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Assessing the eco-compatibility of new generation sunscreen products through a combined microscopic-molecular approach☆

Stefano Varrella [a,b], Roberto Danovaro [a,c], Cinzia Corinaldesi [b,*]

[a] Department of Life and Environmental Sciences, Polytechnic University of Marche, Via Brecce Bianche, 60131, Ancona, Italy
[b] Department of Materials, Environmental Sciences and Urban Planning, Polytechnic University of Marche, Via Brecce Bianche, 60131, Ancona, Italy
[c] Stazione Zoologica Anton Dohrn, Villa Comunale, 80121, Naples, Italy

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ABSTRACT

There is now unequivocal evidence that sunscreen can severely affect marine ecosystems. However, so far, most studies have focused on the impact of single sunscreen ingredients rather than on the whole sunscreen products, which are released into the marine environment. In the present work, we investigated the ecological impact of six formulations, which represent the "new generation" organic UV filters such as diethylamino hydroxybenzoyl hexyl benzoate (DHHB), methylene bis-benzotriazolyl tetramethylbutylphenol (MBBT), ethylhexyl triazone (EHT), and bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT), which are progressively replacing the "old generation" organic UV filters (e.g., oxybenzone, octinoxate) banned in several countries of the world. The six formulations tested were characterized by a different combination of ingredients, on a model species particularly sensitive to environmental alterations: the sea urchin, *Paracentrotus lividus*. We investigated the sea urchin responses both in terms of gene expression and anomalies in embryonic development. We found that all sunscreen products containing only MBBT, DHHB, BEMT, and EHT as UV filters, are more eco-compatible than those also containing also ES, or other ingredients such as emollients and texturizing compounds, which may act synergistically causing molecular stress, morphological anomalies, and ultimately possible death. Overall, the results presented here provide new insights on the effects of sunscreen products based on "new generation" UV filters, and highlights the urgency of testing complete formulations, rather than just specific UV filters to ascertain the eco-compatibility of sunscreen products, to effectively minimize their impact on marine ecosystems.

1. Introduction

The huge tourist flow that reaches the coasts of the Mediterranean Sea every year represents a major threat to coastal marine ecosystems (Batista e Silva et al., 2018; Tovar-Sánchez et al., 2019; World Tourism Organization, 2021). This is also due to the intensive use of sunscreens to the skin by tourists, with the consequent release in the marine environment (Labille et al., 2020a; Tovar-Sánchez et al., 2020a), and/or the input of sunscreen products and UV filters through wastewater treatment systems (Brausch and Rand, 2011; Cadena-Aizaga et al., 2020; Cadena-Aizaga et al., 2022).

Although sunscreens are essential to protect humans from the risks associated with UV radiation, it is now consolidated that these products can have severe negative impacts on a wide variety of habitats and marine organisms, spanning from prokaryotes to large marine animals (Lozano et al., 2020a; Tovar-Sánchez et al., 2020b). The effects are detectable both at the molecular (e.g. gene expression, DNA damage), cellular (e.g. production of reactive oxygen species, antioxidant enzymes), individual/population (e.g. mortality, behavioral alteration), and assemblage level (Caloni et al., 2021; Danovaro et al., 2008; Lozano et al., 2020b; Tovar-Sánchez et al., 2020a and references therein).

These discoveries have stimulated the search for alternative (organic) UV filters to replace impacting chemical filters (e.g., benzophenone-3 (BP-3), benzophenone-4 (BP-4), avobenzone, ethylhexyl-4-methoxycinnamate (EHMC), octocrylene (OCR)) (Cadena-Aizaga et al., 2022; Fagervold et al., 2019; Labille et al., 2020b; Mitchelmore et al., 2019). Due to their persistence, some compounds bioaccumulate in mussels (Bachelot et al., 2012; Castro et al., 2018; Picot Groz et al., 2014; Vidal-Liñán et al., 2018), crustaceans (Cunha et al., 2018; Peng et al., 2017), shrimps (Araújo et al., 2020), squids

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* Corresponding author.
E-mail address: c.corinaldesi@univpm.it (C. Corinaldesi).

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(Cunha et al., 2018; Peng et al., 2017), fishes (Araújo et al., 2018; Fent et al., 2010; Gago-Ferrero et al., 2015; Grabicova et al., 2013; Molins-Delgado et al., 2018), sea urchins (Rocha et al., 2018; Sang and Leung, 2016), dolphins (Gago-Ferrero et al., 2013) and cormorants (Fent et al., 2010). Other adverse effects of sunscreens include: coral bleaching, alteration of the behaviour in mobile species, endocrine disruption, reproductive alteration, neurotoxicity, cytotoxicity, and also organism death (Chen et al., 2018; Danovaro et al., 2008; Jesus et al., 2022; Maipas and Nicolopoulou-Stamati, 2015; Rainieri et al., 2017). Organic UV filters (e.g., BP-3, OCR) once in the marine environment can also produce reactive oxygen species (ROS), which in turn can damage lipids, proteins, and DNA inducing high levels of stress in marine organisms (Lesser, 2006; Sánchez-Quiles and Tovar-Sánchez, 2014). Due to the high lipophilicity and stability of organic UV filters, it has been reported that the harmful effects of these compounds on marine biota might be even exacerbated by global warming (Fastelli and Renzi, 2019; Wijgerde et al., 2020).

The scientific evidence accumulated so far, along with the increasing prohibition of the use of specific UV chemical filters, such as oxybenzone and octinoxate, in several countries of the world has stimulated the search for new eco-compatible UV filters such as triazine UV-filters able to replace the "old generation" of chemical UV filters (Du et al., 2022; Miller et al., 2021; Ouchene et al., 2019; Tovar-Sánchez et al., 2019). However, the environmental fate and biological effects of these "new generation" UV filters have only recently begun to receive scientific attention due to the growing use and increasing concentrations measured in the marine environment (from nanograms to micrograms per liter of seawater or grams of sediments; Apel et al., 2018; Cadena-Aizaga et al., 2022; Du et al., 2022; Fagervold et al., 2019).

Previous studies revealed that the early life stages of marine organisms can be optimal bioindicators to test the eco-compatibility of sunscreen products already on the market and/or new formulations (Corinaldesi et al., 2017; Gambardella et al., 2021). In particular, the embryos and larval development of the sea urchin *Paracentrotus lividus* (Lamarck, 1816), have been used to assess the impact of a wide range of contaminants, including heavy metals, pharmaceuticals, and personal care products (PPCPs; Gambardella et al., 2021 and references therein), and inorganic and organic UV filters (Alijagic et al., 2020; Giraldo et al., 2017; Oliviero et al., 2017; Paredes et al., 2014). *P. lividus* is a species widespread in the coastal ecosystems of the Mediterranean Sea and Eastern Atlantic Ocean, an "ecosystem engineer" (Pagès et al., 2012) playing an important role in marine trophic webs. This species is also frequently encountered in the intertidal zone, which is intensively impacted by blue tourism (Boudouresque and Verlaque, 2020; Burak et al., 2004).

Investigations of the effects of organic UV filters (e.g., 4-methyl benzylidene camphor, BP-3, BP-4 and EHMC, ethylhexyl dimethyl *p*-aminobenzoic acid, and OCR) on *P. lividus,* revealed that these caused malformations of the sea urchin larvae in a similar way to those exposed to other stressors (e. g. trace metals, pesticides; Gambardella et al., 2021; Giraldo et al., 2017; Paredes et al., 2014). Conversely, a low impact on the embryonic development of *P. lividus* was observed when sunscreen products containing organic UV filters, such as diethylamino hydroxybenzoyl hexyl benzoate (DHHB), methylene-bis-benzotriazolyl tetramethylbutylphenol (MBBT), and ethylhexyl triazone (EHT), were tested (Corinaldesi et al., 2017) These results suggest that some chemical filters can have a lower impact than others, or even be eco-compatible.

In addition, previous research showed that bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT) and MBBT do not act as endocrine disruptors, nor do they cause any biological effects (i.e. mortality and growth rate) on *Tetraselmis* and *Artemia salina* (Thorel et al., 2020). These "new generation" organic UV filters (DHHB, MBBT, and EHT) have been already employed in commercialized formulations labelled as eco-friendly (Miller et al., 2021), although their effects on marine organisms belonging to different trophic levels have yet to be elucidated (Tovar-Sánchez et al., 2020a), especially when they interact with the other ingredients present in the whole sunscreen formulation. A number of studies have indeed tested UV filters as single molecules neglecting the potential synergistic effects of these molecules with other ingredients of sunscreen products (e.g., surfactants, emollients, fragrances), which might increase or modify the toxicity of every single ingredient (Tovar-Sánchez et al., 2019).

In the present work, we investigated the effects of novel formulations containing "new generation" organic UV filters, on the embryonic development of the sea urchin *P. lividus*. We tested six sunscreen products containing "next generation" organic UV filters that were characterized by different protection factors (SPFs). For the first time, we also used a multiple approach to assess the stress responses of the sea urchin exposed to sunscreens, by investigating the expression levels of 15 genes coupled with the analysis of the anomalies in the development of the larval stages.

2. Materials and methods

2.1. Sunscreen products

We selected six different sunscreens lotions purchased in Italy, which are characterized by a sun protection factor (SPF) ranging from 30 to 50+ and by a different composition in terms of ingredients such as preservatives, moisturizers, and organic UV filters some of which have been demonstrated to have a low impact on marine life (Corinaldesi et al., 2017; for details about sunscreens composition see Supplementary materials).

In particular, *Sunscreen 1 (SPF-30; SS1), Sunscreen 2 (SPF-50; SS2), Sunscreen 3 (SPF-50; SS3) Sunscreen 4 (SPF-50; SS4)* contained four organic UV filters (methylene bis-benzotriazolyl tetramethylbutylphenol (nano, MBBT), diethylamino hydoroxybenzoyl hexyl benzoate (DHHB), ethylhexyl triazone (EHT), bis-ethylhexyloxyphenol methoxyphenyl triazine (BEMT)). SS1 had the same ingredients as SS4 but with different SPF while SS2 and SS3 had the same composition but the latter did not contain perfume. *Sunscreen 5 (SPF-30; SS5)* and *Sunscreen 6 (SPF-*

Table 1
Composition of sunscreens tested.

INCI	SS1	SS2	SS3	SS4	SS5	SS6
methylene bis-benzotriazolyl tetramethylbutylphenol (nano)	+	+	+	+	+	+
diethylamino hydoroxybenzoyl hexyl benzoate	+	+	+	+	+	+
ethylhexyl triazone	+	+	+	+	+	+
bis-ethylhexyloxyphenol methoxyphenyl triazine	+	+	+	+	+	+
ethylhexyl salicylate	−	−	−	−	+	+
dicaprylyl carbonate	+	+	+	+	+	+
hydrogenated polyisobutene	+	+	+	+	−	−
glyceryl stearate	+	+	+	+	+	+
cetearyl alcohol	+	+	+	+	+	+
sucrose polystearate	+	+	+	+	−	−
dibutyl adipate	−	−	−	−	+	+
propylene glycol dicaprylate/dicaprate	−	−	−	−	+	+
propylene glycol	+	+	+	+	+	+
glycerin	+	+	+	+	+	+
xanthan gum	+	+	+	+	+	+
phenoxyethanol	+	+	+	+	+	+
citric acid	+	+	+	+	−	−
hydroxyacetophenone	+	+	+	+	+	+
decyl glucoside	+	+	+	+	+	+
disodium cetearyl sulfosuccinate	+	−	−	+	+	+
cetearyl glucoside	−	−	−	−	+	+
hydroxyethylcellulose	+	−	−	+	−	−
acrylates/palmeth-25 acrylate copolymer	−	−	−	−	+	+
caprylic/capric triglyceride	+	+	+	+	−	−
perfume	+	+	−	+	+	−
sodium gluconate	+	+	+	+	+	+
sodium hydroxide	−	−	−	−	+	+

50+; SS6) contained the organic UV filter ethylhexyl salicylate (ES) in addition to those present in S1–S4 (Table 1).

Furthermore, SS6 did not contain perfume unlike SS5 but both included ingredients such as emollients, texturizing, surfactants, and buffers lacking in the other sunscreen products.

The ordered complete list of ingredients for each sunscreen product and the relative proportion in the whole formulation is also reported in the Supplementary Material.

Sunscreen products were tested at the concentration of 50 μl L^{-1} (Corinaldesi et al., 2018), which is consistent with estimates of sunscreen products released by tourists during bathing and swimming along the Mediterranean coasts (see Supplementary Materials for details).

2.2. Sampling location, gamete collection, and embryo incubation with sunscreens

Adult specimens of *P. lividus* were collected during the breeding season between October 2020 and March 2021 by scuba-divers along the coast of the Central Adriatic Sea (43°37′11.29″N 13°31′52.9″E) and immediately carried to the laboratory in insulated bags. Animals were maintained for at least 1 week in tanks with circulating filtered seawater (FSW; 0.22 μm pore size) at 14 °C for allowing optimal adaptation. Gametes were collected by injecting acetylcholine chloride 0.5 M diluted in sterile SW filtered with 0.02 μm pore size Anotop® syringe filters (Whatman, Springfield Mill, UK) through the perioral membrane as described by Gambardella et al. (2013). For each experiment, eggs from different females were kept separated, washed three times, and maintained in sterilized glass beakers with FSW until use. Concentrated sperm was collected from the genital pores and maintained undiluted at +4 °C until use. Eggs were fertilized utilizing sperm-to-egg ratios of 1000:1 for both controls and treated embryos. Spawned gametes from three different male and three female specimens were pooled. Fertilization success was on average 99% and was checked by sampling three replicates of 100 eggs to observe the formation of the fertilization envelope with a microscope (Zeiss Axioskop-2 Mot with 10× magnification).

2.3. Effect of sunscreens on P. lividus embryonic development

The impact of sunscreens on embryonic and larval development was tested by exposing *P. lividus* fertilized eggs to six new formulations in final concentration (v:v) of 50 μL L^{-1}, defined according to the analytical procedures reported in Corinaldesi et al. (2017) and Danovaro et al. (2008).

Embryos were grown in sterile tanks filled with 50 mL of FSW, 750 eggs (15 eggs/1 mL FSW), and diluted sperms (1:1000 in FSW) each, and incubated at 18 °C in a controlled temperature chamber on a 12 h:12 h light: dark cycle following the protocol validated by International Organization for Standardization (ISO; (Falugi et al., 2008) until 48 h post fertilization (hpf) to develop *P. lividus* 4-arms larvae (Giudice, 1986)). Three replicated systems (n = 3) from treated and untreated systems (without the addition of sunscreens) used as controls, were collected 20 min after the addition of tested products (T_0), at 3 hpf corresponding to 16 cells stage, after 24 hpf corresponding to prism stage, and after 48 hpf corresponding to pluteus stage fixing samples with a solution of 4% paraformaldehyde (pH 7.4) and 70% ethanol. A total of 150 embryos or larvae for each sample were washed with filtered seawater and analyzed with a counting chambers Sedgewick Rafter (3 sub-replicates of 100 larvae each).

A total of 300 embryos for each system were evaluated under the microscope (Zeiss Axioskop-2 Mot with 10× magnification) to detect morphological abnormalities of larval development compared to controls. The degree of malformations on *P. lividus* developmental anomalies was evaluated based on the classification reported by Gambardella et al. (2021).

Different types of malformations could be identified: crossed, separated tip and fused arms, folded tip, fractured ectoderm, and undeveloped stages. Such malformations were classified according to the degree of larval alteration (level 0: normal development, level 1: incorrect location of skeletal rods, level 2: incomplete or absent skeletal rods, and level 3: development block at the 4-arms pluteus), to determine the degree of the sunscreen impact. Therefore, after 48 h of exposure (t_{48}), the frequency of anomalies for each degree of larval alteration was determined for calculating the index of sunscreen impact (ISI) for each sunscreen tested as reported by Corinaldesi et al. (2017). ISI index ranges from 0 (no impact) to 3 (high impact) along with the levels 1 (slight impact) and 2 (moderate impact).

Photos were taken using a Leica ICC50 W (Leica Microsystems) optical microscope equipped with a digital camera using the AirLab v2.0 application (Leica Microsystems) with 40× magnification for embryos and 10× for larvae.

2.4. RNA extraction and cDNA synthesis

About 3500 eggs in 50 mL of FSW were fertilized and sunscreens were added at 20 min post fertilization (mpf) in triplicate using 3 egg groups collected from 3 different females. Larvae were then collected at 48 h post fertilization (hpf) by centrifugation at 1800 relative centrifugal force (rcf) for 10 min in a swing-out rotor at 4 °C. Samples were washed with phosphate-buffered saline, then were stored in 700 μl of RNA*later*® (ThermoFisher scientific, Monza, Italy) at −80 °C until processing.

Total RNA was extracted using Aurum™ Total RNA Mini Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions, which ensures optimal quality of RNA for gene expression analyses (Ruocco et al., 2017). The amount of total RNA extracted was evaluated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, using a NanoDrop spectrophotometer (ND-1000 UV–Vis Spectrophotometer; NanoDrop Technologies, Wilmington, Delaware). The integrity of RNA extracted was evaluated by agarose gel electrophoresis.

For each sample, 350 ng of total RNA was retrotranscribed with an iScript™ cDNA Synthesis kit using C1000 Touch Thermal Cycler (Bio-Rad, Milan, Italy). To evaluate the efficiency of cDNA synthesis, a PCR was performed with primers of the reference gene, ubiquitin. The reaction was performed on the C1000 Touch Thermal Cycler (Applied Biosystem, Monza, Italy) in a final volume of 25 μl with 5 μl PCR MyTaq HS reaction buffer, 1 μl of cDNA template, 0.3 μM of each oligo, 0.5 μl of MyTaq HS (Meridian bioscience), and nuclease-free water. The PCR program consisted of a denaturation step at 95 °C for 1 min, 35 cycles at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 10 min.

2.5. Gene expression levels by real-time qPCR

Real-Time Quantitative polymerisation chain reactions (qPCR) were carried out with the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX96 Touch Real-Time PCR Detection System (BioRad) with an initial denaturation phase at 95 °C for 1 min, followed by 40 cycles of 15 s at 95 °C and 30 s at 60 °C. Melting curves ranging from 60 °C to 95 °C were evaluated in each reaction to check the specificity of the amplicons. Biological triplicates were considered for the qPCR analysis, and three technical replicates were analyzed for each biological sample. Diluted cDNA (1:2) was used as a template for qPCR reactions.

Changes in expression levels of 15 genes involved in the key stress response cellular pathways of *P. lividus* were analyzed. In particular, relative gene expression levels of Catalase (*CAT*), Multi Drug Resistance Protein 1 (*MDR1*), Manganese Superoxide Dismutase (*MnSOD*), Glutathione Peroxidase (*gpx*), Toll-Like Receptor 4 (*tlr4*), Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (*NF-kB*), heat shock protein 70 (*hsp70*), heat shock protein 60 (*hsp60*), heat shock protein 56 (*hsp56*), catalytic domain of the protein kinase superfamily (*p38 MAPK*), phosphatidylinositide 3-kinase (*pi3k*), 14-3-3 epsilon protein (*14-3-3ε*),

jun-like domain protein (*jun*), C-Jun N-Terminal Kinase (*jnk*), tank-Binding Kinase 1 (*tbk1*). The amplification reaction of these genes was done with the primers used in studies previously published (detailed information is shown in Supplementary Materials (Table S1)). The expression of each gene was analyzed and normalized against the housekeeping genes *Ubiquitin* and *PlZ12-1* (Costa et al., 2012; Romano et al., 2011). Data were analyzed using Bio-Rad CFX Maestro software 2.2 (Bio-Rad).

2.6. Statistical analysis

Morphological and gene expression data were analyzed by one-way ANOVA to identify differences between sunscreens and controls. Where significant effects occurred, Tukey's honest significant difference (HSD) test were carried out with Bonferroni adjustments to P values. Before results interpretation normality and homogeneity of variance assumptions were checked by means of Shapiro-Wilk and Brown-Forsythe tests respectively. The statistical software package Prism 9.0.1 (GraphPad Software, San Diego California USA) was used. Significant differences were considered when $p < 0.05$. All results are presented as mean ± SD.

3. Results

3.1. Effects of sunscreens on sea urchin larval development

The percentages of anomalous *Paracentrotus lividus* embryos detected after the addition of sperm (T_0) in the different treatments ranged from 1.1% to 4.1% with no significant differences compared to the control (1.1%; ANOVA $F_{6, 14} = 1.19$; p-value = 0.3624; Fig. 1A). At the cleavage stage, after 3 h of exposure (Fig. 1B), the numbers of abnormal embryos identified in the different treatments were on average 18.2% and resulted similar to those of the control (16.9%; ANOVA $F_{6, 14} = 0.99$; p-value = 0.4649). At the prism stage (24 h post fertilization; hpf), a significantly higher number of embryos with phenotypic defects was detected when they were exposed to SS3, SS5, and SS6 compared to the control (6.2%; $F_{6, 14} = 117.1$; p-value < 0.0001). In particular, SS3 caused 29.9% of abnormal embryos, which even increased to 89.7% and 100% when exposed to SS5 and SS6, respectively (Fig. 1C).

At the pluteus stage (48 hpf), a significant increase in the abnormalities was observed in the treatments SS1 (51.2%), SS2 (39.7%), SS3 (36.6%), SS5 (99.5%), SS6 (100%) with at least a p-value < 0.01 compared to control (18.4%; Fig. 1D). The different embryonic anomalies encountered as a result of the addition of sunscreens are illustrated in Fig. 2.

In particular, the observations on sea urchin larvae at T_{48} exposed to the sunscreens tested here induced severe malformations, affecting the apex and the arms and the entire larval anatomy (Fig. 3). Specifically, the observed anomalous plutei included crossed and/or separated skeletal tips at the hood apex (Fig. 3B–C), plutei with joined posterior (Fig. 3 D) or missing arms (Fig. 3 E), irregular shape with folded arms (Fig. 3F–G), early plutei (Fig. 3H), embryos whose development is blocked and compromised (Fig. 3I–J).

3.2. Index of sunscreen impact (ISI)

Based on the classification of the abnormal larvae exposed to SS1, ca. 25% of the phenotypic anomalies fell in level 2 and ca. 16% of them were classified in the level 3 with a final index of sunscreen impact (ISI) of 1.08 (moderate impact; Fig. 4 and Table S2). After exposure of embryos to SS2, SS3, and SS4, ISI ranged from 0.43 to 0.81 resulting in a low environmental impact with the fraction of normal plutei ranging from 60.3% to 71.3%. SS5 and SS6 determined 99.5% and 100% of

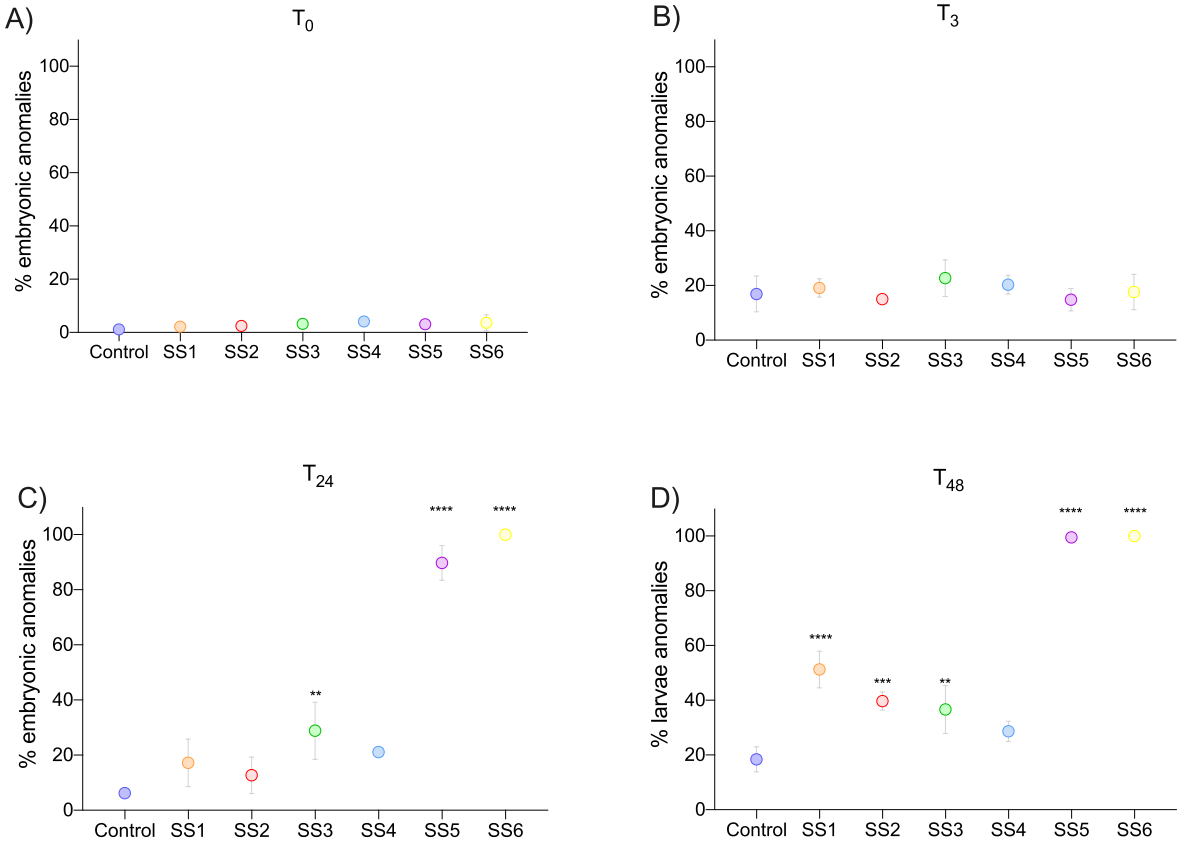

Fig. 1. Percentage of abnormal embryos in control embryos and larvae (embryos grown without sunscreens) and treated embryos and larvae after different exposure times: 20 min (A), 3 h (B), 24 h (C), and 48 h (D) post fertilization. One-way ANOVA (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Data are showed as mean ± SD.

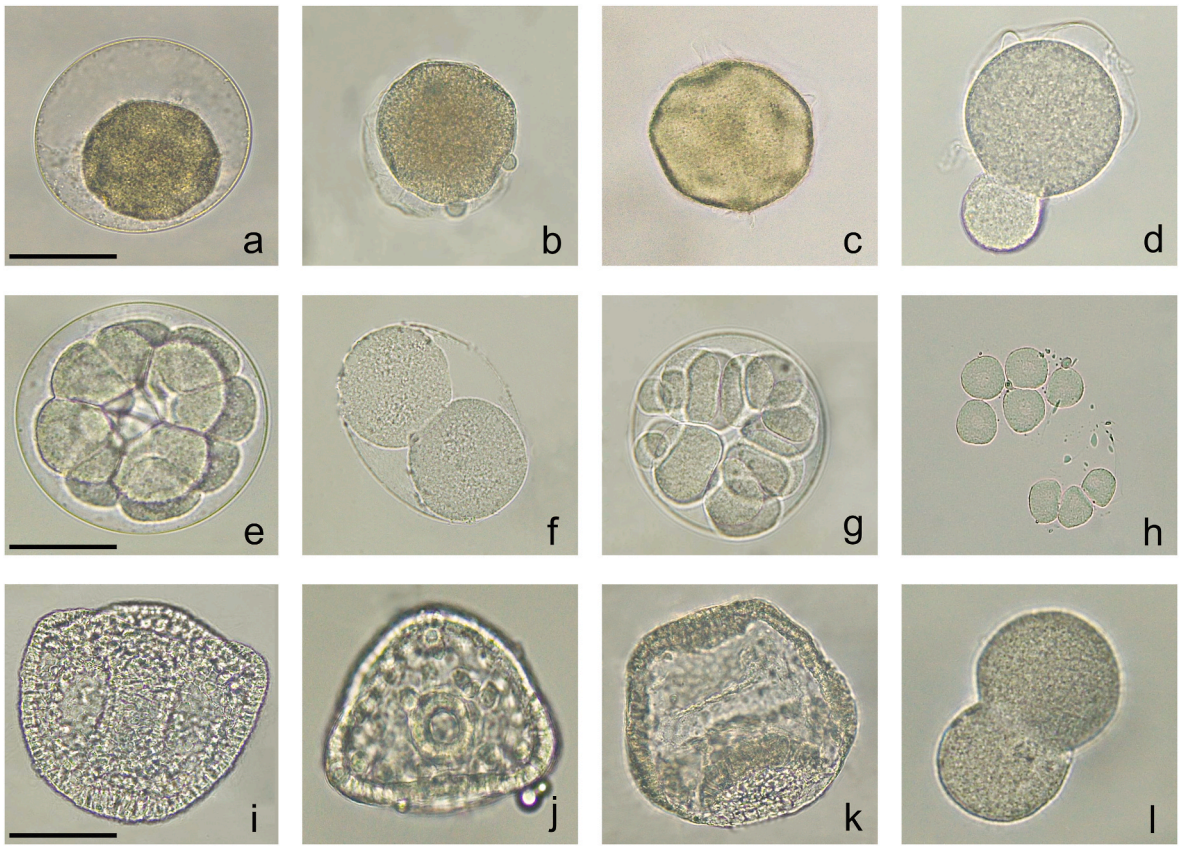

Fig. 2. Anomalies observed in *Paracentrotus lividus* embryos exposed to 50 μL L^{-1} of sunscreens (b-d, f-h, j-l) compared to controls (i.e., not exposed to sunscreens; a-e-i). Embryos with vesicles along the fertilization envelope (b), without fertilization membrane (c), with damaged eggs (d) immediately after sunscreens additions (T_0); embryos at two-cell stage (f), asymmetric division (g) absence of membrane with consequent dispersion of the blastomeres (h) after 3 h of exposure (hpf); embryos with irregular gastrula (j), with altered primary mesenchyme cells migration (k) and exogastrula (l) after 24 (hpf). Photos were taken with Leica ICC50 W (Leica Microsystems) with 40× magnification. Bars, 50 μM.

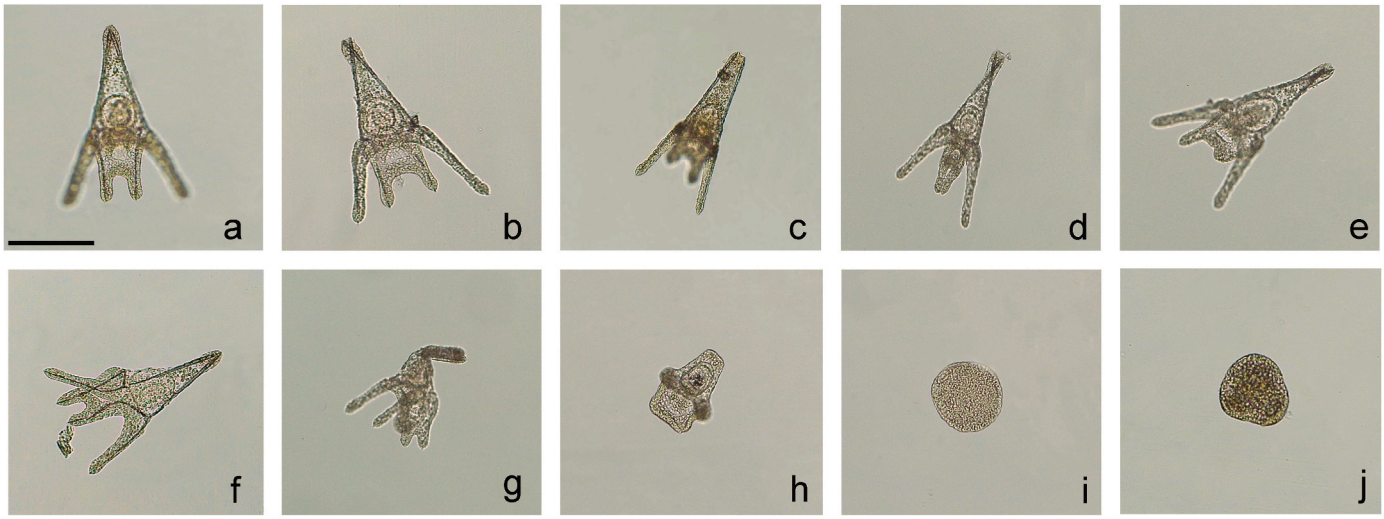

Fig. 3. Malformations of *Paracentrotus lividus* larvae observed after 48-h exposure of embryos to sunscreens (50 μL L^{-1}). Photos were taken with Leica ICC50 W (Leica Microsystems) with 10× magnification. Bars, 50 μM.

anomalous larvae, respectively, which were classified as level 3 resulting in an ISI of ca. 3 (high impact).

3.3. Effect of sunscreens on gene expressions in P. lividus

Overall, the exposure to sunscreen products caused significant responses (One-way ANOVA) in terms of genes' expression (*hsp70*, *hsp60*, *tbk1*, *p38 MAPK*, *gpx*, *14-3-3ε*) (Fig. 5 and Supplementary Table S3). In particular, the *hsp70* exposed to SS1 and SS2 responded with a significant increase of the expression levels than in the control ($p < 0.001$). The expression of other 3 genes (*hsp60*, *tbk1*, and *14-3-3ε*) exposed to SS2 increased significantly ($p < 0.05$). At this stage, SS3

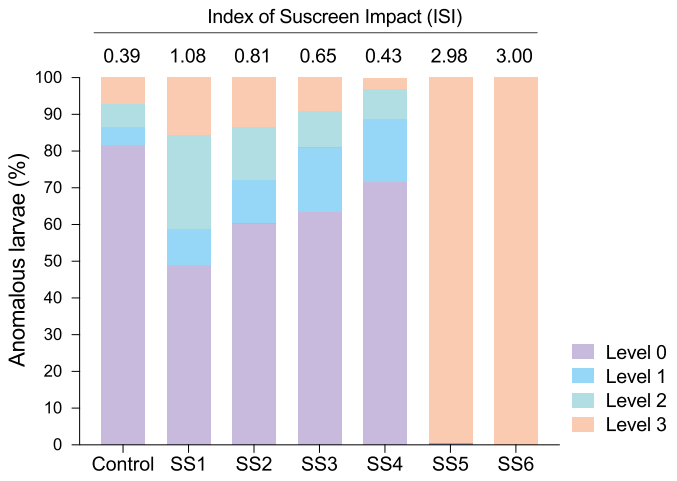

Fig. 4. Index of Sunscreen Impact (ISI) for each sunscreen determined on the types of anomalies identified in larvae and classified according to the level of larval alterations (Corinaldesi et al., 2017; Gambardella et al., 2021). Data and environmental impact of each sunscreen were reported in Supplementary Table S2.

induced significant changes in the expression levels of *14-3-3Ɛ* (*p-value* < 0.05). SS4 did not show any significant changes in the expression levels among stress genes analyzed. The expression levels of *p38 MAPK* and *gpx* changed also under the 48-h exposure of embryos to SS5 and SS6 (*p-value* at least <0.05).

Considering the fold change of genes analyzed in the plutei exposed to six sunscreens in comparison with the control, we found that SS1 treatment induced an increase in the expression levels of *hsp70* with fold changes of 28.13. SS2 determined the reduction of expression levels of *14-3-3 Ɛ* (0.40-fold change), and an increase in the levels of *hsp70*, *hsp60,* and *tbk1* (44.98, 3.98, 1.67-fold changes, respectively) compared to the control. The embryos exposed to SS3 showed a down-regulation of *14-3-3 Ɛ* compared to control (0.43-fold change).

Finally, the SS5 and SS6 treatments induced a down-regulation compared to the control of two genes: *gpx* (in both cases 0.41-fold change), and *p38 MAPK* (0.45 and 0.46-fold changes, respectively).

4. Discussion

The Mediterranean Sea is among the main tourist world destinations and attracts every year hundreds of millions of tourists (ca. 400 million in 2019, with a forecast of 626 million per year by 2025; Fosse et al., 2021; World Tourism Organization, 2021), of which at least 50% spend their vacations at sea (Fosse et al., 2021). We estimated that from 10,000 to more than 15,000 t of sunscreens are released every year, corresponding to a final input of 43–679 μL sunscreen L^{-1} into the Mediterranean coastal waters (see Supplementing Online Materials for details on the calculations). Thus, the concentration used in our experimental systems (50 μL L^{-1}) is close to the most conservative estimate. The values reported here, however, may be an underestimate as we assumed that only 25% of the sunscreens are directly released and many other personal care products can spread through beach showers, industrial discharges, and wastewater treatment plants reaching coastal waters within hours (Casas-Beltrán et al., 2021; Downs et al., 2022; Ramos et al., 2016).

Previous investigations in the natural environment measured the concentrations of "old generation" UV filters (e.g., oxybenzone) in seawater, reporting values up to a few mg L^{-1} (Downs et al., 2016; Tovar-Sanchez et al., 2020c), which are in the same order of magnitude of the UV filter concentrations used in our study (from 0.5 to 4 mg L^{-1}) and already used to test the effects of "old generation" UV filters on marine organisms (Lozano et al., 2020b and references therein). As far as the concentrations of triazine UV filters in marine coastal ecosystems are concerned, the limited information available reported concentrations of hundreds of μg L^{-1} or less (Fagervold et al., 2019). However, these values may be underestimated as the decay time and the decay products of these molecules in the seawater and their toxicity and persistence have to be investigated yet (Du et al., 2022; Tovar-Sánchez et al., 2020a).

In the present study we reported that sunscreen products determined significant embryos' anomalies (range ca. 35–50%), with the highest impacts determined by the products SS5 and SS6 (90–100% of embryos' anomalies within 24 h from the exposure). In some cases, the exposure to sunscreen caused the death of the larvae (e.g. damaged/degraded larvae with degenerating tissues; Gambardella et al., 2021). Only the product SS4 did not cause a significant increase in the number of anomalies. The larval anomalies observed after exposure to the "new generation" UV filters in some cases were potentially reversible (i.e. larvae of smaller size than the controls, or with skeletal rods of the anterior arms fused, or with crossed tips; Gambardella et al., 2021) if the larvae were placed back in natural seawater without sunscreens.

The anomalies observed in *P. lividus* embryos have been consistently reported also in other studies investigating the impact of organic and inorganic contaminants (Gambardella et al., 2021; Varrella et al., 2016, 2014) indicating that some sunscreens can have the same impact of these classical contaminants, causing primary mesenchymal cell migration, alteration of skeletogenesis, changes in the location of skeletal rods and alterations of the gene-regulatory system (Adonin et al., 2021; Gambardella et al., 2021).

Identifying which of the ingredients of a personal-care product are responsible for the negative impact on marine organisms is a complex task as the effect of each ingredient does not always coincide with the synergistic effect with other ingredients of that product (Kudłak et al., 2022; Park et al., 2017).

Several studies reported that "old generation" organic UV filters at the concentrations ranging from 100 μg L^{-1} to 5 mg L^{-1} can exert negative effects on a wide array of marine organisms including *P. lividus* (Fivenson et al., 2021; Giraldo et al., 2017; Lozano et al., 2020b; Paredes et al., 2014). Conversely, recent investigations on the effects of "new generation" organic UV filters (MBBT, DHHB, EHT, BEMT) revealed the lack of significant biological effects on different marine organisms, such as corals (Stien et al., 2020) crustaceans (Thorel et al., 2020), fishes, and algae (Slijkerman and Keur, 2016) at concentrations similar to those tested in the present work (up to 2 mg L^{-1}).

The formulations SS1, SS2, SS3 and SS4 were based on the same eco-friendly UV filters (MBBT, DHHB, EHT, BEMT) with the only difference that SS1 was characterized by the lowest SPF (i.e. 30 vs. 50 or 50+). In addition, SS1 contained perfume like SS2 and SS4 (but unlike SS3), and the surfactant disodium cetearyl sulfosuccinate and the thickening agent hydroxyethylcellulose like SS4 (but unlike SS2 and SS3).

Since SS1, which showed moderate impact, contained a lower concentration of UV filters (overall 13% of the whole product) than the SS2, SS3 and SS4 (overall 22% of the whole product) but with a lower impact, we conclude that the organic filters present in the lotions tested, were presumably not responsible for the different biological response observed. Furthermore, SS1 contained the same concentration of the surfactant disodium cetearyl sulfosuccinate (1%) than SS4, allowing us to exclude also a relevant role of this component in affecting larval development although previous studies hypothesize its dose-dependent effect on the larval development of the sea urchin like previously documented for other anionic surfactants on marine organisms (Cserháti et al., 2002). Another difference in SS1 compared to SS4 could be due to the presence of hydroxyethyl cellulose, a hydrophobic modification of hydroxyethyl cellulose polymers recently documented on aquatic biota (Simões et al., 2021). Although some fragrances have been recently reported as harmful to several aquatic organisms (Bom et al., 2019; Picone et al., 2021), we can reasonably exclude that the impact of SS1 on the embryonic and larval development of *P. lividus* is due to the

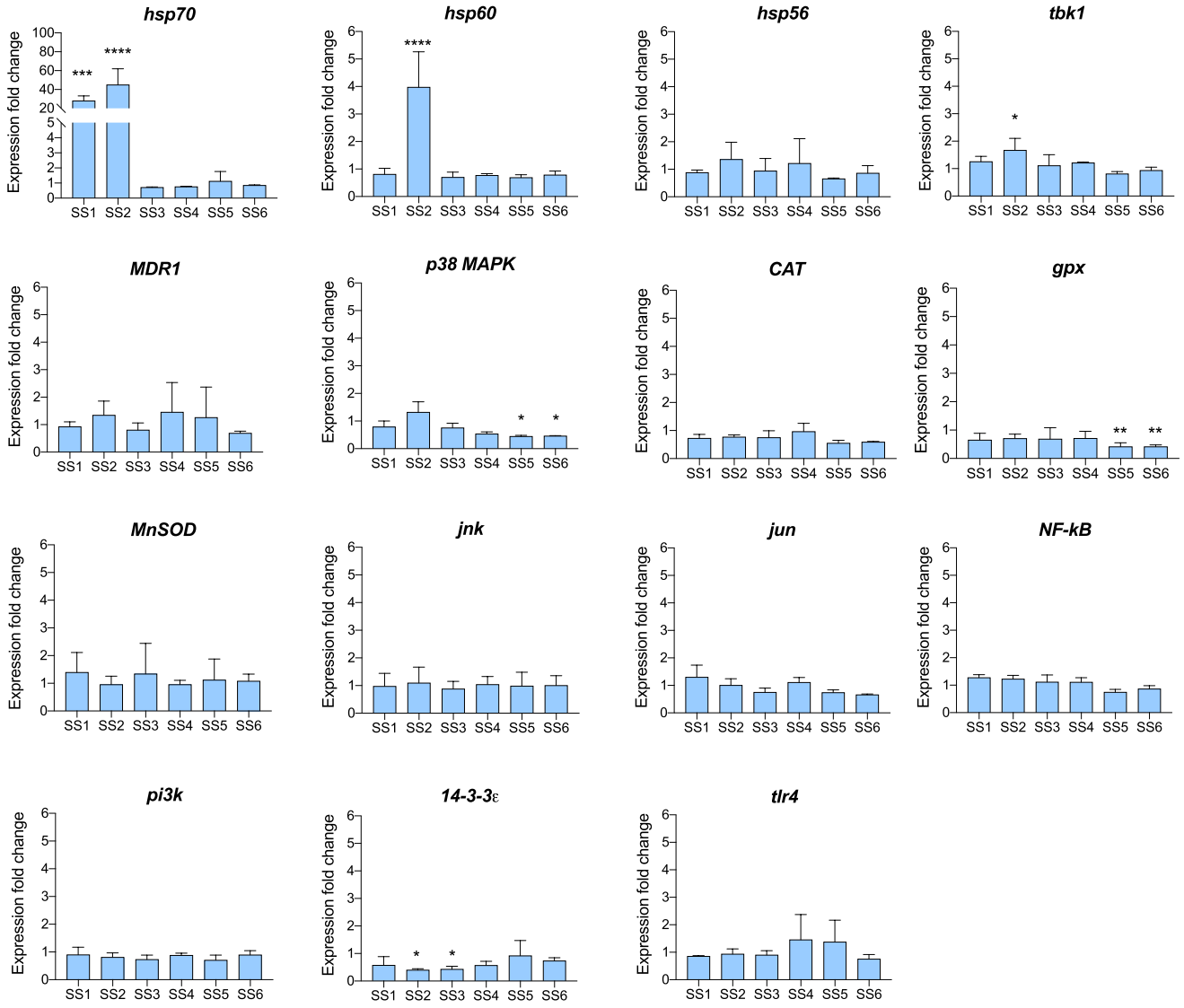

Fig. 5. Expression fold change of stress genes in *Paracentrotus lividus* larvae exposed to sunscreens. Each bar represents the mean of three independent qPCR analyses ±SD, using cDNAs obtained by three independent systems. Data were significantly different according to the one-way analysis of variance ANOVA, followed by Tukey's test with Bonferroni's adjustments. Significant variations are indicated with asterisks: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

fragrances, as they were also present at an equal concentration (0.5%) in SS2 and SS4 (showing a lower impact on embryos and larvae). However, the identification of the ingredients responsible for the higher impact of SS1 should be further investigated also by assessing their combined interaction, their degradation products and persistence in seawater.

The most negative effects on the early developmental stages of *P. lividus* were detected for SS5 and SS6 (index of sunscreen impact, ISI = 3). These sunscreens contained, in addition to the same kind of organic UV filters of SS1, SS2 and SS4, the ethylhexyl salicylate (ES), and other excipients, which apparently acted synergistically causing the most severe biological consequences.

The biological effects of ES on marine organisms are still controversial. Some studies, indeed, reported that this UV filter can have negative effects on the metabolic activity and growth rate of the algae *Tetraselmis* sp., while other studies observed only limited impacts on *Artemia salina* and tropical corals (Danovaro et al., 2008; Thorel et al., 2020).

Our results also suggest that sunscreen products based on the UV filters MBBT, DHHB, BEMT, and EHT (especially, SS4, SS3, and SS2) are potentially more eco-compatible than those also containing ES, and other ingredients such as emollients (e.g. dibutyl adipate, 3%) and texturizing compounds (e.g. sodium polyacrylate, 0.9%; acrylate/palmet-25 acrylate copolymer, 0.6%) present in the composition of SS5 and SS6. Compounds such as copolymers of acrylic acid and dibutyl adipate are toxic or slightly toxic to aquatic organisms (algae and crustaceans; (Duis et al., 2021) and might have contributed to the severe toxicity observed in embryos and larvae (ECHA, 2006)).

Consistent results were obtained from the molecular analyses on genes' expression.

Here, the lowest effects were caused by SS4, while SS1 and SS2 caused an increase in the expression of the *hsp70* gene. Genes encoding for the heat-shock proteins are involved in resistance mechanisms to apoptosis, and their increased expression may be a response to increase the survival of stressed marine organisms (Bonaventura et al., 2005; Roccheri et al., 2004).

SS2 and SS3 determined a transcriptional decrease of *14-3-3 Ɛ*, an adapter protein that plays a key role in a variety of physiological processes (Morrison, 2009). It has been reported that the overexpression of the *14-3-3 Ɛ* gene in *P. lividus* embryos exposed to high UVB radiations can determine a switch to apoptotic pathways within their cells (Russo

et al., 2014, 2010), whereas its downregulation, when *P. lividus* embryos are exposed to different contaminants, can be responsible for reversible malformations on spicules and/or arms (Bonaventura et al., 2022; Chiaramonte et al., 2020; Varrella et al., 2016). The SS2 sunscreen also induced upregulation of *tbk1*, involved in a complex immune defense system to protect the *P. lividus* from pathogenic infections (Chiaramonte et al., 2019).

The SS5 and SS6 products, which caused the worst effects in terms of larval anomalies, down-regulated the expression of *p38 mapk* and *gpx* genes. The *p38 mapk* gene is involved in a wide variety of cellular processes such as inflammation, cell death, and differentiation (Zarubin and Han, 2005), and its modulation indicates skelotogenesis impairment, which may limit the lifespan of embryos, triggering cell-cycle arrest and senescence (Ito et al., 2006; Pinsino et al., 2015). These results provide compelling molecular confirmation of the morphological anomalies observed in the larval development of *P. lividus:* the sunscreens SS1, SS2, and SS3, which caused a slight/moderate impact, did not alter the expression of *p38 mapk,* essential also for a correct skeleton formation (Casano et al., 2008), while this was not the case for SS5 and SS6.

Finally, the *gpx* gene is involved in the defense mechanism against reactive oxygen species (ROS) and contributes to cellular detoxification (Ighodaro and Akinloye, 2018). Therefore, the downregulation of the *gpx* expression in sea urchin larvae exposed to SS5 and SS6 may indicate a reduction in ROS detoxification that can ultimately compromise organisms' survival (Ighodaro and Akinloye, 2018).

5. Conclusions

Among all of the eco-friendly sunscreen products tested, over 25% has been found to be toxic to marine life and a large portion of them has not been tested at all for their eco-compatibility (Lozano et al., 2020b), so that most of the declarations/statements of eco-friendly products are based on assumptions, the presence or not of specific ingredients with known ecological impact or, even less, only on the biodegradability of their packaging.

Overall, the results presented here highlight the importance of testing the entire formulation of the sunscreen products rather than just the presence of specific UV filters to ascertain the sunscreen eco-compatibility. In addition, even a single side ingredient, such as an excipient, which singularly does not cause any significant effect on marine organisms and their habitats, can have significant impacts due to the synergistic action with other ingredients of the formulation (Blasco et al., 2020).

Finally, the simple replacement of one or more UV filters or ingredients of known toxicity (as increasingly observed in various sunscreen brands) does not justify the "eco-friendly" claim of a personal care product.

The Index of Sunscreen Impact (ISI) and its combination with the molecular responses in embryos can represent a reliable and advanced tool to assess in the future the eco-compatibility of sunscreens with marine life.

Author statement

Stefano Varrella: Investigation, Methodology, Data curation, Visualization, Writing - original draft; Writing - review & editing; Conceptualization. **Roberto Danovaro:** Funding acquisition, Writing - review & editing, Conceptualization. **Cinzia Corinaldesi:** Funding acquisition, Project administration, Investigation, Supervision, Writing - review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120212.

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