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Cadmium, lead and their mixtures with copper: *Paracentrotus lividus* embryotoxicity assessment, prediction, and offspring quality evaluation

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Abstract The aim of this research was to assess the combined effects of three heavy metals (copper, lead, cadmium) on the fertilization and offspring quality of the sea urchin *Paracentrotus lividus* at EC50, NOEL, and EC1 concentrations. The observed data were compared with the predictions derived from approaches of Concentration Addition (CA) and Independent Action (IA) in order to evaluate the proper prediction of the observed mixture toxic effect. The *P. lividus* embryotoxicity of trace metals decreases as follows: Cu > Pb > Cd at all toxicity concentration tested. EC50 mixture revealed less toxic only than Cu; EC50 was 0.80 (±0.07) mg/l, the offspring malformations were mainly P1 type (skeletal alterations) up to 20% mixture concentration, and P2 type from 70% concentration. The NOEL and EC1 mixtures evidenced that all compounds contribute to the overall toxicity, even if present at low concentrations: the former EC50 was 0.532 (±0.058) mg/l and the latter was 1.081 (±0.240) mg/l. The developmental defects observed were mainly P1 type in both mixtures. Both CA and IA models did not accurately predict mixture toxicity for EC50 and NOEL mixtures. Instead, EC1 mixture effects seemed well represented by the IA model. The protective action of the CA model, although quite accurate when applied to simple biological systems like algae and bacteria, but failed to represent the worst-case in this study with more complex organisms. It would be useful to introduce in the models one or more factors that take into account the complexity of these biological systems.

Keywords *Paracentrotus lividus* · Heavy metals · Mixture toxicity · Predictive models

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Introduction

Urban and industrial activities in coastal areas introduce significant amounts of pollutants. Heavy metals are of primary concern as they persist in the environment, move up the food chain and could cause several disorders. Among heavy metals, a few, like Cu, are essential for the maintenance of living organism metabolism, others like Cd and Pb do not have any biological role and are toxic even at very low concentrations (Foulkes 2000).

Pollution is rarely due to a single chemical and aquatic organisms are typically exposed to numerous chemicals simultaneously or in sequence. The adverse effects of a mixture of chemicals may not correspond to that predicted from data on pure chemical compounds, in fact, chemical interactions in a mixture can cause complex and substantial changes in their pure chemical properties, including the toxic effects, of its constituents.

Therefore, the assessment of hazardous chemicals in aquatic ecotoxicology has to account for the combination effects on organisms. The biological effects of chemical mixtures are generally expected to be greater than those of the single component (Hernando et al. 2003) so the assessment of multiple chemical toxic effects plays a key role in the risk evaluation. However, chemical combinations may also act antagonistically (Kraak et al. 1994), complicating the identification of specific constituents responsible for the observed biological effects.

Combined effects of metal mixtures have caused much concern. Norwood et al. (2003) reviewed studies in which toxicity tests of metal mixtures were conducted, and concluded that these seemed to be no strict rule, even for the same combination of metals, likewise Phillips et al. (2003) reported on the contradictory interactive effects in the literature data about metal mixture toxicity.

Most compounds, like trace metals, are always present at concentrations far below their individual median effective concentration 50% (EC50), and possibly also below their individual no observed effect level (NOEL), but still they may contribute to substantial toxic effects. In fact, NOELs derived from experimental data by applying statistical hypothesis-testing procedures (Dunnett 1955, 1964), that indicates the upper margins of concentration ranges for which low effects are not excluded to occur. NOELs derived from standard toxicity tests have, typically, been shown to correspond to effects of 10% (Moore and Caux 1997).

The use of regression-base statistical estimates of low-effect concentrations, ECx point estimations (Van Der Hoeven et al. 1997), which are discussed to replace the NOEL in risk assessment procedures (Moore and Caux 1997; Van der Hoeven et al. 1997) could overcome this difficulty.

Sea urchin embryos and gametes are often utilized to assess the toxicity of chemicals in the marine ecosystem (Manzo et al. 2006, 2008; Marin et al. 2000; Kobayashi and Okamura 2002) due to their sensitivity and availability.

Many studies have demonstrated the sensitivity of sea urchin embryos to single metals as pure substances (Filosto et al. 2008; Kobayashi and Okamura 2005; Phillips et al. 2003). In particular the embryotoxicity of essential metals like Cu has been extensively investigated. The Cu EC50 of 0.046 (±0.005) mg/l, reported in Manzo et al. (2008) was similar to the range of EC50s (30–100 μg/l) reported for *Paracentrotus lividus* (Lorenzo et al. 2002; Manzo 2004; Radenac et al. 2001).

For the two non essential heavy metals, Cd and Pb, the EC50 value ranges reported in literature for *P. lividus* was 3400–11300 μg/l (Fernandez and Beiras 2001; Warnau et al. 1996) and 414–680 μg/l (Novelli et al. 2003), respectively.

Only few studies with sea urchins have addressed the interaction of metals (Fernandez and Beiras 2001; Phillips et al. 2003; Kobayashi and Okamura 2005) reporting additive or synergistic effects. To our knowledge the toxic effect of Cu, Pb, and Cd ternary mixture at different concentrations on *P. lividus* embryos development has not been detailed yet. The evaluation and comparison of this mixture toxicity at concentrations corresponding to and below not significant effect concentrations will be particularly interesting to understand. In the last decades, the scientific community has given special attention to the possibility of describing the combined effects of chemicals. To predict the mixture toxicity two models are usually utilized. The Concentration Addition (CA) (Loewe and Muischnek 1926; Loewe 1927) model is based on the idea that chemicals perform a "similar action". A chemical acts like a dilution of another, meaning that any effect can be obtained by replacing one chemical totally or in part by the equieffective amount of another. In fact, it occurs, in a strict sense, only in the special case of competitive and reversible interaction of specifically acting toxicants with an identical molecular binding site (Faust et al. 2001). Then CA could mainly represent the reasonable "worst-case" approach. The Independent Action (IA) (Bliss 1939), model is based on the assumption, that chemicals act upon different subsystems within the same organism, e.g., having different sites and modes of action.

The response variability to metal mixtures could also depend on the test organisms exposed (Braek et al. 1976; Wang et al. 1995) and on the ratio of concentrations utilized.

The aim of this study was to assess the combined effects of three heavy metals (Cu, Pb, Cd) upon the fertilization and the offspring quality of the sea urchin *P. lividus* a concentration corresponding to the calculated EC50s, and at concentrations corresponding to and less than not significant effect concentrations (NOEL, EC1).

The suitability of reference CA and IA models, for the prediction of combined effects of these mixtures, was checked with the purpose of evaluating their efficacy in environmental protection planning.

Materials and methods

Test organisms

Adult *P. lividus* (Lamark) were collected from the Tyrrhenian Sea (Bay of Naples) by the staff of the Zoological Station of Naples (Italy). Sea urchins were then acclimatized for at least 24 h in natural Filtered Sea Water (FSW-0.45 μm ∅) at 18 ± 1°C (salinity 38%, pH 8 ± 0.2). In fact, it was noted that the utilization of the animals immediately after collection produced a decrease of normal plutei in the control, probably because of the stress induced by the collection activity itself. An abrupt increase in temperature or salinity might not only induce spawning, but also seriously harm the gametes (ASTM 2004).

Toxicity test

Gametes were harvested and embryos were reared according to Pagano et al. (1986). Spawning was induced

in sea urchins by injection of 1 ml of 0.5 M KCl through the perioral membrane. Eggs were collected by separately placing each spawning female in a different 250 ml beaker with FSW while "dry" sperm from each male was collected with an automatic pipette and stored in a sterile tube placed on ice. For each experiment, six female individuals were selected for their appropriate egg quality (no immature forms, no debris, and no fertilized eggs) and high amount. Males were selected for sperm motility (checked under the microscope) and amount. Then, the best three male and three females gametes were pooled and filtered through nylon cheesecloth (Ø = 200 μm for eggs and 50 μm for sperm). The egg suspension (stock solution) was diluted in order to obtain the final concentration of 250–300 eggs/ml. Gametes were then employed in embryotoxicity test (T = 18 ± 1°C, exposure time = 48–50 h) conducted at least in triplicate (Pagano et al. 1996a modified).

Detailed embryotoxicity test procedure utilized has been previously described in Manzo et al. (2006). The offspring quality, expressed as frequency of developmental defects, was assessed as previously described (US EPA 1995).

Developmental abnormalities were determined in each replicate by direct observation of 100 individuals, randomly chosen. For each treatment schedule, 100 plutei were scored for the frequencies of: (1) normal (N) larvae, according to their symmetry, shape, and size; (2) retarded (R) larvae with shape and symmetry the same as normal, but with reduced size (<1/2 with respect to N); (3) malformed larvae (P1), affected in skeletal and/or gut differentiation and/or pigmentation; and (4) pre-larval arrest (P2), embryos unable to go to larval differentiation, as abnormal blastula or gastrulae. Mean percentage abnormalities and 95% confidence limits were calculated for all the samples and compared to the results obtained from the controls. If abnormalities in the controls were 20% or more, the test was judged invalid and repeated. However, to evaluate the test's reproducibility a positive control was carried out with a reference toxic substance (Cu) (Arizzi Novelli et al. 2002; Volpi Ghirardini and Arizzi Novelli 2001).

Test solutions

Cu stock and test solutions were prepared as reported in Manzo et al. (2008) and the tested Cu^{2+} concentrations were 5, 15, 25, 35, 50, 62, 75, and 100 μg/l.

Pb stock solution was prepared by dissolving reagent grade lead nitrate (Carlo Erba, Reagents, Italy) in Milli-QTM water to obtain a Pb^{2+} concentration of 1000 mg/l. Test solutions were obtained by diluting the stock solution in FSW, as follows: 0.05, 0.075, 0.1, 0.2, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 mg/l.

Cd stock solution was prepared by dissolving reagent grade cadmium chloride (Carlo Erba, Reagents, Italy) in Milli-QTM water to obtain a Cd^{2+} concentration of 1000 mg/l. Test solutions were obtained by diluting the stock solution in FSW, as follows: 0.1, 0.25, 0.4, 0.45, 0.5, 0.8, 1.0, 1.7, 5.0, and 10.0 mg/l.

Preparing the test solutions the final dilution factor of FSW was always maintained at 10% concentration, approximately corresponding to a final salinity of 34‰ that is near to the value selected by His et al. (1999) for tests on fertilised eggs of *P. lividus* and it is well within the range of salinity "tolerance" (33–38‰) for sea urchin embryos and larvae (Bressan et al. 1995). In previous experiments it was also verified that this procedure doesn't affect the results of the tests.

Seawater used for the test solutions (and for acclimatization) was sampled in an uncontaminated area far from the coast and was already frequently used in the laboratory for ecotoxicological tests and optimization of analytical methods. As a consequence, seawater samples from this area were analyzed several times for trace elements and organic micropollutants using wide-spectrum-screening analytical methods. Cd and Pb concentrations were verified before the test execution, checking those at the lowest and highest concentration (dilution factor 1:100) using electrothermal vaporization-inductively coupled plasma-mass spectrometry (ETV-ICP-MS), according to the procedure of Rosland and Lund (1998) for Pb and Cd (the procedure of Chapple and Byrne 1996, was used for Cu).

Statistical analysis

Differences in development success (comparisons between the control group and each of the experimental groups) were tested for significance using the multiple comparisons Dunnett's test.

The EC50 was calculated using the Linear Interpolation Method (Inhibition Concentration procedure or ICp) (Cesar et al. 2004; US EPA 1993). The bootstrap method is used to obtain the 95% confidence interval, because standard statistical methods for calculating confidence intervals are not applicable. Analysis of variance (ANOVA) was applied, using raw data, to test for significant differences in effects among treatments (significance level was always set at $p = 0.05$); then NOEL was calculated with Dunnett's procedure.

Concentration–response analyses

Concentration–response analysis was performed in the same way for all individual toxicants and for the mixture. Concentration–response functions were statistically determined by applying a best-fit procedure. With this approach,

different regression models (Boltzmann, Logistic, Exponential), provided by Origin® 7 SR2 (Northampton, MA) statistical software, were applied to each data set in order to determine, on the basis of statistical criteria, the regression model that best described the observed data.

Regression curves were obtained and analyzed with Origin® 7 SR2 software, using the least-square method and the analysis of residuals. Models that have passed the residual analysis as reliable candidates are then subjected to a second selection step.

By this procedure, we calculated the sum of absolute residuals (SAE) and the sum of absolute deviations (SAD), the model that showed the minimum SAE and SAD values was selected as the best fitting one; at this stage, the most appropriate model was chosen by applying a goodness of fit criterion. However, first results obtained from a simulation study done by Scholze et al. (2001) indicated that the SAE is much more sensitive than the SAD measure. Regression-based effect concentrations (ECx) were derived from the fitted concentration–response regression models, and the corresponding 95% confidence intervals were estimated by Origin® 7 SR2 software.

The selection of the best-fit model for single chemicals is a very critical point because the best prediction of the toxic effect of the single chemicals in mixture toxicity improves the accuracy of both CA and IA models.

Analysis of mixture toxicities

For the analysis of mixture toxicity, a fixed-ratio design was used. This means that the ratio of the mixture components was kept constant while the overall concentration of the mixture was systematically varied. The resulting concentration–response relationship can thus be biometrically analyzed analogous to the single substances.

The components were mixed in the ratio of their individual EC50 values (''EC50-mixture''), of their individual NOEL values ("NOEL mixture") and of their EC1 values ("EC1-mixture") (Table 1).

The mixtures of three components at their EC50 concentrations were diluted in percentage at the following factors: 10, 20, 30, 40, 50, 60, 70, 80, 90, while the mixtures of three components at their NOEL and EC1 concentrations were diluted in percentage at the following factors: 10, 25, 50, 75, 100, 200, and 300.

According to CA, all components contribute equally to the CA-predicted EC50, NOEL, and EC1 in such mixtures.

The concept of CA is defined for any number of n components by:

$$\sum_{i=1}^{n} \frac{c_i}{EC_{x_i}} = 1 \tag{1}$$

where n is the number of mixture components that produce 1 toxic unit, EC_{x_i} is the concentration of the ith mixture component that provokes x% effect when applied singly, and c_i is the concentration of the respective component in the mixture. Each fraction of c_i/EC_{x_i} represents the concentration of a mixture component scaled for its relative toxicity and is generally termed the ''toxic unit'' of that component. For the calculation of predicted effect concentrations according to CA, Eq. 1 can be reformulated to:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{EC_{x_i}} \right)^{-1} \tag{2}$$

where EC_{x_i} is the concentration of the ith mixture component that provokes x% effect when applied singly, ECx_{mix} is the total concentration of the mixture provoking x% effect, and p_i denotes the fraction of component i in the mixture. Mixture toxicity according to IA is based on the effects of the components and can be calculated by:

$$E(c_{mix}) = E(c_1 + \cdots + c_n) = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \tag{3}$$

where E(cmix) denotes the effect (scaled from 0 to 1) of an n-compound mixture, c_i is the concentration of the ith compound, and $E(c_i)$ is the effect of that concentration if the compound is applied singly. However, for the experimental planning of the concentration range for mixture effects that would be appropriate, a simplified way of calculating the IA prediction may be used (Backhaus et al. 2000a). After the calculation of the mixture effect concentrations ECx_{mix} for CA (Eq. 2), the corresponding concentration c_i of each individual component present at ECx_{mix} can be determined, as the mixture ratio is known. The effect $E(c_i)$ that each component would provoke individually at c_i can then be determined, using the corresponding concentration–response relationship (best-fit model). These values were then used in Eq. 3 for the calculation of the IA predicted mixture effect E(cmix). More details on the calculations of CA- and IA-based predictions, and of the statistical uncertainty associated are available elsewhere (Arrhenius et al. 2006; Faust et al. 2001, 2003).

Table 1 EC50s, NOELs, and EC1 of copper, lead, and cadmium used in mixtures studies

Metals	EC50 (mg/l) ICp	NOEL (mg/l) dunnet	EC1 (mg/l) ICp
Cu^{2+}	0.046 (0.036–0.056)	0.005	0.002 (0.98–10.5 × 10^{-3})
Cd^{2+}	2.630 (2.430–2.69)	0.400	0.320 (0.050–0.400)
Pb^{2+}	1.250 (0.990–1.360)	0.250	0.057 (0.018–0.110)

Results

Reliable concentration–response analyses for single substances are essential for predictions of mixture toxicity. EC50 and EC1 values obtained with ICp EPA method (US EPA 1993), EC50s, calculated by best-fit model, NOEL and LOEC values (Dunnett's procedure), regression models and model parameters, for each tested substances and their mixtures, are given in Table 2.

Regression based EC50 values of single metal span more than one order of magnitude (Table 2): Cu (EC50 = 0.044 mg/l) was revealed as the most toxic metal. The NOEL values exceeded the corresponding EC1 (ICp) by a factor of 1.25 (Cd), 2.5 (Cu), and 4.3 (Pb).

The shape of concentration response curves appears relatively similar (Figs. 1, 2, and Manzo et al. 2008).

Single metals

Cadmium

The effect percentage response data for each test, together with the corresponding regression fit curve are shown in Fig. 1a. The values had a slow increasing trend up to a maximum effects obtained (5 mg/l). The EC50 was 1.003 (0.800–1.220) mg/l and NOEL was 0.40 mg/l ($p < 0.05$). The developmental defects in treated *P. lividus* larvae (Fig. 1b) were mainly P1 type (larvae affected in skeletal or gut differentiation) and R type [larvae with shape and symmetry the same as normal, but with reduced size (<1/2 N)], with an increasing trend from LOEC concentration (0.45 mg/l) until the tested concentration of 5 mg/l.

At the last concentration of 10 mg/l the main effects observed were P1 type.

P2 (total arrest at prelarval stadium) developmental alterations were present but they never exceed the 10% of total exposed larvae.

Lead

The effect percentage response data for each test, together with the corresponding regression fit curve are shown in Fig. 2a. The values had an increasing trend up from the LOEC concentration of 0.5 mg/l to the maximum effect obtained at 3 mg/l. The EC50 was 1.150 (0.880–1.440) mg/l and NOEL was 0.25 mg/l ($p < 0.05$). Until the LOEC concentration the defects observed in plutei were mainly R type (20%). From LOEC concentration the developmental defects in treated *P. lividus* larvae (Fig. 2b) were mainly P1 type (larvae affected in skeletal or gut differentiation) until at the concentration of 2 mg/l, where the P2 type alterations start to increase.

Table 2 Toxic effects on *Paracentrotus lividus* embryos (embryotoxicity) of copper, lead, and cadmium individually and in mixtures

Metals	EC50 (mg/l) ICp	EC50 model	NOEL (mg/l) dunnet	LOEC (mg/l) dunnet	EC1 (mg/l) ICp	Model[b]	α	β	γ
Cu^{2+}[a]	0.046 (0.036–0.056)	0.044 (0.037–0.052)	0.005	0.015	0.002 (0.98–10.5 × 10^{-3})	Boltzmann	45.23	13.11	–
Cd^{2+}	2.630 (2.430–2.690)	1.003 (0.800–1.220)	0.400	0.450	0.320 (0.050–0.400)	Logistic	0.97	0.2306	–
Pb^{2+}	1.250 (0.990–1.360)	1.150 (0.880–1.440)	0.250	0.500	0.057 (0.018–0.110)	Boltzmann	1.12	0.31	–
Mix EC50	0.760 (0.660–0.920)	0.800 (0.660–0.950)	n.d.	0.393	0.029 (0.020–0.068)	Boltzmann	0.813	0.232	–
Mix NOEL	0.560 (0.533–0.600)	0.532 (0.490–0.590)	n.d.	0.066	0.006 (0.004–0.024)	Exp dec 1	119.83	−118.2	1014.7
Mix EC1	1.076 (1.154–0.998)	1.081 (0.606–1.555)	0.189	0.285	0.005 (0.003–0.011)	Logistic	1088.9	0.95	–

The NOELs (No Observed Effect Level) were estimated with Dunnett's procedure ($p < 0.05$), and the EC50s and EC1s were estimated with the EPA ICp procedure (US EPA 1993) as well as by the best-fit model. Values in brackets denote the upper and lower limits of 95% confidence interval of the EC50 and EC1

[a] Data from Manzo et al. (2008)

Logistic[b] $y = \frac{A1-A2}{1+(x/x_0)^{\beta}} + A2$

Boltzmann[b] $y = \frac{A1-A2}{1+e^{(x-x_0)/\beta}} + A2$

Exp dec 1[b] $y = \alpha + \beta e^{-x/t1}$

where, [b] A1 = max (y-data) set 100, A2 = min (y-data) set 0, t1 = 771,837 ± 52,648

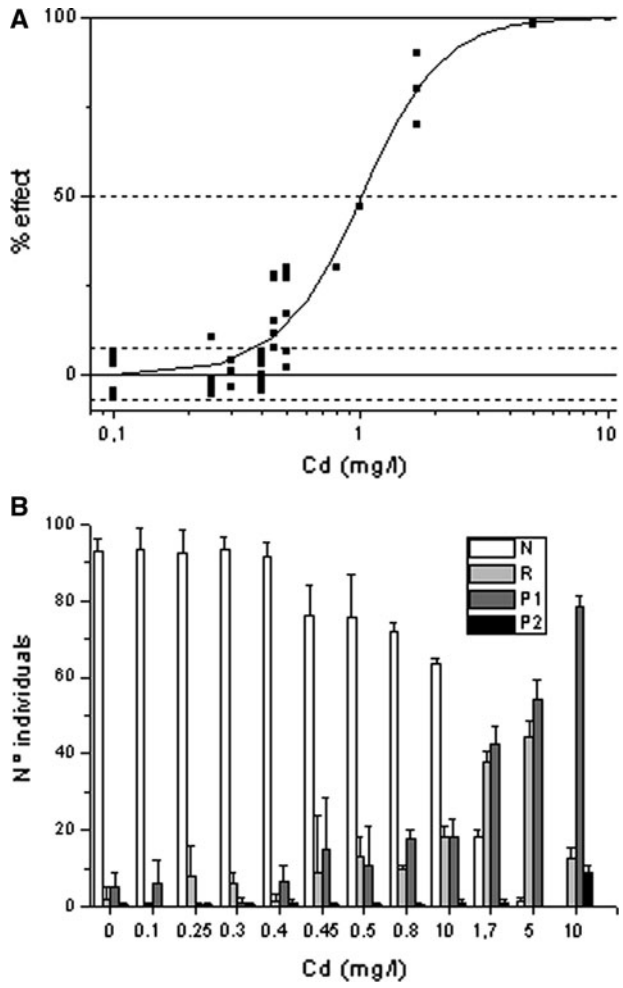

Fig. 1 Cadmium embryotoxicity in *P. lividus*. **a** % of malformed out of 100 individuals normalized with respect to control, as a function of tested concentrations. *Horizontal dotted lines* indicate the 95% confidence limits of the control mean (n = 6). 50% effect level is also represented. *Solid circles* represent Cd treatments (n ≥ 3). *Thick solid line* indicates the regression fit of the observations (Table 2). **b** Number of individuals with different developmental anomalies obtained after 48 h exposure. *N* normal plutei, *R* retarded larvae, *P1* malformed larvae, *P2* blastulae or gastrulae (developmental arrest); see also "Materials and methods"

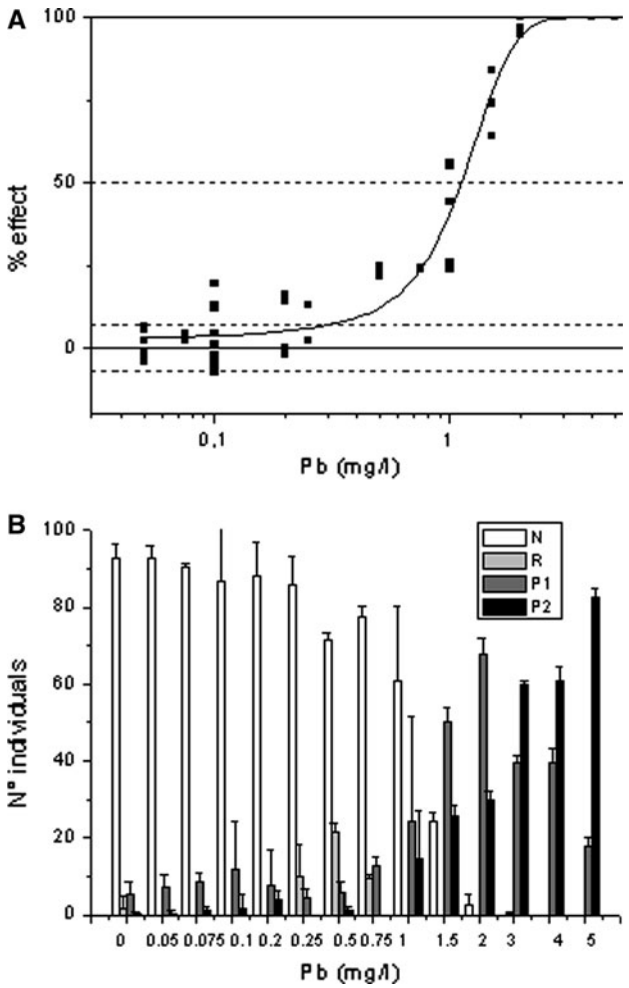

Fig. 2 Lead embryotoxicity in *P. lividus*. **a** % of malformed out of 100 individuals normalized with respect to control, as a function of tested concentrations. *Horizontal dotted lines* indicate the 95% confidence limits of the control mean (n = 6). 50% effect level is also represented. *Solid circles* represent Pb treatments (n ≥ 3). *Thick solid line* indicates the regression fit of the observations (Table 2). **b** Number of individuals with different developmental anomalies obtained after 48 h exposure. *N* normal plutei, *R* retarded larvae, *P1* malformed larvae, *P2* blastulae or gastrulae (developmental arrest); see also "Materials and methods"

Copper

Cu EC50s, NOEL, and LOEC for *P. lividus* embryos (ICp EPA method, Dunnett's procedure) were previously investigated (Manzo et al. 2008) and reported in Table 2.

Three components mixtures

The three metals were mixed in the ratio of their individual EC50, NOEL, and EC1 values obtained with conventional methods (EPA ICp and Dunnett's). Mixture toxicity was tested on their diluted solutions. The effect percentage response data for each biotest, for each mixture, together with the corresponding regression fit curve and CA and IA predicted toxicity, are shown in Fig. 3.

EC50 mixture

The EC50 mixture embryotoxicity could be described by a growth-sigmoid function (Fig. 3a) and showed an EC50 value of 0.80 (±0.07) mg/l, which was not statistically different from the value 0.76 (±0.07) mg/l obtained with EPA ICp-method (Table 2). The NOEL was not determinable with Dunnett's test and EC1 was 0.029 (±0.013). The EC50 value of this mixture was obtained at a

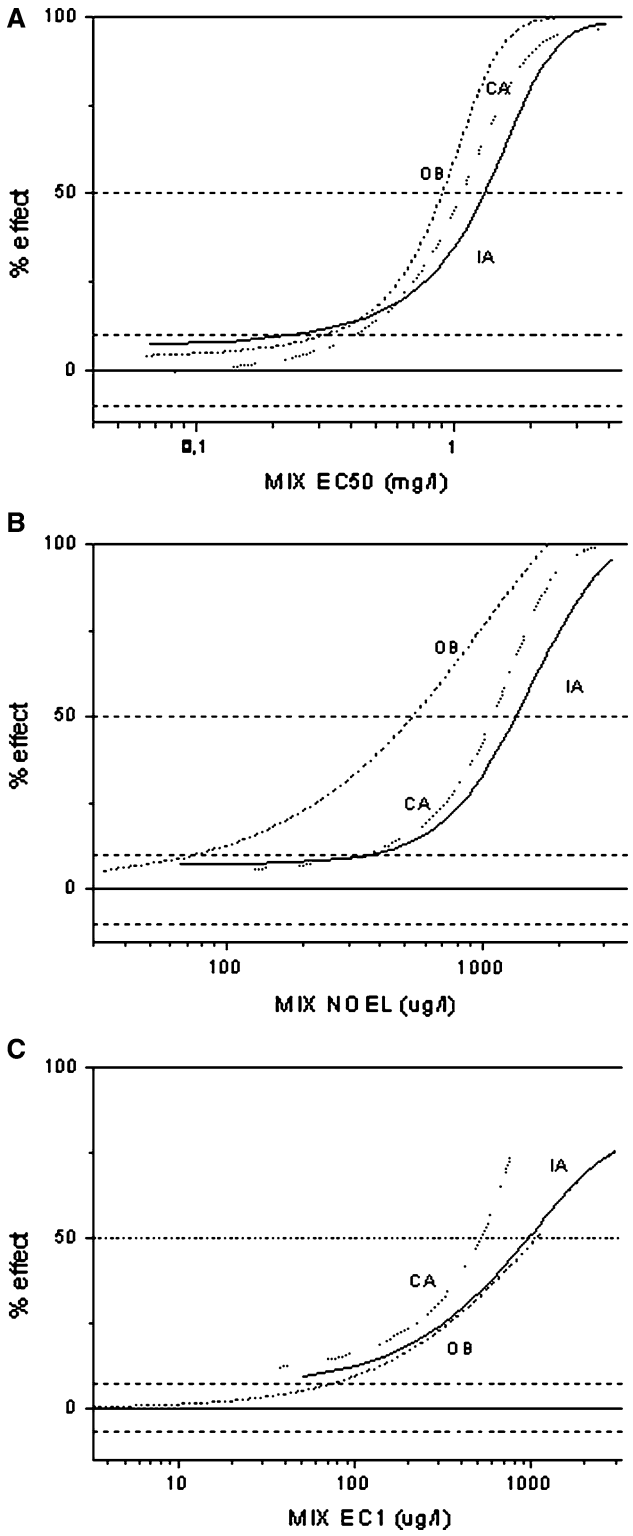

Fig. 3 Observed and predicted mixture toxicity according to CA and IA of EC50 (**a**), NOEL (**b**), and EC1 (**c**) mixtures for *P. lividus* embryos. *Solid lines* indicate the regression fits of the observations (Table 2). The predictions according to Concentration Addition and Independent Action are indicated, confidence limits of the predictions are given in Table 3. *Horizontal dotted lines* indicate the 0 and 50% effect levels (*solid*) and the 95% confidence limits of the controls mean (*dotted*)

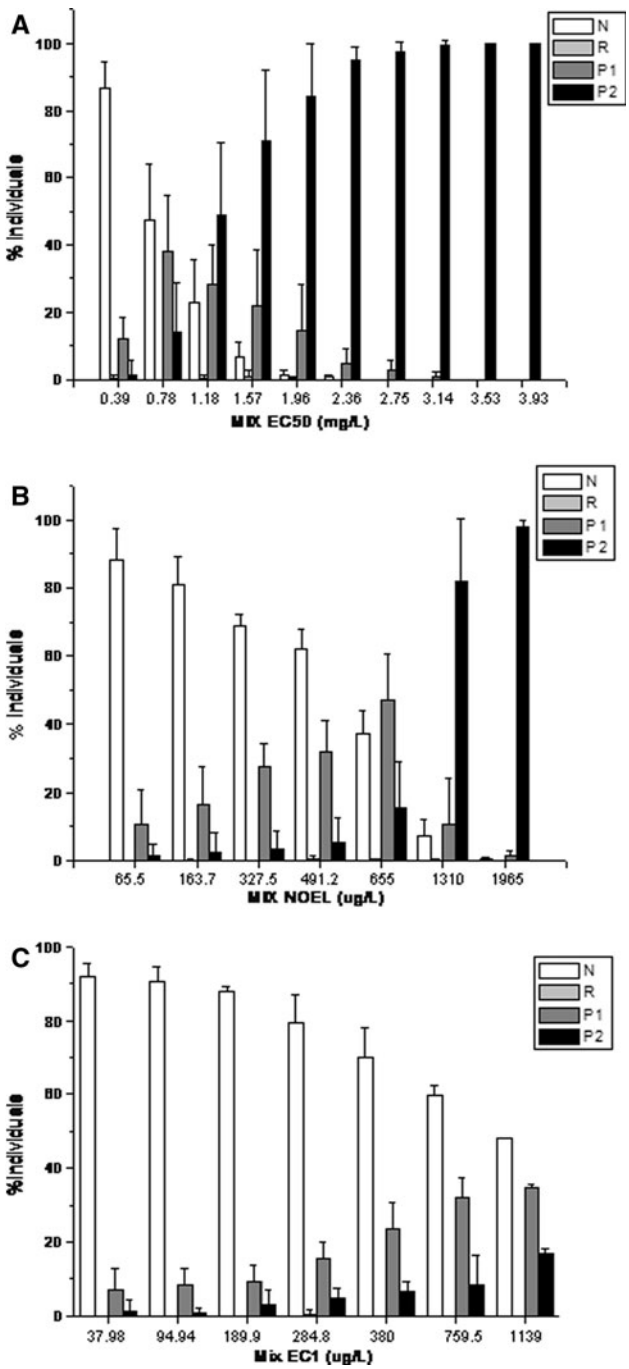

Fig. 4 Offspring quality in *P. lividus* after embryos exposure to EC50 (**a**), NOEL (**b**), and EC1 (**c**) mixtures. Number of individuals with different developmental anomalies obtained after 48 h exposure. *N* normal plutei, *R* retarded larvae, *P1* malformed larvae, *P2* blastulae or gastrulae (developmental arrest); see also "Materials and methods"

concentration of 20%, while a 100% effect was observed on a 50% diluted mixture.

With respect to single metals, the embryotoxicity mixture EC50 obtained was higher only than the Cu EC50 value (Table 2).

Table 3 Observed and predicted EC50, NOEL, EC1 mixture embryotoxicity

Effect concentrations ECX_{mix} (mg/l)				Relative deviation between observed and predicted mixture toxicities				
				CA: observed		IA: observed		IA vs. CA
Effect level X	Observed	Predicted by CA	Predicted by IA	Overest. (antagonism)	Underest. (synergism)	Overest. (antagonism)	Underest. (synergism)	
EC50 (%)								
10	0.30 (n.d.–0.55)	0.32(n.d. –0.53)	0.35 (0.33–0.37)		1.06		1.15	1.10
50	0.80 (0.66–0.95)	1.10 (1.00–1.19)	1.27 (1.24–1.28)		1.37		1.59	1.15
90	1.32 (1.06–n.d.)	1.90 (1.69–2.33)	2.55 (2.50–2.60)		1.44		1.93	1.34
NOEL (%)								
10	0.07(0.05–0.09)	0.32 (0.19–0.43)	0.37 (0.32–0.41)		4.50		5.21	1.15
50	0.53 (0.49–0.59)	1.10(1.06–1.14)	1.36 (1.33–1.37)		2.05		2.60	1.24
90	1.39 (1.28–1.55)	1.87 (1.77–1.99)	2.70 (2.68–2.80)		1.34		1.90	1.44
EC1 (%)								
10	0.10 (n.d.–0.30)	0.007 (n.d.–0.11)	0.07 (0.04–0.09)	0.07		0.67		9.40
50	1.08 (0.61–1.55)	0.52 (0.42–0.62)	0.96 (0.91–1.02)	0.481		0.90		1.80
90	n.d.	0.9 (0.80–1.00)	n.d.					

Relative deviations between observed and predicted effect concentrations. ECX_{mix} are given as ratios that indicate the degree of overestimation (antagonism) and underestimation (synergism) of toxicities. Observed mixture effect concentrations are based on regression fits to experimental data. Values in brackets denote the upper and lower limits of 95% confidence interval of the ECX_{mix}

n.d. Not determined

Up to 20% mixture concentration, the developmental defects in treated *P. lividus* larvae (Fig. 4a) were mainly P1 type (larvae affected in skeletal or gut differentiation), these disappear completely from the 70% concentration, whereas the P2 type alterations, that are always present, are the main effects.

CA and IA predictive toxicity models did not describe the observed data, overestimating the mixture toxicity up to the 10% effect concentration and, at higher concentrations, underestimating the toxicity of the mixture. The embryotoxicity predictions, according to CA and IA, and the relative confidence limits are given in Table 3.

NOEL mixture

The NOEL mixture embryotoxicity could be represented by an exponential function (Fig. 3b) and showed an EC50 value of 0.532 (±0.058) mg/l, which was not statistically different from the value 0.560 (±0.017) mg/l obtained with EPA ICp-method (Table 2). The NOEL was not determinable with Dunnett's test and EC1 was 0.006 (±0.001) mg/l. The EC50 value of this mixture was obtained at a concentration of 100% (0.600 mg/l), while a 100% effect was observed at 300% mixture concentration.

With respect to single substance, the embryotoxicity mixture EC50 obtained was lower only than the Cu EC50 value (Table 2).

From 10 to 100% mixture concentration the developmental defects in treated *P. lividus* larvae (Fig. 4b) were mainly P1 type (larvae affected in skeletal or gut differentiation), that increase within this range to then decrease abruptly from 200% mixture concentration. Contemporarily from 10% concentration the P2 type alterations increase to represent the 100% effect at three times mixture total concentration.

CA and IA predictive toxicity models did not represent the observed data, underestimating the toxicity of the mixture. The underestimating of the NOEL mixture toxicity is higher at low concentration and decrease at high concentration (Table 3). The embryotoxicity predictions, according to CA and IA, and the relative confidence limits are given in Table 3.

EC1 mixture

The EC1 mixture embryotoxicity could be represented by a growth-sigmoid function (Fig. 3c) and showed an EC50 value of 1.081 (±0.24) mg/l, which was not statistically different from the value 1.076 (±0.034) mg/l obtained with EPA ICp-method (Table 2). The NOEL calculated with Dunnett's test was 0.189 and EC1 was 0.005 (±0.002) mg/l.

The undiluted EC1 mixture (0.38 mg/l) produced about 25% effect and the EC50 was obtained at concentration of

three times 100% (3×) (1.14 mg/l), while an 100% effect was never observed. The embryotoxicity mixture EC50 obtained was lower only than the Cu EC50 value (Table 2) compared to the single substance.

From 10% until to 3× mixture concentration, the developmental defects in treated *P. lividus* larvae (Fig. 4c) were mainly P1 type (larvae affected in skeletal or gut differentiation), that increase in this range until a 30% effect. At the same time, from 10% concentration the P2 type alterations increase to represent the 10% effect at 3× mixture concentration.

CA predictive toxicity model did not represent the observed data, overestimating the toxicity of the mixture, while IA well represents the mixture toxicity, overestimating only at low concentration (Table 3). Both models were protective and CA represents the worst-case. The difference between the two models was bigger at low concentrations (≈9.4) than at high ones (≈1.8). The embryotoxicity predictions, according to CA and IA, and the relative confidence limits are given in Table 3.

Discussion

Single metal toxicity

Embryo toxicity results of single metals presented in this study together with Cu embryotoxicity data previously obtained (Manzo et al. 2008) show that the ranking of toxicity of trace metals to *P. lividus* embryos decreases as follows: Cu > Pb > Cd at all toxicity concentrations tested (EC50, NOEL, LOEC) and similar results (EC50, mg/l) were reported for P. *lividus* by Fernandez and Beiras (2001), Radenac et al. (2001), Novelli et al. (2003). The results for Antarctic species *S. neumayeri* showed a different sensitivity (Cu > Cd > Pb, King and Riddle 2001).

Cd EC50 value (2.63 ± 0.076 mg/l) obtained for *P. lividus* with ICp procedure (Table 2), was similar to those reported in literature for sea urchins. In fact 5.2–10.8 mg/l EC50 range for *Dendraster excentricus* (Dinnel et al. 1989) was reported, EC50 value for Cd was 7.38 mg/l for *Arbacia punctulata* (Carr 1996), and 6.9 mg/l for *Sterechinus neumayeri* (King and Riddle 2001). The EC50 quoted for *P. lividus* was 3.8 mg/l (Heyvang 1994); 3.372–11.241 mg/l (Warnau et al. 1996), and 9.24 mg/l (Fernandez and Beiras 2001). Value as low as 0.23 mg/l for *P. lividus* (Novelli et al. 2003) and 0.5 mg/l for *Strongylocentrotus purpuratus* (Dinnel et al. 1989) was also reported. The large differences measured was probably attributable to different methodologies adopted (e.g., whether the sea urchins were cultured or field collected, using different exposure times and heavy metal salts) (Novelli et al. 2003).

The Cd EC50s based on the ICp-method and the best-fit model were different by a factor of more than two because the two methods are based on different Interpolation method of data and can produce different results. The NOEL obtained (0.40 mg/l) was in the large range of values reported for different sea urchin species (from 0.01 mg/l for *P. lividus* (Novelli et al. 2003) to 2 mg/l for *S. neumayeri* (King and Riddle 2001); in this case it is probably due to the different procedure used to calculate it.

For P. lividus exposed to Cd, malformations of pluteus skeletons (P1 type) were mainly found. The increase in skeletal malformations was registered starting from LOEC (0.45 mg/l) value up to 10 mg/l. Also Warnau et al. (1996) reported this trend for *P. lividus* but starting from about 3 mg/l. The capacity of Cd to influence skeletal differentiation was previously suggested (Pagano et al. 1982; Warnau et al. 1996) and Cd might block the uptake pathway of calcium, competing with it at the same site of action (Kamo and Nagai 2008). The total arrest at prelarval stage (P2 type) never exceed the 10% of total exposed larvae, according to Warnau et al. (1996), who described a progressive shift from malformed plutei to gastrula and blastula blockage, only at concentrations of more than 16 mg/l.

The Pb toxicity value (EC50: 1.25 ± 0.1 mg/l) obtained with ICp procedure (Table 2), was comparable to those found by King and Riddle (2001) for a different sea urchin species, and was different from the values reported by Fernandez and Beiras (2001) (0.510 mg/l) and by Warnau and Pagano (1994) (0.414 mg/l) for *P. lividus*.

It could be hypothesized that the Pb concentration (from 0.016 to 20.5 µg/l—UNEP 1996; Manfra and Accornero 2005) in the Southern Tyrrhenian seawater, where the sea urchin *P. lividus* lives and was collected for this study, probably together with other contaminants had build up the tolerance of the species to a series of toxicants including Pb with could have inducted a high level of tolerance (Hunt et al. 1997; King and Riddle 2001; Pavicic et al. 1994) on sea urchin gametes. This underlines a limit on the utilization of organisms collected in a population in a natural habitat. The significant dose–response increase observed in developmental defects, characterized by a progressive shift from retarded larvae, to larval malformation (P1) up to the total arrest of at the prelarval stage (P2), confirms the literature data (Fernandez and Beiras 2001; Radenac et al. 2001; Warnau and Pagano 1994). Pb is described as directly toxic in early life stages (Nacci et al. 2000) and, like Cd, competes with calcium fixation with specific toxicity of this metal to skeletal differentiation (Warnau and Pagano 1994). To our knowledge, toxicity studies on a ternary mixture of Cu, Pb, and Cd have not yet been carried on *P. lividus*.

Mixture toxicity

The EC50 mixture evaluated, compared with single component EC50s, is less toxic only than Cu; it exerts synergistic effects with a rapid increase of embryotoxicity (maximum effect at 50% mixture dilution).

It could be presumed that metal toxic effect interactions can cause an increased toxicity for sea urchin embryos. Even if the mechanisms underlying heavy metal toxicity are not yet well understood (Kamo and Nagai 2008; Wood 2001) we can expect that Cu, being an essential metal, could also contribute to cellular destabilization (Phillips et al. 2003), via metal substitution reactions. In this way the toxic action of the other two non essential metals will be enhanced.

Scarce or no additive effects (*P. lividus*) to a dramatic rise in embryo lethality (*P. microtuberculus*) were reported by Pagano et al. (1996a, b) for a mixture of Al and Fe. Fernandez and Beiras (2001), tested the toxicity for *P. lividus* embryos of Hg in binary combination with the same heavy metals object of this study. The authors observed additive or very slightly synergistic effects for combinations of Hg with Cu, Pb, and Cd.

Binary and ternary metal combinations (Cd, Cu, Ni) tested by Phillips et al. (2003) with sea urchin embryos (*S. purpuratus*) produced diverse types of interactions; differently the mixture of all four metals (Cd, Cu, Ni, Zn) produced synergistic toxicity effects. However, Kobayashi and Okamura (2005) have highlighted that specific effects of Zn were intensified by the presence of other metals such as Pb and Cu (*Anthocidaris crassispina*).

The EC50 value (0.8 mg/l), reached at 20% diluted mixture (Fig. 3a), was constituted by metal concentrations (0.0092 mg/l Cu, 0.250 mg/l Pb, 0.526 mg/l Cd) that are in the range of their respective NOEL. NOELs represent a zero-effect concentration but they derive from experimental data by applying statistical hypothesis-testing procedures (Dunnett's procedure). At and below the NOEL, toxicity may be absent or it may be present but undetected, due to a limited sensitivity of the experimental protocol (Faust et al. 2003).

This shows that heavy metal mixture at NOEL value, could exert an increased effect and this was also confirmed by the NOEL mixture behavior (see below) (Fig. 3b).

The trend of developmental defects produced by the mixture was different from those obtained with each metal. In fact, P1 type malformations, mainly derived by the interaction with calcium homeostasis of Cd, Pb, and Cu (Manzo et al. 2008) were present at high concentrations in mixture only up to 20% dilution, but from 30% dilution, more grave blockage at blastula stage (P2 type) replaced the P1 malformation as main the developmental alteration. Starting from 70% mixture dilution only P2 were observed.

This could be due to the specific nature of metals. Cu, being an essential element, acts also as catalyst for many enzyme systems—increasing amount could inhibit natural cell functions through displacement of other metals at binding site (George 1990); the non essential metals like Cd and Pb could disrupt the ionic balance and alter the permeability characteristic of cell membrane (Belyaeva et al. 2004; Llamas et al. 2000; Viarengo 1985). In addition, metal toxicity could be influenced by many factors that include the interactions of non essential metals with essential one and the formation of metal protein complexes. It's important to remember however that metallothioneins are thought to be involved in the detoxification of essential and non essential trace metals (Amiard et al. 2006); in this case it's possible that the overexpression of MTs is not enough to inhibit the toxic effects caused by metal concentrations in embryos that are probably close to dying.

The comparison of mixture EC50s (Table 2) obtained with Epa ICp and from the "best-fit" curve has shown values not statistically different. In this case, the two procedures of calculation are revealed equivalent.

One aim of this study was to evaluate the behavior of metal mixtures at concentration as low as NOEL and EC1 (Table 2) to understand if toxic effects could derive by combined action at concentration of "no effect" but however present in the environment. This evaluation has been reported in literature only for simple biological systems like algae (Faust et al. 2001; Junghans et al. 2006) and bacteria (Altenburger et al. 2000), but never tested with studied heavy metals (Cu, Pb, and Cd) on sea urchin embryos.

Concentration levels of Cu, Pb, and Cd in seawater in the Mediterranean region vary in a wide range with regard to the contamination of the specific area (proximity to harbours or industrial settlements, large cities…) and to the distance from the coast line. UNEP (1996) gave the following ranges for the concentration levels in the "open" Mediterranean Sea (but "background levels" could be assumed even lower): Cu 40–700 ng/l, Pb 18–140 ng/l, and Cd 4–60 ng/l; while, for a costal waters in the Mediterranean Sea the following ranges were given: Cu 0.01–50 mg/l, Pb 0.016–20 mg/l, and Cd 0.005–0.9 mg/l. Our laboratory obtained (Manfra and Accornero (2002)) the following ranges for coastal sea water in Campania (including industrialized/urbanized areas): Cu 0.60–6.74 (mean 2.58) mg/l, Pb 0.06–2.51 (mean 0.25) mg/l, and Cd 0.01–1.3 (mean 0.12) mg/l. But, again, concentration ranges can substantially vary from area to area. As an example in the Northern Red Sea and Gulf of Aqaba (Shriadah et al. 2004): Cu 0.07–0.29 (mean 0.14) mg/l, Pb 0.02–0.68 (mean 0.25) mg/l, and Cd 0.02–0.78 (mean 0.31) mg/l; while in Greek (contaminated) coastal waters in a small

tidal bay (Dassenakis et al. 1996): Cu 0.03–20.7 mg/l, Pb 0.03–12,2 mg/l, and Cd 0.02–2.3 mg/l.

The metal NOEL/EC1 values determined in this study (Table 2) were in the range of environmental Pb and Cd concentrations. Moreover, Radenac et al. 2001 reported that larval urchin accumulate metals, and in particular, even if Cd had little toxic effects on the initial larval development, its bioaccumulation was comparable to other non essential metal. Instead of, for Cu LOEC was comparable to metal range reported.

The NOEL mixture (Fig. 3b) has shown that all compounds in the mixture contribute to the overall toxicity, even if present only at low statistically not significant concentrations. Actually, NOEL values derived from standard toxicity test have been shown to "represent reductions from the control response of between 10 and 30%" (Moore and Caux 1997). Besides, different studies showed that both mixture of similarly (Arrhenius et al. 2004; Backhaus et al. 2000b) and dissimilarly acting chemicals (Faust et al. 2003) could cause joint effects when present in mixture below or at NOEL values. EC1 mixture (Fig. 3c), where every mixture component was present in concentration well below the individual NOEL value, also clearly evidenced the contribution of each component to the whole toxicity. For all metals tested the EC1 point estimates gave values lower than the NOEL (Table 2) according to that reported by other authors (Arrhenius et al. 2004; Faust et al. 2001, 2003). The toxic effect of mixture at low concentrations (NOEL and EC1), were both mainly attributable to an interference with calcium metabolism, in fact mainly skeletal malformations were produced, with a trend corresponding to the concentration of mixture. At higher concentration the mixture effects were very different; the NOEL mixture effects at concentration of 2× (1.2 mg/l) were mainly of P2 type, that means grave arrest at blastula stage of sea urchin embryos, at a similar concentration (1.14 mg/l) of EC1 mixture (corresponding to 3×) there was no noticeable increment of P2 malformations which, follow an incremental behavior starting from the first mixture concentration. The different behavior of EC1 compared to NOEL mixture, at the same concentration, could be mainly linked to Cu and Pb that are present, in respectively three and fourfold lower concentrations.

Adequacy of model prediction

Due to the chemical nature of the test metals and considering the shape of the dose–response curve (Arrhenius et al. 2004; Faust et al. 2001) it was reasonable to suppose a similar action of metals in this study. However it has to be considered that the shape of does-response curves could vary while scales of the x-axis or y-axis changes, than it would be better to compare the slopes of a certain regression model. There is widespread scientific consensus regarding the prediction of mixture toxicities for chemicals with similar action by the CA model (Backhaus et al. 2000a, b, 2003; Faust et al. 2001, 2003); nonetheless CA best predict mixture toxicity also in the case of not strictly similarly acting substances (Faust et al. 2001). The model CA has also been proposed as a pragmatic default assumption for mixture toxicity predictions (Backhaus et al. 2000a, b; Faust et al. 2001, 2003) also because it could at least represent a reasonable worst-case approach. In our case, CA and IA models equally did not accurately predict mixture toxicity—this was previously observed for dissimilarly acting chemicals (Arrhenius et al. 2006; Manzo et al. 2008; Bellas 2008), with both simple (algae and bacteria) and complex biological systems (sea urchins) but never for similarly acting chemicals. Sometimes a model is considered accurate in predicting toxicity when the differences among the observed and predicted values do not exceed a factor two (Arrhenius et al. 2004), in fact the level of accuracy of acceptance depends on the actual context of an assessment (Arrhenius et al. 2006).

The differences between observed and predicted values were slight pronounced for the EC50 mixture: the ratio between them went from 1.06 to 1.44 for CA and from 1.15 to 1.93 for IA (Table 3). The differences between the CA and IA predictions are quite small and do not even reach a factor of two in any effect level. This is in accordance with the work of Faust (1999) in which it was mathematically proven that the difference between CA and IA for a three-component mixture is always equal to or smaller than three.

The CA model predicts higher, lower, or even identical joint effects than the IA model, depending on the effect level, the number and the concentration ratio of mixture components, the slopes of individual concentration response curves, and the regression models used for their description (Drescher and Boedeker 1995). Typically, however, CA was reported to predict a higher aquatic toxicity than IA, both for binary (Broderius et al. 1995) and multi-component mixtures (Altenburger et al. 2000; Backhaus et al. 2000a, b; Faust et al. 2003; Junghans et al. 2006). The same situation was observed in this study.

The low accuracy of CA and IA models was evident for the NOEL mixture. In fact the ratio among observed and CA-predicted values varies from 1.34 in the upper part of the curve to 4.5 at the lower concentrations and for IA this rate went from 1.90 to 5.21, respectively. On the contrary, the EC1 mixture effect seemed well represented by the IA model. The predictive power of CA was clearly lower for our three metal mixture at EC1 concentration: the curve shape was distinctly different although the deviations at the 50% effect level was less than a factor 1.5. In Arrhenius et al. (2004) the EC1 mixture CA and observed curves

showed the same shape but the CA model curve always overestimated by a factor two, the IA showed a different shape and underestimated and overestimated the observed toxicity.

In this study differences in the capability of model prediction for different ratio mixture was observed. The interaction of metals changed in relation to the concentrations employed in mixture. The variations in the type of combined effects for the different proportions of a mixture have been reported in literature (Cedergreen et al. 2008; Fernandez and Beiras 2001; Otitoloju 2002). Moreover the deviation from predicted combined effects depend on the composition of mixture (Jonker et al. 2005).

The EC50 values predicted by the two models were quite similar (Table 2). The rate IA/CA at 50% effect did not exceed 1.8 for all mixture rates (EC50, NOEL, EC1) investigated, so at 50% effect concentration, neither of the two models appear significantly better than the other. However, sometimes, the two models could give toxicity parameters, as EC50, similar even if the curves were different (Arrhenius et al. 2004). Thus, in this study, neither model could be selected over the other on the basis of accuracy alone. Despite the general approach about the CA model utilized as almost "the worst-case", CA model previsions did not result protective in our study results (Table 3). The protective prevision of the CA model was quite accurate when applied to simple biological systems like algae and bacteria (Altenburger et al. 2000; Arrhenius et al. 2004). As has been pointed out (Barata et al. 2007), extrapolating these results to the effects on whole organisms may be inadequate since unicellular organisms may not reflect the complexity of integral effects on whole organisms, with various target organs and tissues, and with primary and secondary modes of action. Thus, the prediction of mixture toxicity on the basis of the pharmacological mode of action of the toxicant may lead to wrong conclusions when we deal with more complex biological systems such as whole organisms (Bellas 2008). Secondary modes of action, uptake kinetics, transportation, metabolism, and excretion of the chemicals are among the processes that were not considered by the models but that have potentially large impacts on the joint effects (Cedergreen et al. 2008).

The number of mixture components play a key role as well. In fact some authors (Norwood et al. 2003; Phillips et al. 2003) considered the mixture of more than two components as unpredictable.

In this study, the toxicities of the single metals were not assessed at the same time of the mixture toxicity tests of the three metals and it should be noticed that variations could exist between toxicity tests conducted at different times. Then, the regression models of the single chemicals based on toxicity tests conducted at different times might not predict the toxicities of the single chemicals in the ternary mixtures accurately, which will affect the prediction of the mixture toxicities using CA or IA model, and increase/decrease the deviation of the predicted toxicities with the observed ones, thus affecting the accuracies of the CA or IA model.

Any ecologically realistic hazard assessment should take mixture effects into account, as assessing effects individually would underestimate the overall hazard of the mixture. The mechanism-based selection between CA (for mixtures of similarly acting substances) and IA (for mixtures of dissimilarly acting substances) might not be overly important from a pragmatic hazard assessment perspective (Arrhenius et al. 2006). It would be useful to introduce in the models one or more factors that took into account the complexity. Moreover, the setting of water quality criteria of heavy metals in the marine environment should be made considering the effect of a possible joint action at low concentrations upon organisms, mainly at prelarval stadium. Further investigations are clearly necessary to better understand how metals act in mixture, and how to improve the accuracy in predicting their toxicity.

Conclusion

This study evidenced the importance to evaluate the combined toxic effects of multiple chemicals in mixture.

The EC50 mixture revealed synergistic effects with respect to single metals, and a rapid increase of embryotoxicity. Cu might enhanced the toxic action of Pb and Cd, contributing to cellular destabilization.

The NOEL mixture has shown that all compound in the mixture contribute to the overall toxicity, even if present only at low concentrations.

The toxic effect of mixture at low concentrations (NOEL and EC1), were both mainly attributable to an interference with calcium metabolism. In fact mainly skeletal malformation were produced, with a trend corresponding to the concentration of mixture.

CA and IA models equally did not accurately predict mixture toxicity. The differences between the observed and predicted values were slight pronounced for the EC50 mixture, but the low accuracy of models was evident for the NOEL mixture. Moreover, the predictive power of CA was clearly lower for metal mixture at EC1 concentration.

This study suggests that different factors could contribute to the lack of predictability. The evaluated subchronic endpoint (embryos correct development) could be, in fact, influenced in singular phases during the exposition (48–50 h). To better understand the ternary mixture action at different concentrations (EC50, NOEL, EC1), it would need to evaluate the toxic effects in biological systems

simpler than embryos like eggs and sperms and therefore to assess their fertilization capability after exposure. In this test the contact time among cells and toxicants is shorter (20 min) then the embryotoxicity test, where for 48 h the zygote that develops to pluteus is close to metals that can act at different levels.

Thus, the prediction of mixture toxicity on the basis of the pharmacological mode of action of the toxicant may lead to wrong conclusions when we deal with more complex biological systems such as whole organisms (Bellas 2008). Secondary modes of action, uptake kinetics, transportation, metabolism, compartment, and excretion of the chemicals are among the processes that were not considered by the models but that have potentially large impacts on the joint effects (Cedergreen et al. 2008).

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