. Considering the complex mixture of chemical compounds present in the elutriate of marine sediments, it is evident that in this case their effects on HSPs production are not reflected on embryo morphology. For this reason, the evaluation of HSPs did not add any information to the ITI estimation.

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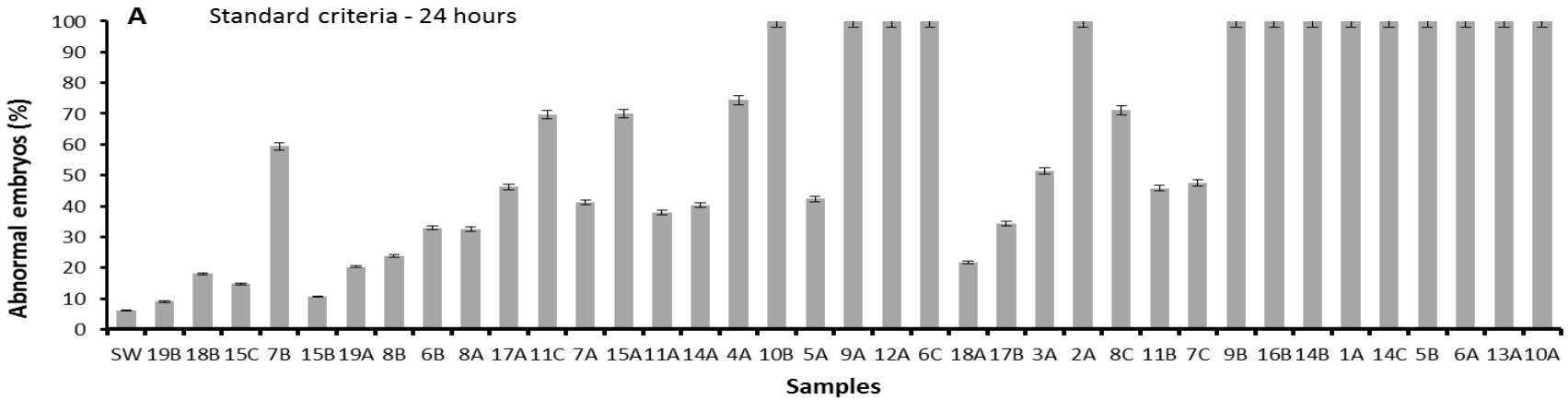
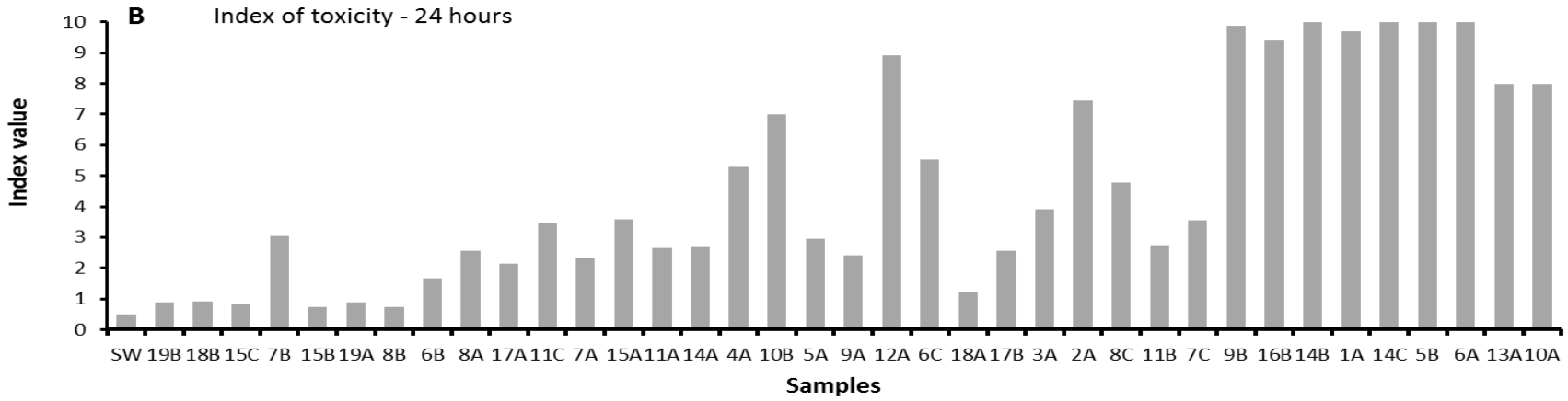
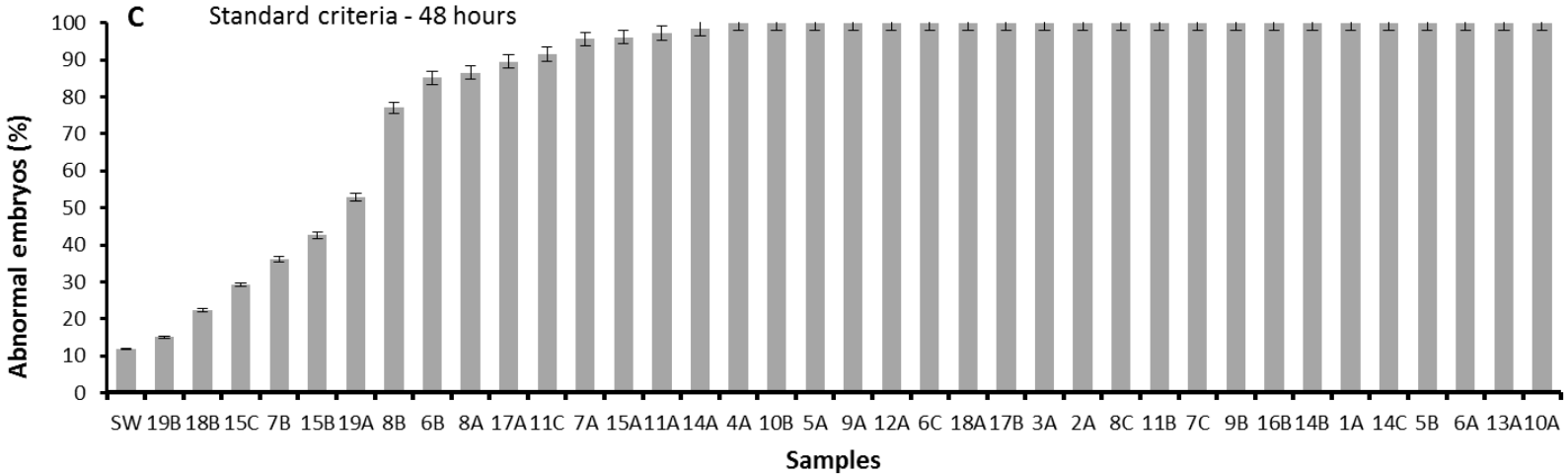
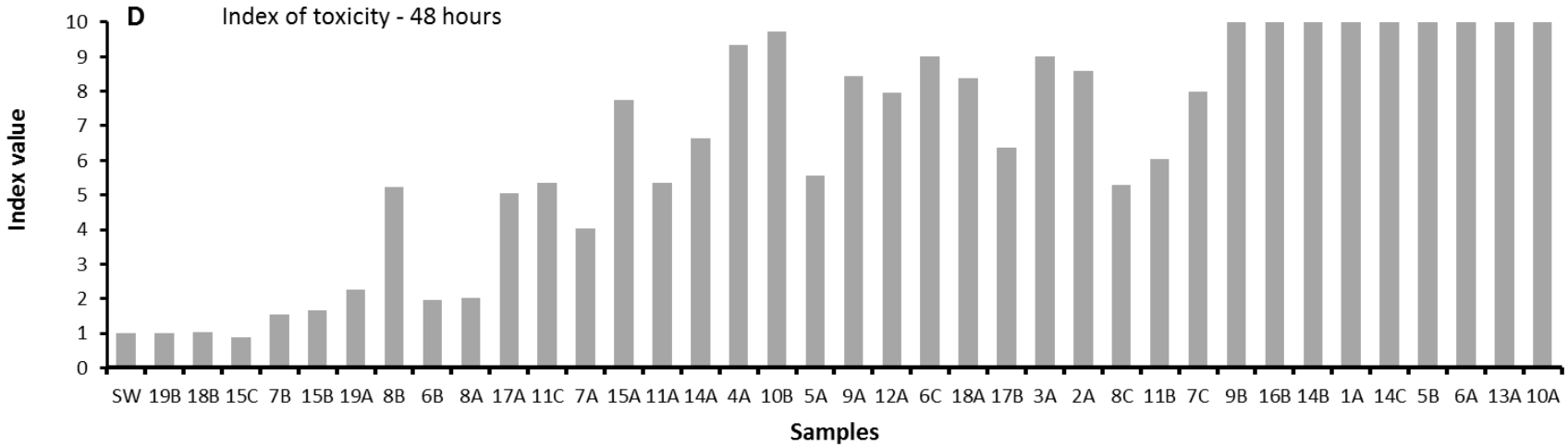

A
Standard criteria - 24 hours
Abnormal embryos (%)
0
10
20
30
40
50
60
70
80
90
100
SW 19B 18B 15C 7B 15B 19A 8B 6B 8A 17A 11C 7A 15A 11A 14A 4A 10B 5A 9A 12A 6C 18A 17B 3A 2A 8C 11B 7C 9B 16B 14B 1A 14C 5B 6A 13A 10A
Samples


B
Index of toxicity - 24 hours
Index value
0
1
2
3
4
5
6
7
8
9
10
SW 19B 18B 15C 7B 15B 19A 8B 6B 8A 17A 11C 7A 15A 11A 14A 4A 10B 5A 9A 12A 6C 18A 17B 3A 2A 8C 11B 7C 9B 16B 14B 1A 14C 5B 6A 13A 10A
Samples


C
Standard criteria - 48 hours
Abnormal embryos (%)
0
10
20
30
40
50
60
70
80
90
100
SW 19B 18B 15C 7B 15B 19A 8B 6B 8A 17A 11C 7A 15A 11A 14A 4A 10B 5A 9A 12A 6C 18A 17B 3A 2A 8C 11B 7C 9B 16B 14B 1A 14C 5B 6A 13A 10A
Samples


D
Index of toxicity - 48 hours
Index value
0
1
2
3
4
5
6
7
8
9
10
SW 19B 18B 15C 7B 15B 19A 8B 6B 8A 17A 11C 7A 15A 11A 14A 4A 10B 5A 9A 12A 6C 18A 17B 3A 2A 8C 11B 7C 9B 16B 14B 1A 14C 5B 6A 13A 10A
Samples

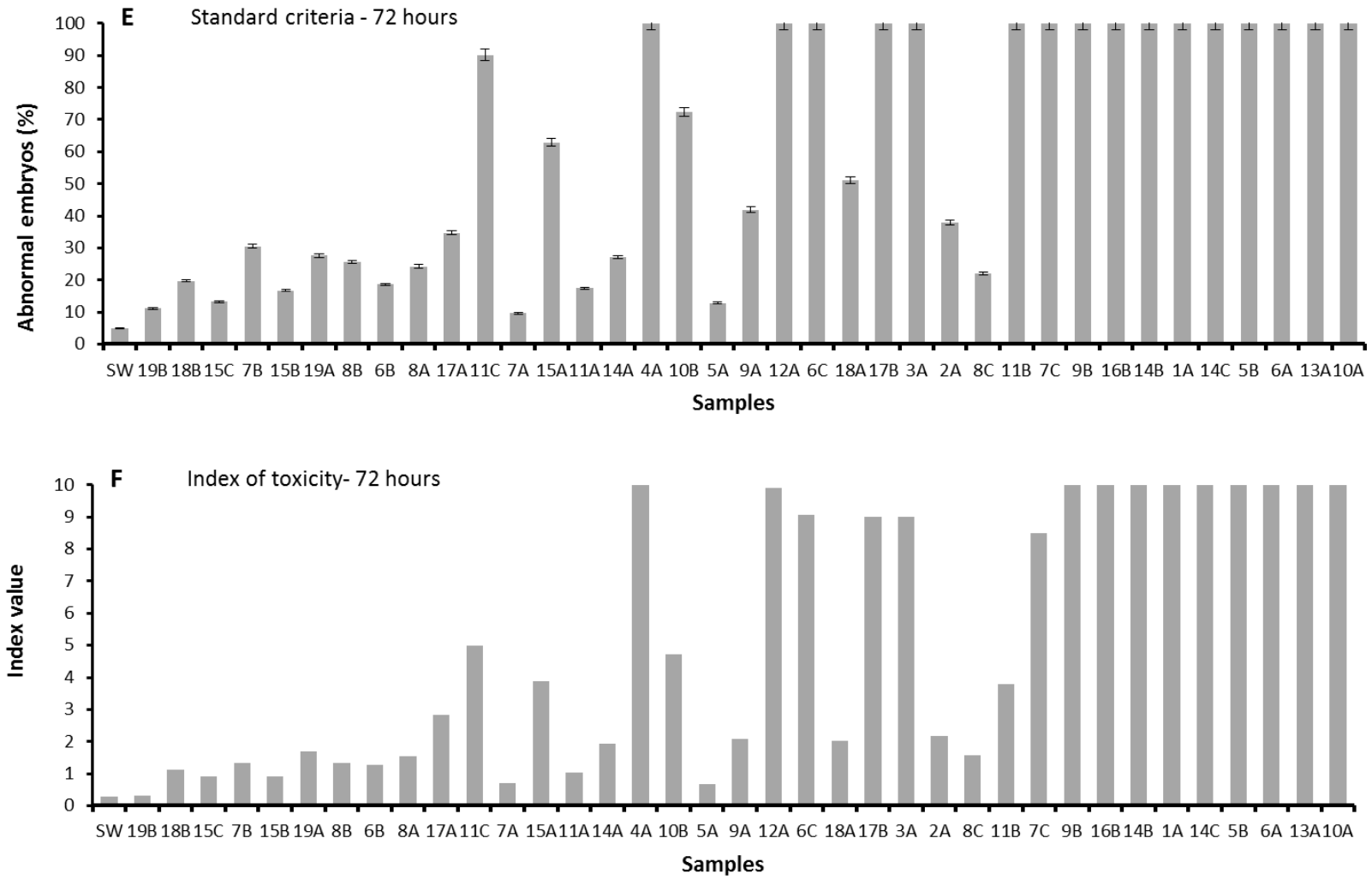
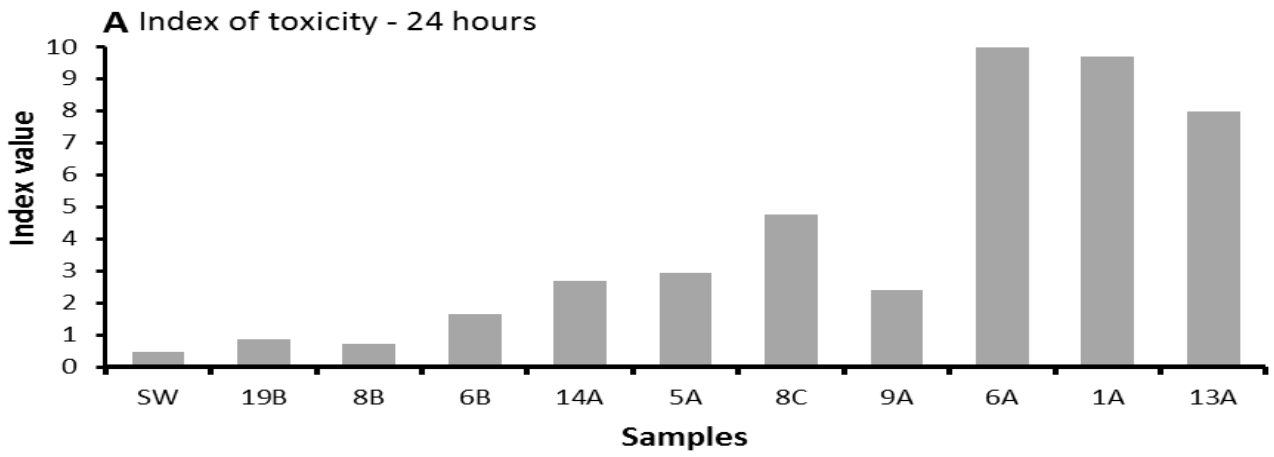
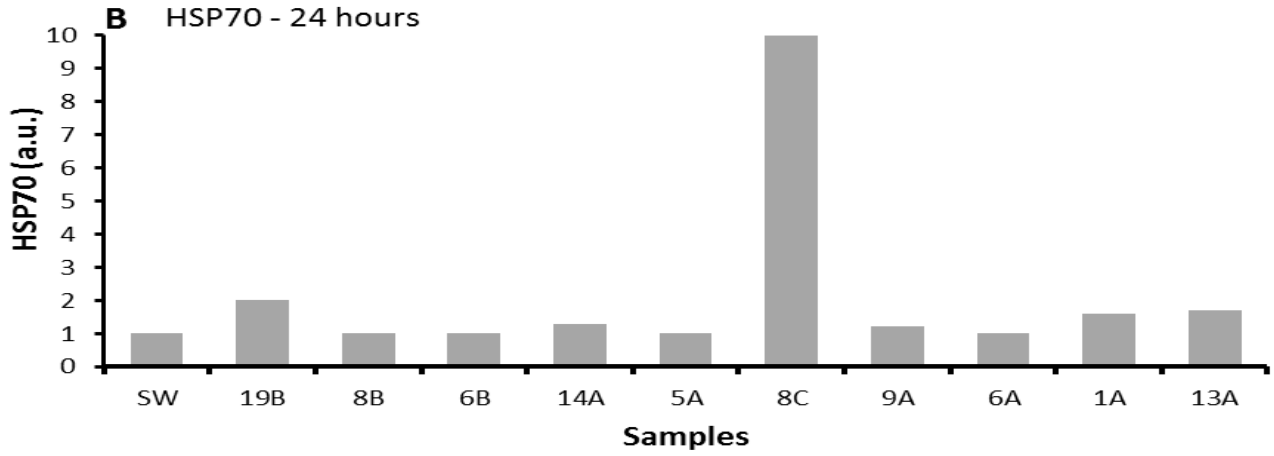
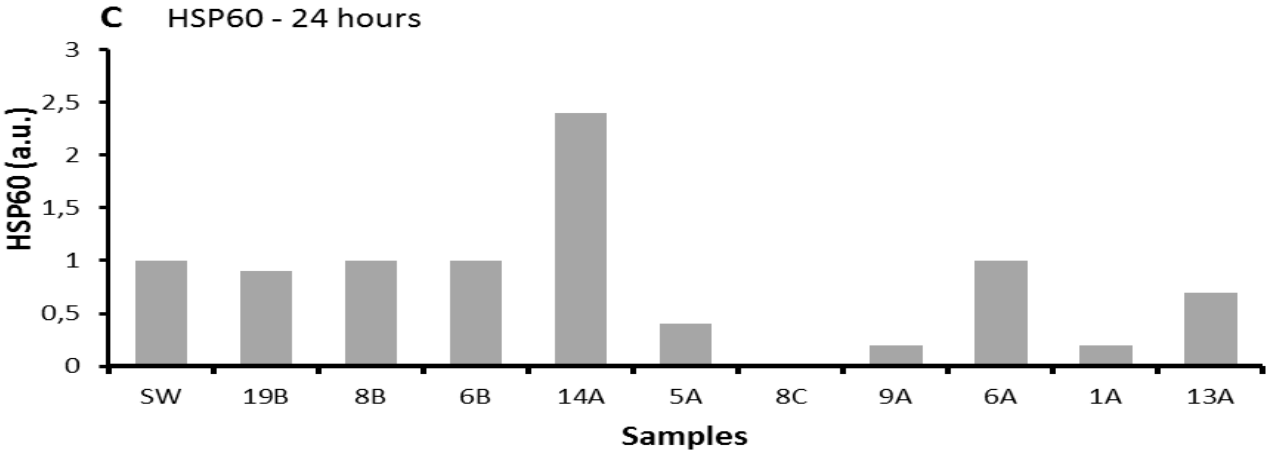


Fig. 25 – Sea urchin embryonic development evaluated according to standard criteria (A, C, E) and a new ITI (B, D, F) 24 hours (A, B), 48 hours (C,D) and 72 hours (E,F) after fertilization. Histograms represent the results from the morphological analysis expressed as mean percentage (%) of abnormal embryos ± standard deviation (A, C, E) and values of ITI (B, D, F), calculated as reported in Materials and Methods (Table 4). Data refer to each elutriate sample, reported on the X axis. SW: control.


A Index of toxicity - 24 hours
Index value
0
1
2
3
4
5
6
7
8
9
10
SW
19B
8B
6B
14A
5A
8C
9A
6A
1A
13A
Samples


B HSP70 - 24 hours
HSP70 (a.u.)
0
1
2
3
4
5
6
7
8
9
10
SW
19B
8B
6B
14A
5A
8C
9A
6A
1A
13A
Samples


C HSP60 - 24 hours
HSP60 (a.u.)
0
0,5
1
1,5
2
2,5
3
SW
19B
8B
6B
14A
5A
8C
9A
6A
1A
13A
Samples

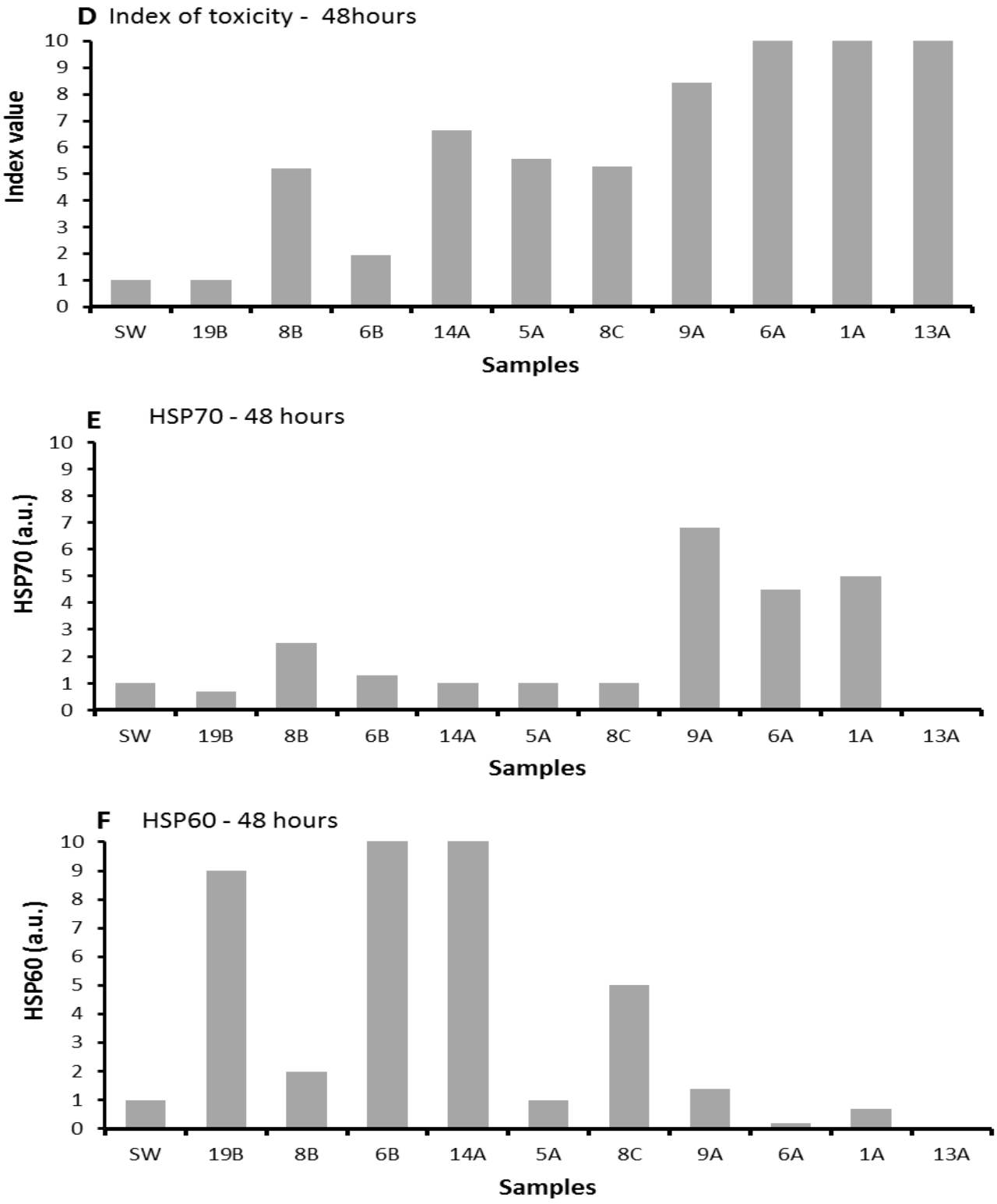


Fig. 26 – The results of semi-quantitative analysis of protein levees of HSP60 and HSP70 in embryos exposed to 10 sub-samples. Histograms refer to 10 elutriate samples (sample codes are reported on the X axis) analyzed at 24 hours (A,B,C,) and 48 hours (E, F, G,) after fertilization. In particular,a comparison is reported between the values of the new integrative toxicity index (A, D), the protein level of HSP70 (B,E) and HSP60 (C,F) in exposed embryos. SW: control

Comet and diffusion assay

The analysis of DNA alterations in aquatic organisms has been shown to be a highly suitable method for evaluating the genotoxic contamination of environments, being able to detect exposure to low concentrations of contaminants in a wide range of species. For this reason, tests directly assessing DNA strand breaks are commonly used to assess genotoxic impact in aquatic animals. The Single Cell Gell Electrophoresis (SCGE) or Comet assay was first applied to ecotoxicology about 15 years ago, and it has become one of the most popular tests for detecting strand breaks in aquatic animals through in vitro, in vivo and in situ exposures (see Frenzilli *et al.* 2009 for a review). However no protocol is available for application to sea urchin embryos, but only to gametes (Nahon *et al.* 2008; Pruski *et al.* 2009). Consequently the present study required the development of a new methodology. After the selection of pH of solution and the size of the nylon mesh, the first step, the removal of the fertilization envelope, was concluded (Fig. 27 A). The subsequent operation of cell dissociation was also successfully concluded at 4 cell, morula, blastula and early gastrula stage, as indicated in Fig. 27 B. Regarding comet assay protocol, after the definition of dissociated cell density to use, the main problem was the absence of lysis of the nuclear envelope. To overcome this problem, in addition to testing different times in lysis solution, the best results were obtained exposing dissociated cells to a solution of 0.075 M KCl in order to distend the membrane by osmosis and facilitate cell lysis, as indicated in "Materials and Methods". The results were partly positive after 20 minutes with the removal of the membrane only in some of the cells, which appear visible at fluorescent microscopy but not completely able to detect apoptosis events and DNA strand breaks. (Fig. 27 C). For this reason, further studies are needed to ameliorate the methodology and there are still no clear associations (if any) with embryotoxicity endopoints.

Chemical and Physico-chemical characterization of samples

The results of chemical analyses conducted on each sample of sediment are reported in Table 7. More than half of the analyzed samples (55%) exhibit a diffuse presence of trace metals (especially Cu, Zn, Cd and V) in concentrations higher than the Base Chemical Limit (BCL) fixed by the guidelines of ICRAM-APAT (2007). 22% present at least 1 metal which exceeds the Limit Chemical Level (LCL) reported by ICRAM-APAT (2007). The same percentage (22%) of total sediment samples also present organic pollutants with values higher than LCL. However, comparing these data with morphological analysis of exposed *P. lividus* embryos, the chemical contamination did not appear to be sufficiently important to explain the high toxic effects observed. As an example, sample 19B presents the

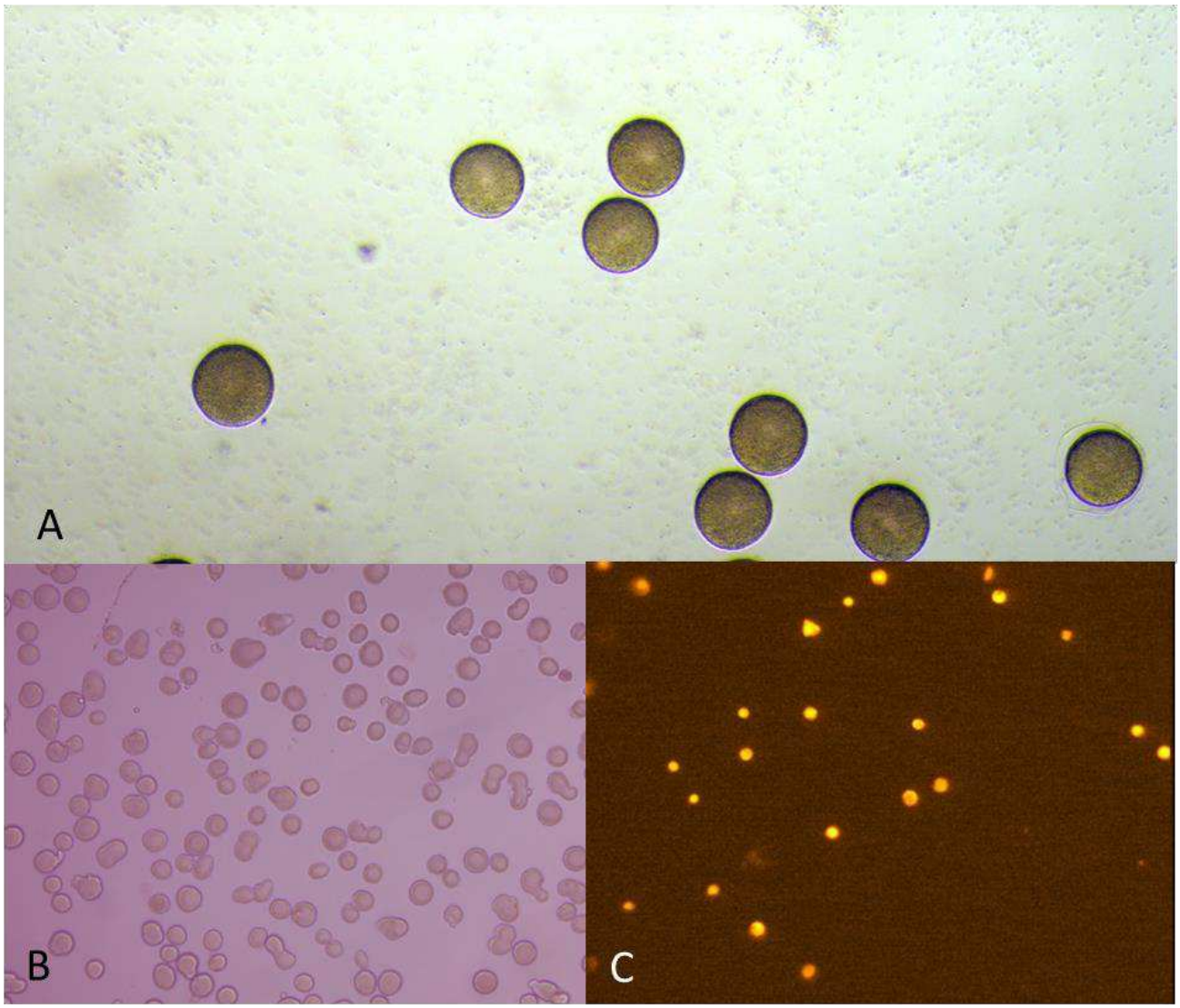


Fig. 27 – Comet assay protocol. A: Embryos 20 minutes after fertilization and before the first cell division with removed fertilization envelope. B: Dissociated cells. C: The appearance of sea urchin embryos nuclei after preparation for comet assay

lowest toxicity at 48 hours on sea urchin embryos, but at the same time exhibits the highest observed value of PCB. A possible explanation is that some of the chemicals measured in elevated concentrations do exert more chronic than acute effects.

Physico-chemical parameters were investigated on elutriate samples and are reported in Table 7. The temperature was maintained at 20°C, and the pH, salinity and dissolved oxygen data are comparable for almost all the samples with those recommended for standard procedure (pH: 7.8-8.2; salinity: 35-38‰; dissolved oxygen >70%) (Arizzi Novelli *et al.* 2006). It is also well known that the sea urchin embryo test is sensitive to sulfide and ammonia concentrations, which can represent confounding factors or pollutants depending on whether they derive from a natural or an anthropogenic source (Losso *et al.* 2007). For this reason the concentrations of these compounds in elutriate samples were also investigated. The results (reported in Table 8) suggest the absence of sulfide but a diffuse presence of ammonia. In contrast to metal and organic pollutants in sediment samples, the relatively high values of ammonia can partially explain the toxicity observed in the sea urchin embryo test. In fact, the values range from 0.004 mg/l (14A) to 0.413 mg/l (8A). Losso *et al.* (2007) estimated a NOEC of 0.5 mg/l for ammonia, a value close to those found in this study. In fact, the analysis of correlation revealed a significant correlation between developmental endopoint and ammonia content, as reported in the next paragraph

Analysis of correlation

Table 9 reports the analysis of correlation between HSPs (24 and 48 h), ITI (24, 48 and 72 h) and the chemical parameters, and also the specific morphotypes which revealed the presence of significant correlation. The chemical parameters Al and Cr are partially correlated with developmental endpoints and HSPs, although this correlation is not significant. In contrast, V exhibits a moderate and significant correlation with the ITI calculated at 24 h and 72 h, as does Zn with toxicity index estimated at 24 hours and ammonia with the index calculated at 24, 48 and 72 h (Table 9). However, V and Zn, despite their significant correlation with the ITI, are present at low concentrations in most of the samples and hence probably cannot account for the high and diffuse toxicity observed with morphological analysis on sea urchin embryos. Conversely ammonia, besides being correlated with ITI during all the incubation period, is present in relatively high concentrations, which can partially explain the developmental results.

Sample code	Al	Cd	Cr	Cu	Ni	Pb	V	Zn	Hg	∑PAHs	∑PCBs	Endrin	Eptaclore epoxide	TPhT + TBT (Sn)	OSn (Sn)
1A	2.75	0.05	52.53	9.25	31.21	4.82	83.48	58.46	0.05	94.65	<0.5	<0.5	<0.5	<0.2	<0.5
2A	2.75	0.25	43.96	53.27	42.13	24.03	73.32	178.47	0.41	183.72	<0.5	<0.5	<0.5	<0.2	<0.5
3A	1.57	0.04	44.31	25.81	22.86	12.01	70.95	108.03	0.49	95.46	<0.5	<0.5	<0.5	70.44	54.72
4A	1.48	0.12	48.49	25.39	24.74	11.63	79.29	115.78	0.45	88.4	<0.5	<0.5	<0.5	133	133
5A	2.78	0.46	61.51	60.30	42.59	40.11	81.8	162.43	0.33	193.14	<0.5	<0.5	<0.5	<0.2	<0.5
5B	4.01	0.76	65.65	62.34	54.32	25.53	92.57	196.95	0.19	228.72	<0.5	<0.5	<0.5	<0.2	<0.5
6A	3.14	0.23	50.01	46.25	26.41	37.67	72.39	219.97	0.29	316.61	<0.5	<0.5	35.87	<0.2	<0.5
6B	4.85	0.24	34.77	19.06	29.11	26.42	53.87	66.752	0.29	157.24	<0.5	<0.5	<0.5	35.97	35.97
6C	4.64	0.76	30.13	17.25	29.15	28.83	50.84	67.161	0.23	200.72	<0.5	<0.5	<0.5	<0.2	<0.5
7A	6.02	0.83	41.73	48.60	44.43	42.89	63.43	122.60	0.20	153.71	<0.5	<0.5	<0.5	<0.2	<0.5
7B	7.38	0.99	27.97	24.01	66.84	33.40	41.74	94.744	0.29	181.59	<0.5	<0.5	<0.5	<0.2	<0.5
7C	4.62	0.25	23.49	7.36	68.66	10.77	35.76	49.132	0.14	21.11	<0.5	<0.5	<0.5	<0.2	<0.5
8A	4.29	0.98	81.06	37.36	84.36	21.81	61.04	87.82	0.15	49.22	<0.5	<0.5	<0.5	<0.2	<0.5
8B	1.16	0.11	10.76	2.59	43.20	13.35	12.01	28.58	0.08	23.15	<0.5	<0.5	<0.5	<0.2	<0.5
8C	8.50	0.37	71.84	14.21	70.71	22.68	99.97	77.05	0.11	65.04	<0.5	<0.5	<0.5	<0.2	<0.5
9A	1.85	0.21	38.15	32.02	36.12	32.02	122.4	82.45	0.12	325	<0.5	<0.5	<0.5	<0.2	<0.5
9B	4.45	0.48	70.07	28.7	44.30	33.17	102	119.46	0.14	154.45	<0.5	<0.5	<0.5	<0.2	<0.5
10A	2.07	0.13	47.25	35.89	26.41	26.96	63.84	96.98	0.16	179.3	<0.5	<0.5	<0.5	<0.2	<0.5
10B	1.40	0.21	42.28	23.59	21.30	11.80	59.55	115.96	0.16	46.96	<0.5	<0.5	<0.5	<0.2	<0.5
11A	0.97	0.23	42.82	32.72	64.37	26.77	78.14	58.73	0.14	188.79	340.2	20.62	<0.5	<0.2	<0.5
11B	1.89	0.40	28.44	24.75	32.81	18.54	46.98	96.72	0.01	52.59	<0.5	46.64	<0.5	<0.2	<0.5
11C	1.21	0.09	22.93	4.76	24.60	7.91	28.67	23.36	0.00	135.87	<0.5	<0.5	<0.5	<0.2	<0.5
12A	3.79	0.18	82.12	16.81	40.35	17.43	109.80	81.78	0.02	102.97	<0.5	<0.5	<0.5	<0.2	<0.5
13A	2.83	0.25	66.47	21.42	44.25	29.78	95.55	99.06	0.01	38.92	<0.5	<0.5	78.37	<0.2	<0.5
14A	0.97	0.23	42.82	32.72	64.37	26.77	78.14	58.73	0.14	188.79	<0.5	<0.5	<0.5	166	61
14B	1.28	0.16	53.52	12.30	52.90	11.63	79.3	64.13	0.11	110.01	<0.5	<0.5	<0.5	<0.2	<0.5
14C	4.02	0.90	69.92	30.40	84.33	12.40	98.2	107.40	0.13	100.35	<0.5	<0.5	<0.5	<0.2	<0.5
15A	1.53	0.11	31.62	34.80	21.16	16.99	51.4	57.06	0.19	146.67	<0.5	<0.5	<0.5	<0.2	<0.5
15B	2.41	0.37	18.85	16.50	18.72	21.53	20.4	31.76	0.16	45.09	<0.5	<0.5	<0.5	<0.2	<0.5

15C	2.14	0.34	19.21	23.2	23.05	25.53	22.4	41.14	0.14	37.04	<0.5	<0.5	<0.5	<0.2	<0.5
16B	1.15	0.09	48.57	19.6	25.96	18.96	70.5	53.14	0.18	263.72	<0.5	<0.5	<0.5	<0.2	<0.5
17A	1.62	0.12	41.15	36.1	25.15	11.15	48.1	47.45	0.15	97.99	<0.5	248.54	<0.5	<0.2	<0.5
17B	1.63	0.08	41.16	29.8	22.94	21.51	66.7	65.01	0.31	32.66	<0.5	<0.5	26.1	<0.2	<0.5
18A	3.39	0.46	25.64	23.00	31.56	33.52	42.6	81.29	0.05	197.91	<0.5	<0.5	<0.5	26.34	26.34
18B	2.37	0.08	6.69	3.15	18.38	29.37	14.7	23.48	0.1	135.59	<0.5	<0.5	<0.5	<0.2	<0.5
19A	1.01	0.07	29.63	7.34	2.38	8.24	33.6	65.03	0.31	203.87	<0.5	332	<0.5	<0.2	<0.5
19B	0.33	0.01	12.28	2.54	2.34	1.46	19.2	34.74	0.16	453.6	<0.5	453.46	<0.5	<0.2	<0.5

Table 7 – Chemical parameters recorded in sediments from Trapani harbor. Values highlighted in light blue are higher than Base Chemical Level (BCL) and lower than Limit Chemical Level (LCL), values highlighted in dark blue are higher than LCL. Values highlighted in yellow are higher than the acceptable limit fixed by Italian legislation (152/06) relating to soils.

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Sample code	pH	S (‰)	DO (%)	S^{2-} (mg/l)	NH^{4+} (mg/l)
1A	7.8	38	85	<0.001	0.029
2A	8	37	88	<0.001	-
3A	8.3	37	76	<0.001	0.125
4A	8.04	38	90	<0.001	0.059
5A	8.43	39	91	<0.001	0.05
5B	8.38	38	88	<0.001	0.166
6A	8.45	38	82	<0.001	0.097
6B	8.3	39	79	<0.001	0.046
6C	7.25	36	93	<0.001	0.106
7A	8.2	38	83	<0.001	-
7B	7.8	38	82	<0.001	-
7C	9.08	37	83	<0.001	0.289
8A	8.32	38	88	<0.001	0.413
8B	7.9	37	80	<0.001	0.054
8C	8.02	38	88	<0.001	0.07
9A	7.85	37	83	<0.001	0.084
9B	8.63	38	77	<0.001	0.184
10A	7.89	38	82	<0.001	0.173
10B	7.22	39	81	<0.001	0.109
11A	8.24	39	90	<0.001	0.076
11B	8	39	90	<0.001	-
11C	8.06	38	86	<0.001	0.157
12A	8.29	39	81	<0.001	0.037
13A	8.05	38	82	<0.001	0.064
14A	8.64	38	70	<0.001	0.004
14B	8.38	37	86	<0.001	0.07
14C	8.2	38	80	<0.001	0.083
15A	7.8	38	83	<0.001	0.015
15B	8.4	36	86	<0.001	0.063
15C	8.22	38	79	<0.001	0.064
16B	8.34	37		<0.001	0.164
17A	7.9	37		<0.001	0.315
17B	8.36	38		<0.001	0.044
18A	8.44	38		<0.001	0.086
18B	7.88	38		<0.001	0.215
19A	8.32	39		<0.001	0.124
19B	7.7	38		<0.001	0.107

Table 8 – Physical and chemical parameters recorded on elutriates of sediment form Trapani harbor. S: Salinity, DO: Dissolved Oxygen, S^{2-}: Sulfides, NH^{4+}: Total Ammonia.

Considering the protein levels of HSPs, they are not significantly correlated either with the developmental endopoint (classical and new ITI) or with the chemical parameters. However, correlating the amounts of HSP60 and HSP70 with the specific morphotypes observed at each time of incubation (24 and 48 hours), a significantly strong and positive correlation was found between HSP70 at 24 hours and the presence of embryos at the gastrula stage with mesoderm defects. Moreover, at the same time of incubation the levels of HSP70 are also positively correlated with the abundance of embryos at the gastrula stage with endoderm defects (moderate and significant correlation) (Table 9).

	CHEMICAL PARAMETERS					Morphotypes	
	Al	Cr	V	Zn	NH4	G with mesoderm defects at 24h	G with endoderm defects at 24h
ITI 24h	0.125	0.668	0.650	0.520	0.691	0.101	0.133
ITI 48h	-0.069	0.131	0.133	0.021	0.426	-	-
ITI 72h	-0.050	0.467	0.472	0.287	0.689	-	-
HSP60 24h	-0.462	-0.307	-0.335	-0.077	0.206	-0.238	-0.237
HSP70 24h	0.782	0.389	0.260	-0.108	-0.058	0.817	0.656
HSP60 48h	0.206	0.123	0.015	0.478	0.359	-	-
HSP70 48 h	-0.058	0.163	0.345	0.132	0.237	-	-

Table 9 – Matrix of Pearson correlation coefficients among the ITI (at 24, h, 48h, 72h), HSP levels (24h, 48h), each observed morphotype, physical and chemical parameter. Only chemical parameters and morphotypes which exhibit a significant correlation with ITI or HSPs are reported . The presence of correlation is shown in bold and the significant correlation ($p<0.05$) is highlighted in yellow.

3.4 Conclusions

Besides the biological relevance of evaluating embryotoxicity giving a different weight according to severity and/or reversibility of observed effects, the results of validation and application of ITI indicate the importance of this approach in order to better evaluate the embryotoxicity risk caused by different contaminants. In fact, the complex mixture of pollutants in contaminated sediment may cause very differentiated effects on embryo development, and often the non-diluted sample gives rise to 100% of the effect using standard criteria. In this context, the results obtained in the present Thesis support the possibility to discriminate between various intensities of adverse effects, and the provision of different scores appears particularly relevant for moderately and highly toxic sediments. In this typology of samples, it can be demonstrated that the embryotoxicity is generally overestimated using the classical approach of normal embryos. Thus the application of such an integrative toxicity index might also have important applied consequences such as the sediment classification in harbor areas. In fact, following

the current Italian guidelines the ecotoxicological classification is based on the worst results within the battery of bioassays, often represented by the sea urchin embryo test, which is one of the most sensitive bioassays. Moreover, owing to the widespread distribution of sea urchins in European seas, it is largely used and recommended in several guidelines for toxicity assessment in environmental samples. Hence by applying the new integrative index of toxicity in quality sediment assessment the discriminatory ability of the *P. lividus* embryo test is enhanced, providing a substantial improvement in the classification of dredged material and considering different classes of marine sediments as being less toxic. As a consequence, a more accurate classification of dredging material will allow for the selection of more appropriate management options in harbor areas.

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