1   Title

2   **Coexposure to sulfamethoxazole and cadmium impairs development and attenuates transcriptional**

3   **response in sea urchin embryo**

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26   Highlights

27   • SMX and CdCl2 exposure affected sea urchin development.

28   • Coexposure dramatically impaired embryo development.

29   • Coexposure induces alterations in mRNA expression of stress response.

30

Abstract

Among sulfonamides, sulfamethoxazole represents one of the most widely employed. A considerable amount of sulfamethoxazole is introduced into the marine environment after utilization in aquaculture. The cytotoxicity of sulfamethoxazole relies mainly on arylhydroxylamine metabolites and it is associated with the production of reactive oxygen species. Cadmium represents a metal largely employed in several anthropic activities and it is toxic for all living organisms even at low concentrations. Since it is not degraded, cadmium irreversibly accumulates into cells.

In order to understand the mechanisms of response to changes in the chemical environment, we investigated by light microscopy observations and RT-qPCR assays the impact of sulfamethoxazole and cadmium in *P. lividus* sea urchin embryos. During development, embryos were exposed to sulfamethoxazole amount comparable to that usually used in aquaculture procedures and/or sublethal levels of cadmium chloride. Impairment of development and biomarkers for inflammation, detoxification, metal scavenging and cell death were inspected.

Even though treatment with sulfamethoxazole apparently did not affect development, it stimulated a remarkable molecular response to oxidative stress. Moreover, combined exposure seriously compromised development and the defense mechanisms to cadmium were blocked.

This study leads to the conclusion that coexposure to sulfamethoxazole and cadmium induces neutralizing effects on sea urchin embryos. Thus, in marine areas nearby aquaculture farms, where sulfamethoxazole discharge represents an important environmental contaminant, cadmium occurrence may alter population dynamics of *P. lividus*.

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1. Introduction

Sulfamethoxazole (SMX) represents a broad spectrum antimicrobial drug which blocks the folic acid metabolism inhibiting by competition the bacterial enzyme dihydropteroate synthetase (Munson et al., 1998). In human and animal metabolism, approximately 43% of the SMX is decomposed to the corresponding metabolite N4-acetyl-sulfamethoxazole (N4-AcSMX), 9–15% is present as sulfamethoxazole-N 1 glucuronide, while 15–25% is excreted as unchanged molecule (Hoeltge and Kreuzig, 2007; Radke et al., 2009).

Interestingly, at wastewater treatment plants, the hydrolysis of the N4-AcSMX and the recreation of the parental drug occurred; thus, SMX concentration in effluents results higher than in the influent (Joss et al., 2005; Göbel et al., 2007). Significant amounts of antibiotics into groundwater and marine environments are usually released (Kemper, 2008) and large variety of sulphonamide concentrations spanning from 6 µg/l to 50 mg/l were detected (Qiting and Xiheng, 1988; Hirsch et al., 1999; Tadkaew et al., 2010). In addition, SMX has been used for veterinary intents in several aquatic farms of the Mediterranean basin (Costello et al., 2001; Rigos and Troisi, 2005). Hence, non-target organisms are exposed to antimicrobial drug residues accumulating it in the aquatic trophic chain (Samuelsen et al., 1992; Coyne et al., 1997).

However, data on the effects of antibiotics for non-target organisms were produced for a limited number of species, especially freshwater or estuarine crustaceans (Rigos and Troisi, 2005; Baran et al., 2011; Nicosia et al., 2014a).

Usually several pollutants are present at the same time in the aquatic environment, and some of them exert synergistic, additive or neutralizing effects (García-Galán et al., 2008; De Liguoro et al., 2009; Pomati et al., 2006); thus, different degrees of toxicity usually occurred.

Metals represent one of the major sources of pollution in marine environments and among them, cadmium (Cd) has been considered as a priority hazardous substance (Directive 2000/60/EC).

Cadmium ions are transported to target tissues and act by a molecular and ionic mimicry mechanism substituting the proper ions in their metabolic sites (Bridges and Zalups, 2005). Cd is accumulated by organisms causing oxidative stress, DNA damage, and macromolecular damage (Shimizu et al., 1997; Ercal et al., 2001).

Cd is also a natural constituent of ocean water, with average levels between 5 and 20 ng/l in open seas (Kremling and Streu, 2001), while higher levels between 1.49 µg/l in the Galician coasts (Beiras et al., 2003) and 73.8 mg/l in the Dardanelles Strait (Yılmaz and Sadikoglu, 2011) were reported in highly polluted coastal areas. The stress response effects of Cd exposure have been widely described in different marine

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86   organisms including bivalves (Jo et al., 2008; Nardi et al., 2017), *Octopus vulgaris* paralarvae (Nicosia et al;

87   2015) and sea urchin *Paracentrotus lividus* embryos (Ragusa et al., 2013; Bonaventura et al., 2015;

88   Migliaccio et al., 2015; Chiarelli et al., 2016). However, few data are available on the effects of toxic chemical

89   mixtures or coexposure to multiple stressors at developmental and molecular level (Steevens et al., 1999,

90   Bellas, 2008; Bonaventura et al., 2015).

91   The sea urchin embryo is a model organism for toxicological studies exploiting the morphological

92   perturbations in embryo development exerted by several pollutants (Kobayashi, 1991; Warnau and Pagano,

93   1994; Pagano et al., 2017).

94   It has been shown that embryos are able to absorb and accumulate several metals (Agnello, et al., 2007;

95   Pinsino et al., 2010); moreover, it has been reported that SMX exposure could operate by attenuating the

96   expression of stress response and antioxidant genes (Nicosia et al., 2014a). Thus, it could be hypothesised

97   that combined exposure may result in severe reactions. To date, no study is available on the combined

98   effects of cadmium and SMX on *P. lividus* embryos.

99   In order to characterise the sea urchin embryo stress response to SMX and to assess the joint-action toxicity

100   of the binary mixture Cd/SMX, we evaluated the effects of SMX alone and in combination with Cd.

101   *P. lividus* embryos were continuously exposed to non-lethal SMX and $CdCl_2$ concentrations from fertilization

102   to 48 hours post fertilization (hpf) and morphological analyses were carried out during development.

103   The molecular effects of these compounds were evaluated by means of mRNA expression analysis of

104   multiple genes whose expression is associated to stress response. The transcription of inflammation,

105   detoxification, metal scavenging and apoptotic related genes were profiled in response to single and joint

106   challenges.

107

108   **2. Materials and Methods**

109   *2.1. Embryo Culture and Experimental Design*

110   Gametes were collected from gonads of the sea urchin *P. lividus* collected in the South-West coast of Sicily,

111   nearby Capo Granitola. Eggs were fertilised and embryos reared at 18℃ in Millipore filtered seawater

112   (MFSW). Considering the routine procedures used in the aquaculture farms and metal dosage used in other

113   studies (Khan et al., 2013; Ragusa et al., 2013; Chiarelli et al., 2014), SMX-exposed embryos were

114   continuously cultured in the presence of 50 mg/l of SMX (S7507 Fluka)- theoretical concentration; while Cd-

115   exposed embryos were continuously cultured in the presence of 0.1 mM or 1 mM $CdCl_2$ (Sigma-Aldrich)-

theoretical concentrations. Cd/SMX-exposed embryos were exposed in different tanks to combinations of the two pollutants herein analysed.

Unexposed embryos from the same batch of eggs were used as control. Each experiment was performed three times.

2.2. Morphological Analysis

Development was monitored from fertilization to pluteus stage (48 hpf). The percentages of embryos with normal or abnormal development in each treatment were determined by counting about 100 embryos/experiment using Olympus BX50 optical microscope and representative images were recorded by a Nikon digital camera.

2.3. RNA Extraction and First-Strand cDNA Synthesis

Total RNA was extracted from control and exposed *P. lividus* embryos at 25 hpf with the PureLink RNA Mini Kit (Ambion) following the manufacturer's instructions. RNA concentrations and quality were spectrophotometrically verified, while RNA integrity was checked using a 1.5% agarose denaturing gel. The RNA was stored at $-80°C$ for future use. An amount of total RNA corresponding to 2 µg was treated with Deoxyribonuclease I, Amplification Grade (Invitrogen) to remove any residual genomic DNA contamination, and DNase I was inactivated by adding 25 mM EDTA.

First-strand cDNA was synthesised from 1 µg DNase I treated total RNA samples using QuantiTect Rev. Transcription (Qiagen), following the manufacturer's instructions. The cDNA mixture was tested by PCR using 18S rRNA primers, then stored at $-20°C$ until needed.

2.4. Relative Quantification Using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR).

RT-qPCR experiments were performed using the BIO-RAD CFX96 system with QuantiFast SYBR Green PCR (Qiagen) as detection chemistry. Gene-specific primers used to determine target gene expression levels are listed in Table 2. The *18S* ribosomal RNA target was used as internal control to verify the quantitative real-time PCR reactions. Quantitative real-time PCR amplifications were conducted according to the manufacturer's recommended procedures, and every reaction was repeated three times. The amplification conditions included an initial denaturation at $95°C$ for 5 min, 40 cycles of $95°C$ for 30 s, $60°C$ for 35 s, followed by a melting curve from 65 to $95°C$. Amplicons were detected by agarose gel analysis after

146   each PCR reaction to confirm the specific gene amplification. Data analysis was carried out according to the

147     $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

148

149   **Table2**

150   Genes and oligonucleotide primers used in this study

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Gene name	Abbreviation	Primers (5'-3')
ATP-binding cassette A3	*ABCA3*	GCTTGGCGTAATCATGGTCATAGC[a] TCACTCATTAGGCACCCCAGG[b]
Protein inhibitor of nitric oxide synthase	*PIN*	TCGGGAATGAGTTTGTTAGGACG[a] GCACGAGACCAAGCATTTCATC[b]
Protein Tyrosine Phosphatase Receptor Type D	*PTPRD*	TCCTCACATAACCTTACAACATACAGCT[a] AATAGCAAGCAAAATACTTCCCTTCC[b]
TGF-β-activated kinase 1	*TAK1*	GGATTCTCTTGTGTGGGTGATACAC[a] TGTGATATTCCTTTCACGATTTCCG[b]
BCL2 antagonist/killer	*BAK*	CCTGATTAGAGCCAGTATGTCTTCTGG[a] ATTGCAGAATACACAACTATGCCTGC[b]
Caspase 8	*CASP8*	GAACCTGAGGATACGACGAGC[a] GGCGTGCTACTGTCCGAAC[b]
Manganese Superoxide dismutase	*SOD*	CTCCGTTCTCAACCTGCTG[a] CGCTGACTAAGCCAAACATATCC[b]
Glutathione peroxidase	*GPX*	ATACAGTACGCAGCACACCAGC[a] AGAATCTCCGAGCCAGGTATG[b]
Fas-associating death domain-containing protein	*FADD*	CGTCACGGAGAACCATAGTAGG[a] GGTGCCAAGATGTAACTGTCG[b]
Metallothionein 4	*MT4*	GCTCAAAATCTTCAACATGGCTAATGA[a] AGCACTTTCCAGTTTCACAACAAGC[b]
Metallothionein 5	*MT5*	CGACTTTAGCTCAAATTCATCACCATG[a] TCCACAGCATTTACCATCCTTGC[b]
Metallothionein 6	*MT6*	CACGATTTGTGCTCAATCCTTCAT[a] TTTGTGCATGATGTTCCACAGC[b]
Metallothionein 7	*MT7*	CGTCAAGAGATCAAAATCATCAACCA[a] ACAGCACTCGCCAGTAATACAGCAC[b]
Metallothionein 8	*MT8*	GATGGTTGTCGTCGCTCCTAACA[a] TCAAGAAAGGCTGGTATCAAATCTGAC[b]
18S ribosomal RNA	*18s*	GAATGTCTGCCCTATCAACTTTCG[a] TTGGATGTGGTAGCCGTTTCTC[b]

152   [a]Forward primer, [b]Reverse primer

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2.5. Statistical Analysis

Experiments were performed in triplicate. The results were expressed as a mean value ± Standard Deviation (SD). Conditions for applying parametric tests have been checked and data were statistically analyzed by t-test or one-way analysis of variance (one-way ANOVA) using Statistica 6.0 (StatSoft, Tulsa, OK, USA). *p* values less than 0.05 were considered statistically significant and were indicated by ∗.

3. Results and Discussion

3.1. Effects of SMX and Cd on sea urchin development

In order to investigate the effects of SMX on sea urchin development, fertilised eggs were exposed to antimicrobial in MFSW for 48 h (corresponding to pluteus stage in the controls) using a nominal concentration corresponding to 50 ppm SMX. No lethal effect was recorded in the control group. Thus, embryonic cultures were observed under microscope at 4, 8, 12, 24, 38 and 48 hpf corresponding to morula (16 blastomeres), blastula, swimming/mesenchyme blastula, gastrula, prism/prepluteus and pluteus stage, respectively (Figg. 1 and 2). SMX exposure did not grossly affects the survival rate and no defects in embryo development were observed even at pluteus stage (48 hpf). Even if no reports are currently available on SMX cytotoxicity in sea urchins, it has been reported that SMX exposure did not affect survival and prolonged the lifespan of the non-target organism *Caenorhabditis elegans* (Liu et al., 2013). Additionally, similar results were also obtained for the daggerblade grass shrimp *Palaemonetes pugio* in which no effect was observed during embryo development following SMX maternal exposure (Garcia et al., 2014).

Cadmium treatment effects on sea urchin embryos were already known (Russo et al., 2003;, 2004; Roccheri et al., 2004) and our results confirm that embryos developed with no significant differences with respect to controls until the swimming blastula stage (12 hpf). After continuous treatment with 0.1 mM $CdCl_2$, at 24 hpf, when controls are at the gastrula stage, embryos showed developmental defects: retard in endoderm invagination and no spicule rudiment formation, moreover 14% of embryos were so-called abnormal blastulae (Figg. 1-3). At 38 and 48 hpf (prisma and pluteus stage in the controls) developmental defects and skeletal malformations were more evident. As expected, abnormalities were more severe in those embryos that received higher cadmium concentrations, this is particularly evident at 24 hpf, corresponding in untreaded embryos to gastrula stage. However, percent of abnormal embryos observed were lower than previously reported (Russo et al., 2003). This could be explained by the different culture conditions; indeed in our experiments embryos were cultured without the antibiotic mix usually supplemented to sea water, to avoid any bias due to the presence of other chemicals in the culture.

185 Cotreatment with SMX and 0.1 mM CdCl$_2$ caused significantly higher percentages of abnormalities with

186 respect to the treatment with 0.1 mM CdCl$_2$ only (Figg. 1-3). At 4 hpf several morulae showed defects in cell

187 division. At 12 hpf a percentage of blastula cells were not tightly connected. At 24 hpf a high percentage of

188 embryos displayed retard in endoderm invagination and primary mesenchyme cells were not localised in the

189 ventrolateral region of the blastocoel. Moreover, embryos showed ectoderm anomalies and no triradiate

190 spicules elaboration. A low percentage of embryos were blocked at blastula stage. This co-exposure led to

191 more than 90% of embryos with skeletal alterations that became evident at 38 hpf and later. At 48 hpf

192 variable phenotypes were obtained: undeveloped embryos (blastula, abnormal gastrula), and prepluteus

193 stages (shortening of the apex and reduced length of the arms and spicules as if retarded in growth, but

194 quite normal gut differentiation) and pluteus stage larvae with incomplete or absent skeletal rods. A low

195 percentage of them showed fractured ectoderm.

196 Higher Cd concentration was also used in combination with SMX. Embryo coexposure to SMX and 1 mM

197 CdCl$_2$ displayed high toxicity of the treatment, characterised by blockage of development at the blastula

198 stage (Figg. 1-3). At 4 hpf number and dimensions of blastomeres were altered; at 8 hpf more than 70% of

199 embryos were malformed: no blastocoel was developed and cell dimensions were abnormal. Blastomeres

200 were loosely connected and a lot of single cells appeared in the background at 12 hpf. At 24 hpf there were

201 no gastrulae in the culture, as embryo development appeared to be blocked at the blastula stage or

202 abnormal morula, the so-called occluded blastulae (Figg. 1-3). Later, at 38 hpf, embryos were completely

203 degenerated.

204 As already known, cadmium impaired the skeletogenic pathway, conversely SMX did not seem to alter any

205 specific developmental pathway. As a result, embryos developed quite normally. On the other hand, SMX/Cd

206 co-occurrence caused gastrulation block, cell-cell junction weakening and survival reduction. These results

207 show that embryos were unable to cope with the simultaneous presence of these chemicals and suggest

208 alteration of the mechanisms that usually lead to their survival.
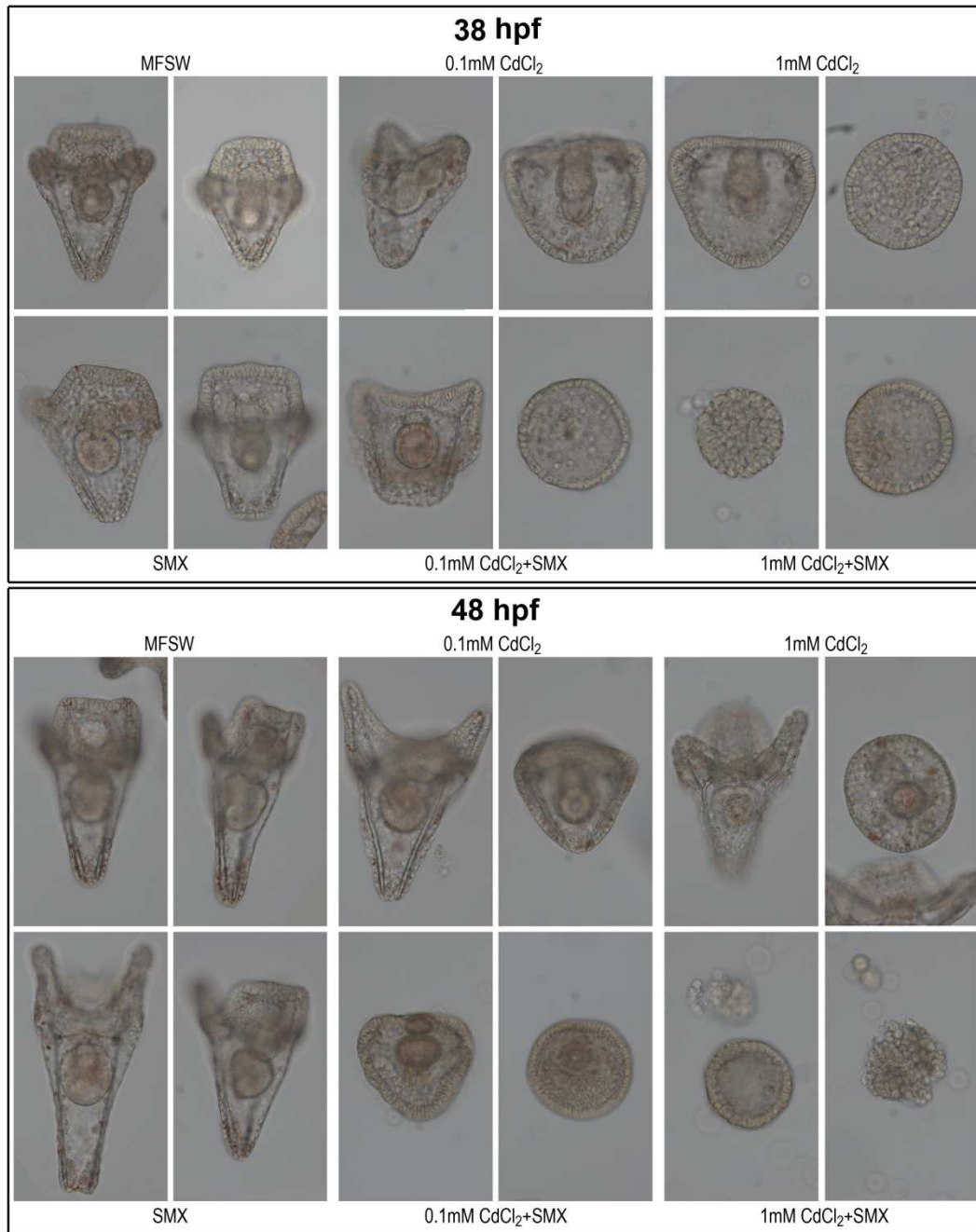
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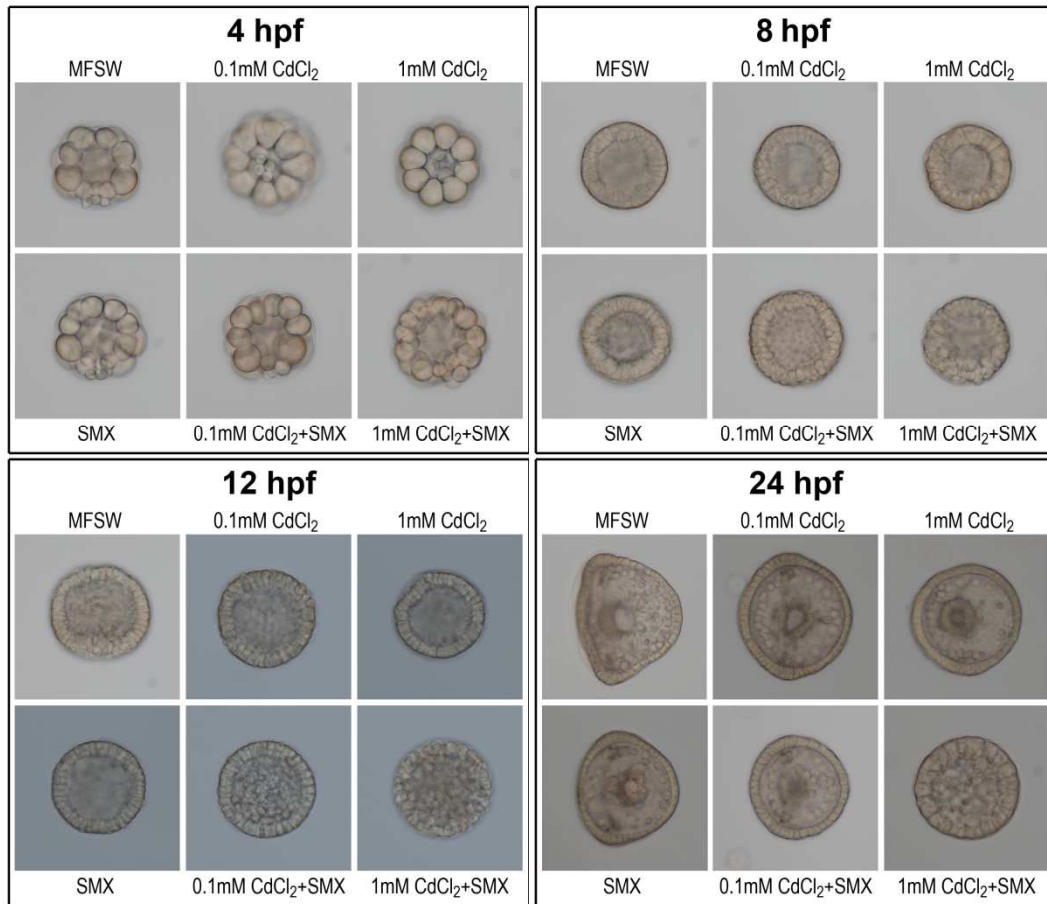
Fig. 1. Sea urchin embryo early development in response to different treatments. Embryos were separately exposed to SMX, $CdCl_2$ or SMX and $CdCl_2$ applied in combination as reported in Table 1. Treatments differently affected sea urchin development. Joint exposures resulted in higher percentages of abnormalities including defects in cell division at 4hpf (morula stage in control embryos), delay in endoderm invagination and mislocalization of primary mesenchyme cells at 24 hpf (gastrula stage in control embryos).

Fig. 2. Sea urchin embryo late development in response to different treatments. Embryos were separately exposed to SMX, CdCl$_2$ or SMX and CdCl$_2$ applied in combination as reported in Table 1. Treatments differently affected sea urchin development. Coexposure resulted in undeveloped embryos, pluteus larvae with fragmented spicules and larvae with incomplete or absent skeletal rods.
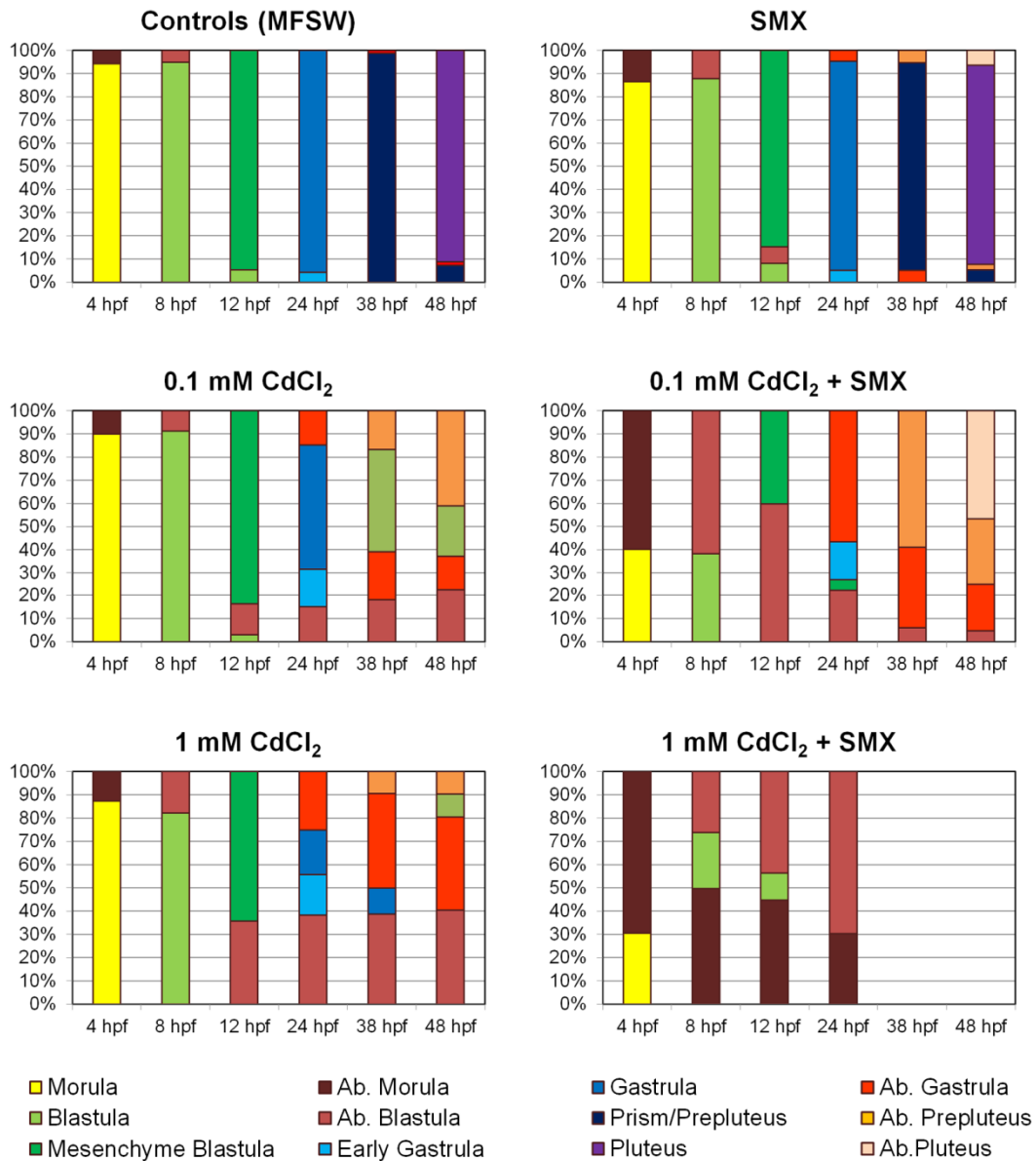
Fig. 3. Embryo morphological abnormalities and percentages in response to the different treatments. Bar chart corresponding to embryos exposed to 1mM $CdCl_2$ and SMX at 38hpf and 48hpf are not reported since no living embryos were retrieved. Means of three experiments are shown; SD were lower than 5%. Ab.: abnormal.

*3.2. Transcriptional Effects of SMX and Cd on sea urchin gene expression pattern*

It is widely accepted that oxidative stress that occurs as a result of the effect of xenobiotics severely modifies canonical transcriptional patterns (Martín-Díaz et al., 2009; Nicosia et al. 2014a, Nicosia et al. 2014b). It is also well known that the main transcriptional modifications associated to ontogenesis in sea urchin occur

234 during blastula-gastrula transition (Lyons et al., 2012). Therefore, we investigated the transcriptional changes

235 (induction or downregulation events) affecting the inflammation related genes (*PIN, PTPRD* and *TAK*),

236 detoxification-related genes (*ABCA3*, *SOD* and *GPX*), metal scavenging (*metallothioneins MT*s) and

237 apoptotic genes (*FADD, CASP8* and *BAK)* at 24hpf, corresponding to the gastrula stage in normal embryos.

238 The expression and release of inflammatory mediators is usually associated with ROS and can be regulated

239 by the activation of redox-sensitive signaling factors (Mittal et al., 2014).

240 Among them, NOS regulation based on the activity of protein inhibitor of NOS-1 (PIN) (Adcock et al., 1994;;

241 Semenova and Ozernyuk, 2016; Sawa et al., 2013;), phosphothyrosin phosphatase (PTP)-mediated

242 regulation of the JAK-STAT pathway (Yang et al., 2007; Böhmer and Friedrich, 2014) as well TGFbeta

243 activated kinase (TAK1) activation of JNK, p38, and NF-κB signaling (Ma et al., 2011; Sakurai, 2012)

244 represent remarkable mechanisms in cell signaling.

245 Thus, to unveil the possible involvement of these factors in response to SMX, the mRNA levels of *PIN,*

246 *PTPRD,* and *TAK* were analysed and results are shown in figure 4. The relative mRNA expression of the

247 selected genes increased in embryos exposed to SMX. In particular, *PIN* mRNA accumulated to 8.1-fold

248 greater than the control, *PTPRD* transcript was overexpressed approximately 3.3-fold greater than the

249 control group and *TAK1* expression was increased to 3.6-fold greater than the control.

250 Conversely, considerable negative variations appeared in response to 0.1mM and 1mM $CdCl_2$; indeed,

251 significant down-regulation in the mRNA levels of the selected genes was observed in sea urchin embryos

252 exposed to cadmium salt. To investigate the perturbation in mRNA levels determined by the joint effects of

253 pollutants, the expression of these genes was also investigated in Cd/SMX exposed embryos. The qPCR

254 analysis revealed that Cd/SMX coexposure negatively affected gene expression: all the transcripts were

255 downregulated or slightly induced (*PIN*) in embryos exposed to this mixture with respect to SMX treatment,

256 thus suggesting that Cd presence abolished the transcriptional induction exerted by SMX treatment.

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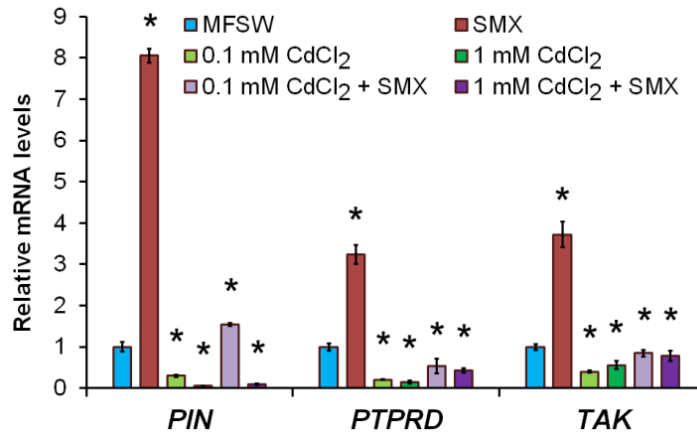
Fig. 4. Treatments induce alterations in mRNA expression of inflammation related genes. RTqPCR results showing *PIN*, *PTPRD* and *TAK* mRNA levels in *P. lividus* embryos, with respect to *18S* at 24 hpf. Bars represent mean ± SD, and asterisks denote responses that are significantly different from the controls (*P<0.05).

It has been shown that different metal combinations produce neutralizing, additive or synergistic effects thus resulting in variable levels of toxicity (Wah Chu and Chow, 2002; Xu et al., 2011). Therefore, it is reasonable to suppose that neutralizing mechanisms have herein acted to downregulate mRNA expression of pro-inflammatory genes.

Another well-known response mechanism to xenobiotics is the activation of antioxidant systems. Members of such mechanisms, including ABC transporters, superoxide dismutase (SOD) and glutathione peroxidase (GPX), are induced by various oxidative stresses and appear to possess protective roles (Viarengo et al., 1995; Canesi et al., 2007).

To evaluate the activation of antioxidant systems in response to sulphonamidic exposure, the transcriptional expression levels of *ABCA3*, *SOD* and *GPX* genes were investigated (Fig. 5). These mRNAs were significantly induced after exposure to 50ppm SMX: the *SOD* and *GPX* transcripts accumulated to 2.3-fold and 1.5-fold respectively in SMX treated embryos. Therefore, the expression of the mRNA for antioxidant genes suggests that ROS were induced by SMX in sea urchin embryo and that the antioxidant system was transcriptionally enhanced to remove ROS.

Similarly, *ABCA3* expression resulted 2.6-fold greater than the control. Members of the ABC family are known to act as organic anion transporters extruding a variety of xenobiotcs (Schinkel and Jonker, 2003). Therefore it is reasonable to suppose that during embryo development similar mechanisms are used to reduce the source of ROS-related compounds.

282   In embryos exposed to $CdCl_2$ different results were obtained. The *ABCA3* mRNA was reduced both at

283   0.1mM and 1mM $CdCl_2$ exposures. *SOD* and *GPX* transcripts were down-regulated at 0.1mM; while they

284   peaked (1.4 and 1.8-fold higher than the control group, respectively) after exposure to 1mM $CdCl_2$. Our data

285   are in agreement with other works showing the induction of antioxidant genes in fish exposed to Cd (Roméo

286   et al., 2000, Hansen et al., 2006). Additionally, similar results were obtained in *Crassostrea gigas* (Jo et al.,

287   2008). Therefore, the expression of the mRNA for antioxidant genes confirm that ROS were induced by high

288   Cd concentrations in sea urchin embryos and that the antioxidant system was transcriptionally enhanced to

289   remove the ROS.

290   The SMX/Cd combined exposure exerted a negative effect on the overall mRNA expression level of these

291   genes also. The qRT-PCR analysis showed a significant downregulation of *ABCA3* and *GPX* in presence of

292   Cd and SMX at every tested concentrations; while *SOD* levels remained grossly unchanged whit respect to

293   cadmium treatment alone.

294   Thus, as occurred for inflammation-related genes previous analysed, it could be hypothesised that the

295   decrease in the mRNA levels of the antioxidant genes may likely be associated to neutralising activity

296   exerted by Cd/SMX blend.

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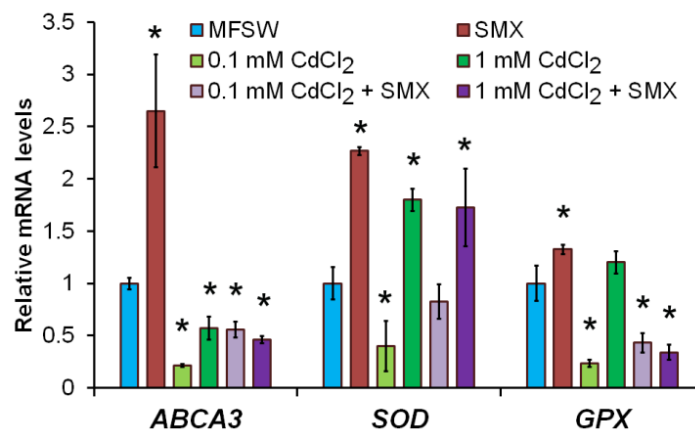
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299   **Fig. 5.** Treatments induce alterations in mRNA expression of detoxification-related genes. RTqPCR results

300   showing *ABCA3*, *SOD* and *GPX* mRNA levels in *P. lividus* embryos, with respect to *18S* at 24 hpf. Bars

301   represent mean ± SD, and asterisks denote responses that are significantly different from the controls

302   (*$P < 0.05$).

303

304   Metallothioneins (*MT*)s are efficiently induced by a mechanism involving the upstream antioxidant responsive

305   element resulting in DNA protection from oxidative damage blocking the Fenton reaction (Dalton et al., 1994;

306   Chiaverini and Ley, 2010;). Thus, the effects of antimicrobial drug exposure, alone or in combination with

307   metal, were analysed on the expression of inducible (*MT4*, *MT5* and *MT6*) and constitutive (*MT7* and *MT8*)

308   sea urchin metallothioneins (Ragusa et al., 2013).

309   The *MT*s mRNA levels were measured in control and exposed embryos and data are presented in figure 6.

310   SMX exposure negatively affected the mRNA expression level of inducible metallothionein MT4; while the

311   other inducible metallothionein mRNAs, *MT5* and *MT6,* maintained their levels or were slightly reduced.

312   Interestingly, constitutive *MT7* and *MT8* were upregulated after SMX exposure. Thus, the over-expression of

313   constitutive homologues raise the possibility that the transcriptional patterns of metallothionein response to

314   pollutants are variable according to the induction of selected members. Moreover, our results are consistent

315   with findings reported for the red swamp crayfish *Procambarus clarkii* (Nicosia et al., 2014a) in which SMX

316   also down-regulated metallothionein expression. The transcriptional activation of such class in response to

317   metal exposure has been described in sea urchins (Ragusa et al., 2013; Russo et al., 2014); in this study

318   using sea water without canonical antibiotics, a similar gene expression pattern was obtained and *MT*s were

319   found to be up-regulated following cadmium exposures.

320   The expression of these genes was also investigated in Cd/SMX exposed embryos. The qRT-PCR analysis

321   revealed that all the transcripts, constitutive and inducible ones, were up-regulated in sea urchin embryo

322   exposed to such mix and the overall mRNA expression levels resemble those observed in metal exposed

323   embryos. In particular, co-treatments unaffected the response raised by Cd for inducible MTs and MT8;

324   conversely, the MT7 transcriptional response appears negatively affected. A recent study (Russo et al.,

325   2014) reported that during sea urchin development Cd/UVB combined exposure upregulates *Pl-MT* mRNA

326   (corresponding to *MT8*). Thus, according to MT's protective role, sea urchin embryos cope with Cd/SMX

327   exposure by means of metal-scavenging as well ROS-quenching activities.

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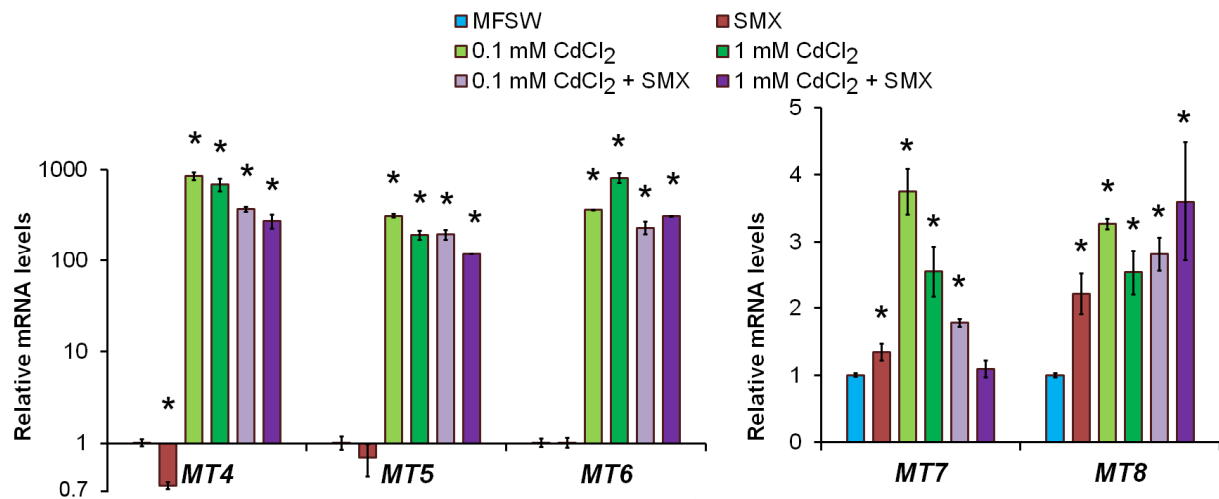
Fig. 6. Treatments induce alterations in *metallothionein* gene expression. RTqPCR results showing inducible (*MT4*, *MT5* and *MT6*) and constitutive (*MT7* and *MT8*) *metallothionein* mRNA levels in *P. lividus* embryos with respect to *18S* at 24 hpf. Bars represent mean ± SD, and asterisks denote responses that are significantly different from the controls (*$P<0.05$).

Finally, it is well established that oxidative stress and apoptosis are closely related and members of stress signaling pathway also regulate the extrinsic and intrinsic cell death pathways (Kannan and Jain, 2000; Circu and Aw, 2010). To investigate the perturbation in mRNA levels of apoptosis related genes, the transcript levels of *FADD* (extrinsic pathway), *CASP8* (initiator caspase) and *BAK* (intrinsic pathway) were analysed.

It has been reported that FADD is significantly up-regulated in TNFα induced apoptosis (Gupta et al., 2004; Zhang et al., 2008); additionally BAK overexpression has been associated to activation of caspase-3 and apoptosis (Tong et al., 2004; To et al., 2011).

Analysis of *BAK*, *FADD* and *CASP8* mRNA levels in response to SMX is shown in figure 7. In particular, the *FADD* and *BAK* mRNA levels were significantly overexpressed (1.6-fold and 2.3-fold, respectively, than the control); while a reduction in *CASP8* mRNA level was observed.

Differently, sea urchin embryos exposed to metal showed a reduction in *CASP8* and *BAK* transcripts while *FADD* mRNA remained overexpressed suggesting the activation of death pathways.

During the last decade several reports have been produced unveiling the activation of cell death machinery in sea urchin embryos exposed to Cd. These pathways, including apoptosis and autophagy, have been described as key players in stress response (Agnello et al., 2007; Chiarelli et al., 2014).

16

350 Therefore, it is likely to hypothesise that during embryo development similar death mechanisms are activated

351 in response to different stressors. Moreover, the activation of cell death machinery in response to Cd and

352 SMX may occur through the involvement of other caspases than the initiator one.

353 Interestingly, SMX and Cd co-exposure abrogates the transcriptional induction of all the analysed genes.

354 Indeed, the qPCR analysis revealed that *FADD* and *BAK* mRNA levels were severely down-regulated in sea

355 urchin embryo exposed to such mix. Conversely, *CASP8* mRNA peaked (1.7-fold than the controls) after

356 exposure to 50ppm SMX/1mM CdCl$_2$.

357 It is known that the removal of damaged cells from embryos exposed to toxic compounds or other stressors,

358 throughout apoptosis or autophagy, represents a conserved mechanisms among aquatic species

359 (Huettenbrenner et al., 2003, Chiarelli et al., 2016).

360 Thus, combined exposure to Cd and SMX once again seems to abrogate the actions of specific members of

361 these pathways during sea urchin development. Therefore, the impairment of this system could be

362 considered as responsible for the altered developmental program resulting in embryos with aberrant

363 phenotypes.
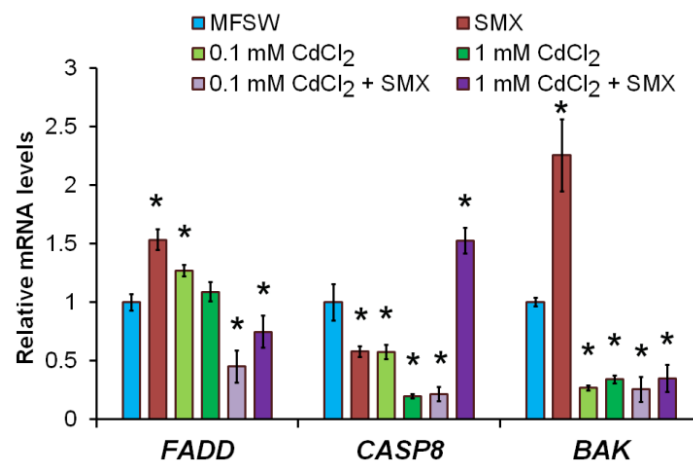
364

365 **Fig. 7.** Treatments induce alterations in mRNA expression of cell death genes. RTqPCR results showing

366 *FADD*, *CASP8* and *BAK* mRNA levels in *P. lividus* embryos with respect to *18S* at 24 hpf. Bars represent

367 mean ± SD, and asterisks denote responses that are significantly different from the controls (*P<0.05).

368

369 **4. Conclusions**

370 Herein, we evaluated for the first time the effects of SMX and Cd/SMX exposure on *P. lividus* sea urchin

371 embryos in order to characterise the defence mechanisms activated in response to these compounds.

372 We chose concentration levels measured in polluted marine environment and already used in other studies

373 to provide results easily comparable with previous data. Moreover, these concentrations were checked for

17

374 the absence of any lethal effects when used singularly. Our experiments show a lethal effect of the SMX/Cd

375 mix strongly suggesting a dramatic impairment of the molecular response. Indeed gene expression profiles

376 resulted in the block of the SMX response when Cd waterborne exposure occurred during the development.

377 Every pathway analysed undergoes abrupt modifications impairing embryo ability to cope with the stressors

378 and survive. These data also provide additional evidence supporting the necessity to associate single

379 contaminant/stressor exposure data with combined analysis.

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