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Effects of four food dyes on development of three model species, *Cucumis sativus*, *Artemia salina* and *Danio rerio*: Assessment of potential risk for the environment☆

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ABSTRACT

Food dyes, or color additives, are chemicals added to industrial food products and in domestic cooking to improve the perceived flavor and attractiveness. Of natural and synthetic origin, their safety has been long discussed, and concern for human safety is now clearly manifested by warnings added on products labels. Limited attention, however, has been dedicated to the effects of these compounds on aquatic flora and fauna. For this reason, the toxicity of four different commercially available food dyes (cochineal red E120, Ponceau red E124, tartrazine yellow E102 and blue Patent E131) was assessed on three different model organisms, namely *Cucumis sativus*, *Artemia salina* and *Danio rerio* that occupy diverse positions in the trophic pyramid. The evidence collected indicates that food dyes may target several organs and functions, depending on the species. *C. sativus* rate of germination was increased by E102, while root/shoot ratio was ~20% reduced by E102, E120 and E124, seed total chlorophylls and carotenoids were 15–20% increased by E120 and 131, and total antioxidant activity was ~25% reduced by all dyes. Mortality and low mobility of *A. salina* nauplii were increased by up to 50% in presence of E124, E102 and E131, while the nauplii phototactic response was significantly altered by E102, E120 and E124. Two to four-fold increases in the hatching percentages at 48 h were induced by E124, E102 and E131 on *D. rerio*, associated with the occurrence of 20% of embryos showing developmental defects. These results demonstrated that the food dyes examined are far from being safe for the aquatic organisms as well as land organisms exposed during watering with contaminated water. The overall information obtained gives a realistic snapshot of the potential pollution risk exerted by food dyes and of the different organism' ability to overcome the stress induced by contamination.

1. Introduction

Food dyes are substances deliberately added to food and beverages, and even to pharmaceuticals and cosmetics, to impart color. Available as liquids, powders, gels and pastes, dyes make products aesthetically more attractive, influencing the perceived flavor and simulating a more natural color. The international health authorities such as European Food Safety Authority (EFSA), Organization for Economic Cooperation and Development (OECD) and World Health Organization (WHO) are engaged in security certification of natural or synthetic food dyes being widely available and used by consumers. Besides, the food dyes are also subjected to strict legislative provisions (see, for example, the regulation EC no 1333/2008 and EC no 257/2010). Interferences with behavior in children have been determined since the late '70s (Weiss et al., 1980), later confirmed in laboratory animals such as rat (Kamel and El-lethey, 2011) and mouse (Tanaka, 2006), in which neurotoxicity was

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demonstrated (Gao et al., 2011). More recently, the increasing use of synthetic azo-dyes (Feng et al., 2012) has focused safety concerns toward cytotoxicity (Mpountoukas et al., 2010), genotoxicity and mutagenicity (EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), 2013) and on possible interference with the immune (Kus and Eroglu, 2015) and reproductive (Mehedi et al., 2009) systems. Accumulation in tissues and bioenergy effects have also been demonstrated (Abe et al., 2018).

As predictable, lists of common side effects are now available for humans and usually used animal model species (Vliet, 2011); however, these efforts have increased rather than reduced the uncertainty on effects, often giving contrasting results even in closely related species (Shimada et al., 2010; Thomas and Adegoke, 2015). Few studies are also available on plants. Test-organisms such as *Allium cepa* L. and *Brassica campestris* L. have been treated with different food dyes and the toxicity assessed on root development and cell proliferation (de Oliveira et al., 2013; Dwivedi and Kumar, 2015; Marques et al., 2015). Studies on dyeing factory effluents have demonstrated inhibition of seed germination, stem elongation and root development in maize (Nirmalarani and Janardhanan, 1988; Puvaneswari et al., 2006).

Despite their evident potential toxicity, the global food colors market, estimated at USD 1.8 billion in 2016, is expected to grow by about 6% per year in the next decade ("Global Food Colors Market Size & Share|Industry Report, 2018–2025," 2018). A growing consumption means an increasing release in the environment, particularly in water bodies and watered soils, of both dyes and their biotransformed derivatives. Therefore, a potential interference of food dyes on wild flora and fauna cannot be neglected.

In opposition to the widespread knowledge on some classes of pollutants, as for example the heavy metals (An et al., 2004; Avallone et al., 2015, 2017; Favorito et al., 2017; Ferrandino et al., 2009; Sorrentino et al., 2018), at present scarce attention has been directed to the possible risks associated to the massive dissemination in the water environment of such huge amount of food dyes. The main reason is probably the common thinking that exposure in nature is not relevant due to dilution; however, for aquatic organisms, the exposure might be relevant being continuous, from *ovo* to death, and multiple, being water contaminated by several different colorants.

Therefore, in order to give a comprehensive insight of the possible consequences of aquatic dye contamination on different biological targets, the developmental toxicity of four common food dyes was determined in laboratory toxicity tests in three representative models: *Cucumis sativus* L., an orchard species potentially exposed to contaminated watering and two aquatic animal models, the nauplii of the marine Crustacea *Artemia salina* L. and the embryos of the freshwater teleost *Danio rerio* (Hamilton 1882).

The food dyes selected for this study are common shelf products, used to prepare domestic foods, mainly for kids. One, cochineal red E120, is natural and three, Ponceau red E124, tartrazine yellow E102 and Patent blue E131, are synthetic. Concentrations used in the toxicity tests were the same suggested for cooking (600 mg/500 ml milk or cream) and higher than those present in water since the aim was to identify their potential targets of toxicity. The end-points considered were, for *Cucumis sativus*, the percentage of germination, root and shoot lengths (Motta et al., 2016), seed coat morphology in germinated seeds, total chlorophylls (a+b), carotenoids (x+c) and antioxidant concentration (Arena et al., 2013, 2017). The end-points for *Artemia salina* were mortality (Ates et al., 2013), reduced mobility (Kokkali et al., 2011), the occurrence of developmental defects (Motta et al., 2016) and changes in the phototactic response (Bartolomé and Sanchez-Fortun, 2005). For *Danio rerio* embryos, the hatching time, the heart rate (Hill et al., 2005) and the occurrence of developmental defects (Lancieri et al., 2002) were determined.

The information obtained provides a realistic snapshot of the potential pollution risk associated with the spread of food dyes in the different compartments of the environment and suggests a more careful consumption of these products always superfluous and overused in human diet.

2. Materials and methods

2.1. Water sample preparation

Commercially available food dye solutions, containing cochineal red E120, Ponceau red E124, tartrazine yellow E102 or Patent blue E131, were diluted in mineral water for human consumption at a final concentration of 1.2 g/L. The four solutions were prepared and immediately used to water the seeds. The water salinity for *Artemia salina* and *Danio rerio* was adjusted according to the species requirement as specified in Krishnaraju et al. (2005) and Westerfield (2000) respectively. The control for each model species was represented by the organisms (seeds and animals) not exposed to dyes but only to mineral water considered pure from contaminants.

2.2. Seeds germination tests

Cucumis sativus is widely used as an efficient plant test organism for toxic effect evaluation of metals or chemical compounds (An et al., 2004; Wang et al., 2001; Motta et al., 2016). *Cucumis sativus* seeds (germination reported in label to exceed 95% of seeds) were surface-sterilized by washing few seconds in 5% H_2O_2, rinsed in distilled water and arranged randomly in groups of 13, in 90 mm Petri dishes containing tissue paper wetted with 8 ml of mineral water or water added with the four food dyes. The Petri dishes, set in triplicate, were incubated at 22 °C in a climatic chamber and examined daily; when needed, few drops of distilled water were added to the tissue paper to preserve the humidity. The germination experiment was repeated five times, for a total of 195 observations (13 seeds × 3 petri dish × 5 replicates).

For *C. sativus* the following end-points have been considered: daily percentage of germinated seedlings (presence of a visible radicle, n = 195), the primary root and shoot lengths (n = 25); the root/shoot ratio at 7 days from seeding (n = 25). The measures were made on digital photos of randomly chosen control and treated seedlings and analyzed using the software Image J 1.45.

2.3. Seed coat structure

Germinated seeds were collected and fixed according to Ciancio et al. (2008). Briefly, the samples were prefixed in 4% glutaraldehyde and 0.05% OsO_4 in 0.2 M cacodylate buffer, pH 7.2 for 10 min; fixed in 4% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2, for 1 h at 4 °C and post-fixed in 1% OsO_4, in cacodylate buffer, for 1 h. They were dehydrated in a graded series of ethanol and embedded in Epon resin. Semi-thin sections were prepared for light microscopy observations after staining with 1% toluidine blue in 1% sodium tetraborate buffer to show general morphology.

2.4. Photosynthetic pigment content and antioxidant capacity determination

Photosynthetic pigment content and antioxidant activity are considered a proxy of plant health status and may give information about the mechanisms involved in the plant response to stress (Gonçalves et al., 2007; Puvaneswari et al., 2006). For this reason, total chlorophyll and carotenoid content were determined as

reported in Arena et al. (2014). Briefly, pigments were extracted in ice-cold 100% acetone and centrifuged at 5000 rpm for 5 min (*Labofuge GL, Heraeus Sepatech*, Hanau, Germany). The absorbance of the supernatants was quantified by a spectrophotometer (*UV-VIS Cary 100*, *Agilent Technologies*, Palo Alto, CA, USA) at 470, 645 and 662 nm, according to Lichtenthaler (1987). Measurements were carried out on 8 leaves for each different treatment; chlorophyll and carotenoid concentration were expressed in $\mu g/cm^2$. The experiment was repeated two-fold for a total of 16 measurements.

In order to evaluate the antioxidant capacity, the ferric reducing/antioxidant power assay (FRAP) was performed according to George et al. (2004). An amount of 250 mg of fresh sample was ground in liquid nitrogen and treated with methanol/water (60/40, v:v) solution. Samples were then centrifuged, and the supernatant was collected for the assay. The FRAP reagent was added to each sample extract and the mixture incubated in darkness for 1 h. The sample absorbance was read by a spectrophotometer (UV-VIS Cary 100) at 593 nm. Measurements were carried out on 8 leaves for treatment, and total antioxidant capacity was quantified and expressed as μmol Trolox equivalents mg^{-1} FW by using a Trolox standard curve. The experiment was repeated two-fold for a total of 16 measurements.

2.5. Toxicity tests on Artemia salina larvae and naupliar phototactic response

Nauplii of *Artemia salina* have been considered a reliable and accurate invertebrate model for research and applied toxicology for more than four decades (Persoone and Wells, 1987). In this study, *Artemia salina* cysts were incubated in artificial seawater for 24 h at 22 °C; at hatching the nauplii were collected with the aid of a Pasteur pipette and a light source (Solis et al., 1993). About 500 nauplii [instar I stage; (Sorgeloos et al., 1978)] were transferred in a dish containing 30 ml of one of the solutions to be tested (mineral water as control and mineral water with one of the four food dyes). Incubations were carried out for 7 days, at 22 °C, under constant aeration with a 16 h light photoperiod; if necessary, few drops of mineral water were added to adjust for evaporation loss. Animals were fed ad libitum with unicellular algae and yeast (Hannas et al., 2010). The experiments were carried out in quadruplicate using different batches of cysts. Mortality percentage in control samples never exceeded the 10% at 2 days (Vanhaecke et al., 1981).

Three different end-points were considered. Mortality (acute effects) and immobility (sub-acute effects) were determined daily on 1 ml samples of naupliar suspensions. Under a dissecting microscope, the total number of nauplii present was recorded together with the number of death or poorly motile nauplii. These were intended as animals not swimming or moving appendages for 10 s of observation (Piazza et al., 2014). The structural alterations were assessed by light microscopy at the day 5, after counting, fixing nauplii in 2% formaldehyde for 20 min, thoroughly washed in ethanol 75% (Motta et al., 2016).

For the phototactic response, a testing chamber (16 × 2 × 2 cm) was set, which allowed testing the increased density of a population (5000–7000 individuals in 25 ml seawater) of instar I stage nauplii (Sorgeloos et al., 1978) towards a source of light focused on one extremity in a dark box. After a 10 min period of acclimation in the dark (during which nauplii distribute randomly in the chamber), the light was switched on to induce the naupliar phototactic response. Density was measured at time zero (dark density) and after 20 min of illumination, both at the illuminated (L) and the dark (D) extremities. A density increase in L would indicate a positive phototactic response, while a density increase in D would indicate a negative phototactic response. The response was quantified as the ratio between the final and initial density. The test was performed on control nauplii maintained in pure seawater and on nauplii pre-exposed for 48 h to each of the four food dyes.

2.6. Toxicity tests on Danio rerio embryos

Danio rerio embryos are considered a suitable vertebrate model for toxicological investigations of chemicals (Nagel, 2002) due to their fast development and transparent eggs (Hill et al., 2005).

In this study, embryos, generated from healthy adult fish, were collected with a siphon from the fish tanks and housed in dishes containing E3 medium in a water bath at 28.5 °C (Westerfield, 2000). After 6 h from fertilization (hpf), 50 embryos at the shield stage were randomly distributed into plates containing 10 mL of the four solutions to be tested or, for the control group, containing E3 medium. Embryos development was followed under a Leica Zoom 2000 stereomicroscope, at a magnification of 10× and 20×, for 24, 48 and 72 h after the beginning of the treatment. Mortality, hatching time and morphological alterations were recorded. The heart rate was assessed 72 h after the beginning of treatments and expressed as beats per minute (Monaco et al., 2017a). All the experiments were carried out in quadruplicate.

2.7. Statistical analysis

Statistically significant differences among treatments were assessed using one-way ANOVA (McDonald, 2008) followed by Holm Sidak test for multiple comparison tests with the aid of Graph Pad Prism 7 (GraphPad Software, La Jolla California USA, www.graphpad.com). Time course data of *Cucumis sativus* seedlings germination, and *A. salina* nauplii mortality and motility percentages were fit to a logistic model. The significant differences between treated and control curves were tested via a sum-of-squares F test. Minimum accepted significance level was $p < 0.05$. Arcsine transformation was applied to percent values.

3. Results

3.1. Effect of food dyes on Cucumis sativus

3.1.1. Germination percentage

All seeds were germinated after 72 h, both under control condition and in presence of dyes. However, a significant ($p < 0.05$) reduction of the time to get a 50% germination (T_{50}) was observed in seeds exposed to yellow E102 (Fig. 1).

3.1.2. Seed coat structure

Coats obtained from control seeds and from seeds exposed to the four food dyes showed the same gross morphological organization of the outer integument and no significant differences in thickness (Fig. 2A–E).

3.1.3. Root and shoot development

One week after germination, seedlings treated with blue E131 showed longer ($p < 0.05$) roots compared to control and other treatments, whereas no significant difference was found among treatments in shoot length. Moreover, blue E131 seedlings exhibited a root/shoot ratio comparable to that of control while for yellow E102, red E120 and red E124 values were significantly lower compared to control ($p < 0.05$; Table 1).

3.1.4. Chlorophyll, carotenoid and antioxidant activity

The total chlorophyll ($a+b$) and carotenoid ($x+c$) contents and total antioxidant capacity in seedlings of *Cucumis sativus*, at one week after germination, are reported in Table 1. Total chlorophyll and carotenoid amount significantly ($p < 0.05$) increased in red

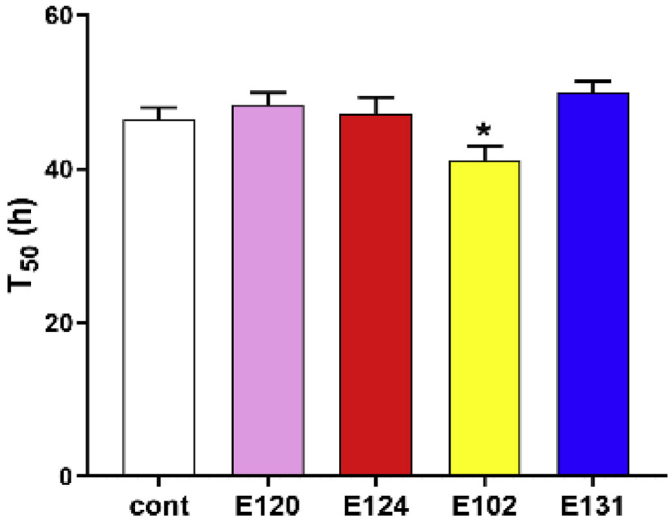

Fig. 1. Effect of the four food dyes (E120, E124, E102, E131) on the time (h) to obtain a 50% of *Cucumis sativus* seedlings germinated (T50). Data are means ± SE (n = 75). A statistically significant difference (*p < 0.05) is evident for tartrazine yellow E102.

E120 and blue E131 treated seedlings compared to the respective controls. The other food dyes did not determine any significant change compared to controls. The antioxidant activity significantly ($p < 0.05$) decreased in all samples exposed to food dyes.

3.2. Effects of food dyes on Artemia salina nauplii

3.2.1. Mortality, low mobility and anatomical malformations

A significant increase in mortality occurred since day 4 among nauplii exposed to red E124 (Fig. 3, left panel) and, to a lesser extent, to yellow E102 and blue E131. On day 7, end of the experiment, mortality raised to 21.5%, 20.0% and 20.5% respectively, values significantly higher than those registered in control (14%) or red E120 treated (15.2%, Fig. 3, left panel) nauplii.

The percentages of nauplii with low mobility increased in samples exposed to red E124 (Fig. 3, right panel), yellow E102 and blue E131: by day 7, in fact, values were 20.4%, 29.9% and 21.5% respectively, significantly higher than those registered in control (14.5%) or red E120 treated (14.1, Fig. 3, right panel) nauplii. Noteworthy is the evidence that low mobility of nauplii increased from day 4 in red E124 treated animals while in yellow E102 and blue E131 the increase occurred since day 5 or 6 with an apparent decrease of percentages in the first 4 days.

The overall analyses revealed that all nauplii, dead, poorly motile or vital, have regularly developed heads, antennae II and body parts, with no sign of swelling or gross deformities (Fig. 4). In about one third of nauplii exposed to red E124 (Fig. 4E) or yellow E102 (Fig. 4H and I), however, the thoracopods were more developed than expected (Fig. 4D and G); in addition, in about 5% of the nauplii exposed to red E124 thoracopods were also disproportioned, appearing longer but also thinner than expected (Fig. 4F). Nauplii exposed to red E120 (Fig. 4B) or blue E131 (Fig. 4C) never showed such defects.

3.2.2. Phototactic response

The phototactic response of nauplii treated with blue E131 did not show appreciable differences compared to controls (Fig. 5). On the contrary, the treatment of nauplii with red E120 and yellow E102 induced a significantly higher response, while a significantly reduced response was prompted by treatment with red E124.

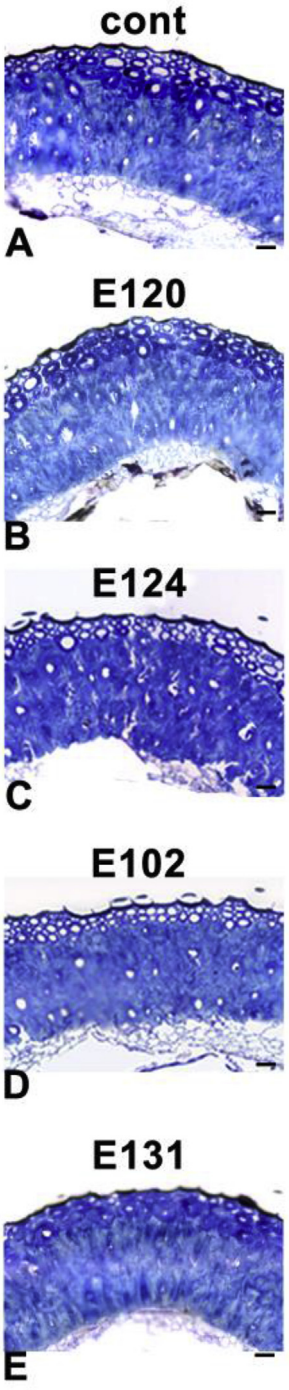

Fig. 2. Seeds coat structure of *Cucumis sativus* seedlings germinated in presence of the four food dyes (E120, E124, E102, E131). No significant differences in gross morphology and thickness are observed. Magnification 20×. Bar: 20 μm.

3.3. Effects of food dyes on Danio rerio

3.3.1. Hatching rate and mortality

At 30 hpf control and treated embryos were still in the chorion; at 78 hpf all the larvae, control and treated, hatched as expected (Kimmel et al., 1995). At 48 h of treatment (Fig. 6A) embryos exposed to red E124, yellow E102 and blue E131 showed a hatching percentage of 71.54%, 63.65% and 31.19% respectively, while no significant differences were registered between controls and red E120 exposed embryos showing hatching percentages of 15.81%

Table 1
Growth (shoot and root length and root/shoot ratio) and physiological (Total chlorophyll, *a*+*b*, total carotenoid, *x*+*c*, and total antioxidant activity) parameters in seedlings of *Cucumis sativus* L. treated with different food dyes (E120, E124, E102, E131) after one week from germination.

	Growth (N = 25)			Physiology (N = 16)		
	Root length (cm)	Shoot length (cm)	Root/shoot ratio	Total Chlorophylls *a*+*b* ($\mu g/cm^2$)	Total Carotenoids *x*+*c* ($\mu g/cm^2$)	Total Antioxidant activity (μmol Trolox Equivalents mg^{-1} FW)
Cont	7.24 ± 0.29^a	1.19 ± 0.07^a	$6.08 \pm 0.10a$	85.78 ± 3.55^a	16.49 ± 0.57^a	10.33 ± 0.28^a
E120	6.15 ± 0.37^a	1.32 ± 0.11^a	4.66 ± 0.13^b	99.39 ± 6.92^b	18.96 ± 1.31^b	7.39 ± 0.23^b
E124	7.03 ± 0.48^a	1.39 ± 0.12^a	5.06 ± 0.14^b	87.65 ± 3.67^a	16.92 ± 0.69^a	7.53 ± 0.24^b
E102	6.54 ± 0.33^a	1.26 ± 0.11^a	5.19 ± 0.14^b	81.91 ± 2.56^a	16.06 ± 0.43^a	7.99 ± 0.28^b
E131	8.15 ± 0.32^b	1.37 ± 0.14^a	5.95 ± 0.11^a	112.17 ± 3.67^b	20.02 ± 1.28^b	7.49 ± 0.13^b

Values are means ± SE. Different letters indicate statistically significant differences among treatments ($p < 0.05$). Cont: control; E120: cochineal red E120, E124: Ponceau red E124, E102: tartrazine yellow E102; E131: Patent blue E131.

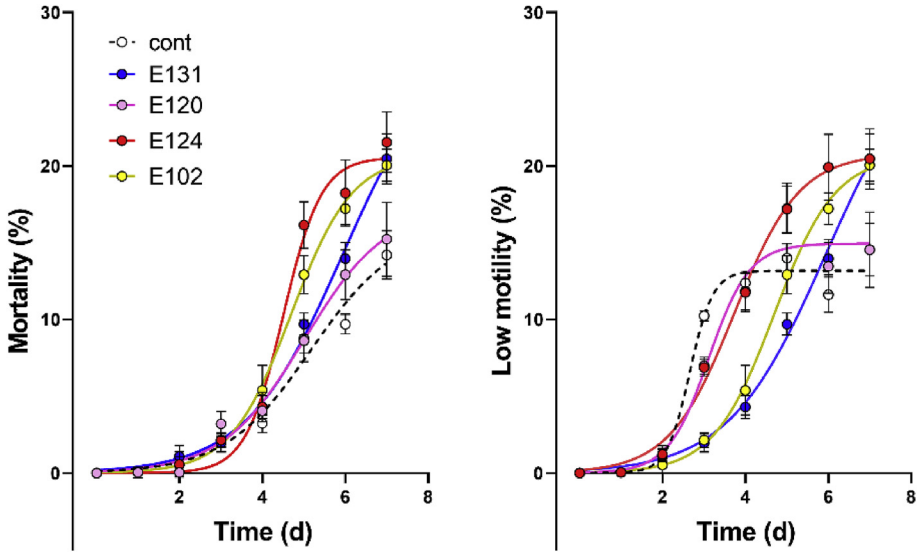

Fig. 3. Mortality (Left) and reduced mobility (Right) of *Artemia salina* nauplii exposed to the four food dyes. Data were fitted to a logistic model and the differences among curves were tested by the sum-of-squares F test ($p < 00$). Data are means ± SE ($n = 9$), $p < 0.05$. A significant increase in maximum percent mortality and low motility was observed in nauplii treated with red E124, yellow E102 and blue E131.

and 15.0%. No mortality was recorded during the experiment, in control and treated embryos.

3.3.2. Developmental defects

A percent of embryos exposed to red E124, yellow E102 and blue E131 showed marked phenotypic alterations. These occurred respectively in 21.03%, 18.15% and 23.03% of embryos (Fig. 6B). No alterations were observed in control embryos and embryos exposed to red E120. Percentages did not change significantly during the 72 h experiments, and the altered embryos appeared vital.

The alterations observed consisted mainly in axis and/or tail deviation and, especially in embryos exposed to red E124 in edema and for exposed to blue E131 in alterations of the head (Fig. 7). Most of the alterations are already visible at 24 h of treatment, becoming more pronounced as the body plan sets.

3.3.3. Heart rate

At 72 h of treatment the heart rate significantly increases ($p < 0.01$; Fig. 6C) in red E124 and blue E131 treated embryos, with values rising from 150 ± 1.5 bpm of controls to 166 ± 2 bpm and 172 ± 2 bmp respectively. No changes are registered in embryos treated with yellow E102 or red E120.

4. Discussion

The chosen end-points, currently adopted in environmental toxicity testing, clearly demonstrate that food dyes have significant effects on the three model systems examined. They also indicate that these effects vary from model to model and from dye to dye, suggesting that food dyes may interfere on multiple targets. The concentration used in this study (1.2 g/L) is higher than the levels registered in the environment but is in the range of values utilized in simulated effluents examined in literature (O'Neill et al., 1999). It was a deliberate choice aiming at amplifying and/or accelerating the toxic effects thus allowing acute exposure, an experimental condition suitable for the rapidly developing models chosen.

Several interesting evidences emerge from results summarized in Table 2. First, red E120, red E124 and yellow E102 modify the phototactic response of the *Artemia* nauplii, a response to light driven by photoreceptors and regulated by the light absorbing pigments. As far as we are aware, no data is available on the effects determined by these three dyes on eye structures. By contrast, several reports demonstrate that blue E131, used as an intraocular dye to facilitate surgery, causes significant dysfunction in retinal post-synaptic neurons (Lüke et al., 2006) and even reduced viability and proliferation in retinal pigmented cells (Morales et al., 2010). The relevance of the topic suggests that further and accurate investigations on food dyes eye toxicity should be carried out.

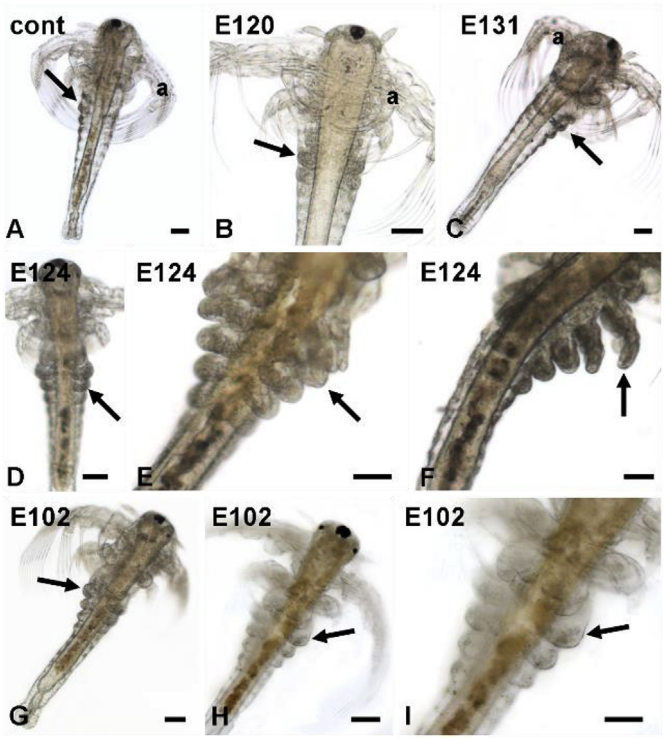

Fig. 4. Morphological characteristics of *Artemia salina* nauplii exposed to the four food dyes (E120, E124, E102, E131) for four days. (A-D, G) Normally developed nauplii with antennae II (a) and segmented trunks presenting the first rudiment of the thoracopod (arrows). (E, H–I) Over developed thoracopods (arrows). (F) Thin and long thoracopods (arrow). Bar: 50 μm.

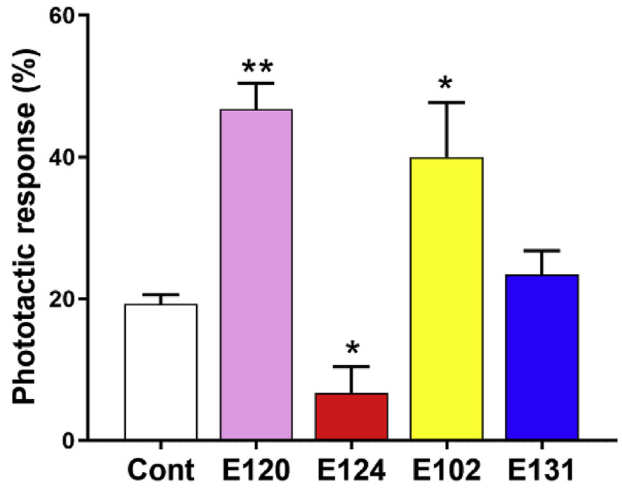

Fig. 5. Phototactic response of *Artemia salina* nauplii exposed to the four food dyes (E120, E124, E102, E131) for 48 h *p < 0.05; **p < 0.01. A significant increase in the response is observed after exposure to red E120 and yellow E102 dyes, while a significant reduction is registered after red E124 treatment.

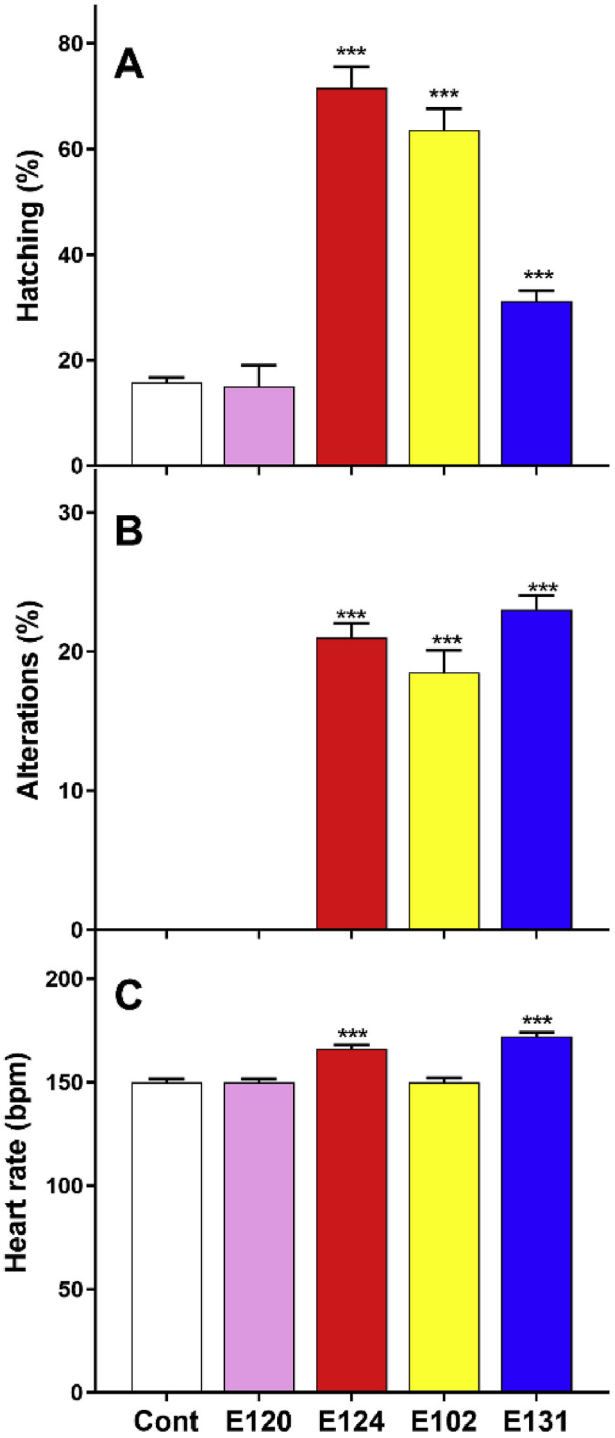

Fig. 6. Effects of four food dyes (E120, E124, E102, E131) on *Danio rerio* embryos after 48 (A) or 72 (B–C) h of exposure. Red E124, yellow E102 and blue E131 anticipate hatching (A) and induce developmental defects (B). Heart rate is accelerated by red E124 and blue E131(C). Data are means ± SE. ***p < 0.001.

The teratogenic effect exerted by the tested food dyes is demonstrated on the two animal models. In *Artemia*, damage is moderate and apparently limited to an acceleration in the formation of the thoracic appendages, but the increased mortality indicates that other alterations, not detectable in full investigations, might have occurred. In *Danio rerio* axes bending and cardiac edema occurs, two defects usually resulting in embryo toxicity testing, including with dyes (Joshi and Katti, 2018; Shen et al., 2015) or metal contaminants (Favorito et al., 2011; Monaco et al., 2016, 2017a, 2017b). They have been related to severe oxidative stress (Sheu et al., 2017) and/or to genotoxicity (Mpountoukas et al., 2010; Soares et al., 2015).

By contrast, beat rate data is discordant with literature that associate edema to a decreased rate (Shen et al., 2015). Complex mechanisms of action can be hypothesized and among these,

Fig. 7. Developmental defects found in *Danio rerio* embryos exposed for 48 h to the four food dyes (E120, E124, E102, E131). (A, E, I): normally developed control embryos. (C, F, G, J, L): embryos with edema (arrows); (B, D, F, H, J, K, L): embryos with axis and/or tail alteration (arrows); (J–K): embryos with microcephaly. Bars: 450 μm.

Table 2
Summary of the effects registered on *Cucumis sativus*, *Artemia salina* and *Danio rerio* after exposure to the four food dyes (E120, E124, E102, E131). (+): values significantly increased compared to control; (−): values significantly decreased compared to control; (0): values not different from the control ($p < 0.05$).

	Cochineal red E120	Ponceau red E124	Tartrazine yellow E102	Patent blue E131
Cucumis sativus				
Germination at 36 h	0	0	+	0
Germination at 72 h	0	0	0	-
Shoot length	0	0	0	0
Root length	0	0	0	+
Root/Shoot Ratio	-	-	-	0
Seed coat thickness	0	0	0	0
Chlorophyll concentration	+	0	0	+
Carotenoids concentration	+	0	0	+
Antioxidant activity	-	-	-	-
Artemia salina				
Mortality	0	+	+	+
Motility	0	-	-	-
Phototactic response	+	-	+	0
Developmental defects	0	+	+	0
Danio rerio				
Hatching rate	0	+	+	+
Heart beat rate	0	+	0	+
Morphological alterations	0	+	+	+

alterations in the ionic currents at the level of the cardiac pacemaker (Baker et al., 1997) and/or, as suggested for other dyes, variations in protein expression (Shen et al., 2015).

As regards *Cucumis*, as reported for other contaminants (An et al., 2004; Cargnelutti et al., 2006; Munzuroglu and Geckil, 2002), exposure to dyes induces modifications in germination rate and seedling development. The photosynthetic apparatus is targeted, and the synthesis of chlorophyll and carotenoids is stimulated. Similar responses are observed after exposure to heavy metals or dyes from industrial wastewater (Arena et al., 2017; Chaoui et al., 1997; Puvaneswari et al., 2006; Tauqeer et al., 2016; Vafaei et al., 2012). Chlorophyll and carotenoids are involved in light harvesting, and thus it is tempting to hypothesize that the food dyes exert a positive outcome on photosynthesis in *Cucumis*. The increase in carotenoids, also involved in photoprotection of photosynthetic apparatus against reactive oxygen species (Halliwell, 2006), would also positively contribute to the seedling' health status. However, our data indicate that modifications at plant growth level in part offset these positive effects: in fact, the decrease of root/shoot ratio indicates an adverse effect of food dyes on root development and a reduced absorption capacity of water and nutrients by seedlings. Different studies performed on *Allium cepa* and *Brassica campestris* have demonstrated that the addition of food dyes (tartrazine, sunset yellow and Ponceau 4R) to roots determines a time and concentration-dependent inhibition of root meristematic cell division, affecting root biomass formation (de

Oliveira et al., 2013; Dwivedi and Kumar, 2015; Marques et al., 2015).

Further evidence of the action of food dyes on *Cucumis* seedlings is the decrease of total antioxidant capacity in developing leaves. This capacity either increases or decreases in responses to diverse stress factors depending on plant species (Arena et al., 2014; Chaoui et al., 1997; Gallego et al., 1996; Gonçalves et al., 2007; Jadhav et al., 2012; Nagajyoti et al., 2010). The observed reduction in *Cucumis* can be explained hypothesizing a consumption of cell stock reserve to counteract the oxidative stress induced by food dyes. Whichever the cause, the drop of antioxidant capacity will determine in *Cucumis* seedlings a weakening of the cell defence machinery and an increased sensitivity to the environmental injuries.

Another interesting aspect emerging from data is the potential effect of food dyes, yellow E102 in particular, in anticipating development: hatching in *Danio rerio* and germination in *Cucumis*. A possible hypothesis is that the food dye alters the osmotic balance increasing the pressure inside the seed coat and chorion envelope facilitating their break. A further supposition is that the food dye directly modifies the seed coat and chorion structure and/or composition reducing their resistance. Preliminary evidences collected on germinated seeds indicate that treated coats maintain thickness and general organization and therefore that this would not be the case, at least for *Cucumis*. Finally, it cannot be excluded that anticipation is caused by an acceleration of metabolic pathways. Investigations on seed oxygen consumption during germination are now in progress; however, evidences indicate that the exposure of *Cucumis* seedlings to food dyes alters carbon balance between epigeal and hypogeal biomass likely resulting in an inhibition of root meristematic cells division and root biomass formation, as previously demonstrated for yellow E102 and red E124 (de Oliveira et al., 2013; Dwivedi and Kumar, 2015; Marques et al., 2015). The hypothesis of acceleration is, therefore, the least plausible for seedlings.

In *Danio* two mechanisms may be hypothesized to explain the anticipated hatching: a positive interference of dyes with choriolysin secretion and/or with the spontaneous movements of the developing larva (De la Paz et al., 2017; Kimmel et al., 1995; Schoots et al., 1982). We suppose that in our case this second eventuality is the more realistic considering that synthetic dyes are known to induce hyperactivity (Doguc et al., 2013) by influencing the level of catecholamines in the brain (Eagle, 2014). Moreover, in previous studies it was showed a clear correlation between the time of hatching and the movements made by larvae after the hatching (Capriello et al., 2019).

The faster swimming of *Danio* larvae exposed to food dyes sharply contrasts with reports indicating that some food dyes interfere with energy consumption (Abe et al., 2018). In *Artemia*, in effect, the decreased mobility of nauplii is congruent with the hypothesis of energy problems since no alterations are detected in swimming structures. Investigations on metabolic performances would possibly clarify these aspects.

Based on our results, it is evident that the natural cochineal red E120, affecting only 5 of 16 end-points examined, appears the less toxic dyes among those tested (Table 2). In addition, it is noteworthy that toxicity was exerted mainly on *Cucumis* and *Artemia* but not on *Danio*. Further investigation, at a molecular level, will be useful to clarify the real toxicity of this specific dye on this animal model and the risks in replacing the natural product with the synthetic one.

In conclusion, the evidence here collected indicates that food dyes target several organs and functions, in both plant and animal species and highlights the importance to utilize a set of organisms to assess the potential risks associated with their use. Concern should raise, and more accurate investigation addressed to fully understand the toxic responses induced in the aquatic organisms by these so far neglected but very diffused pollutants.

Conflict of interest

The authors have no financial or personal relationships that could inappropriately influence or bias the content of the paper.

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