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1 **High-content analysis of larval phenotypes for the screening of xenobiotic toxicity using**  
2 ***Phallusia mammillata* embryos**

3

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14

15 **Abstract**

16 In recent years, pollution of surface waters with xenobiotic compounds became an issue of concern  
17 in society and has been the object of numerous studies. Most of these xenobiotic compounds are  
18 man-made molecules and some of them are qualified as endocrine disrupting chemicals (EDCs)  
19 when they interfere with hormones actions. Several studies have investigated the teratogenic  
20 impacts of EDCs in vertebrates (including marine vertebrates). However, the impact of such EDCs  
21 on marine invertebrates is much debated and still largely obscure. In addition, DNA-altering  
22 genotoxicants can induce embryonic malformations. The goal of this study is to develop a reliable  
23 and effective test for assessing toxicity of chemicals using embryos of the ascidian (*Phallusia*  
24 *mammillata*) in order to find phenotypic signatures associated with xenobiotics. **We evaluated**  
25 **embryonic malformations with high-content analysis of larval phenotypes by scoring several**  
26 **quantitative and qualitative morphometric endpoints on a single image of *Phallusia* tadpole larvae**  
27 **with semi-automated image analysis.** Using this approach we screened different classes of  
28 toxicants including genotoxicants, known or suspected EDCs and nuclear receptors (NRs) ligands.

29 The screen presented here reveals a specific phenotypic signature for ligands of retinoic acid  
30 receptor/retinoid X receptor. Analysis of larval morphology combined with DNA staining revealed  
31 that embryos with DNA aberrations displayed severe malformations affecting multiple aspects of  
32 embryonic development. In contrast EDCs exposure induced no or little DNA aberrations and  
33 affected mainly neural development. Therefore the ascidian embryo/larval assay presented here  
34 can allow to distinguish the type of teratogenicity induced by different classes of toxicants.

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38 **Keywords:** ascidian; embryo; toxicity; morphological analysis; endocrine disrupting chemical  
39 (EDC); genotoxicity

40

## 41 1. INTRODUCTION

42 Anthropogenic activities have a large impact on aquatic ecosystems from the discharge of chemical  
43 substances, many of which are supposed to be present in trace amounts (i.e. heavy metals) or even  
44 absent in natural conditions (i.e. pharmaceuticals, pesticides) (Mnif et al., 2011; Desbiolles et al.,  
45 2018). Endocrine disrupting chemicals (EDCs) are xenobiotic substances that can interfere with  
46 the endocrine system through two main pathways: the genomic pathway, via binding to nuclear  
47 receptors (NRs); or the non-genomic pathway, via binding to cell membrane receptors or affecting  
48 hormone metabolism. Thus endocrine disruption can be induced by xenobiotics that bind nuclear  
49 receptors such as AR, ER, TR, PPAR, RXR, PXR, CAR or ERR (termed NR-binding EDCs) (Mnif  
50 et al., 2011). Upon NR binding, EDCs can either activate a receptor (agonist action) or block it for  
51 normal hormone (antagonistic action).

52 Many phenotypes of EDCs exposure have been described in invertebrate species (Urushitani et al.,  
53 2018; Castro et al., 2007; le Maire et al., 2009; Nishikawa et al., 2004; Matsushima et al., 2013),  
54 but the mode-of-action of these EDCs remains mainly unclear. One approach to predict whether a  
55 xenobiotic might target a NR is to compare its phenotype against the phenotypes produced by a  
56 specific NR agonist/antagonist (Tohmé et al., 2014, Gomes et al., 2019b). If a phenotypic signature  
57 can be associated with a specific NR agonist/antagonist, then it can be potentially used as a  
58 biomarker of NR-signaling disruption.

59 In the past years, ascidian species have been used as toxicological models (reviewed in Dumollard  
60 et al., 2017; Zega et al., 2009; Gallo and Tosti, 2015; Battistoni et al., 2018). Ascidians are marine  
61 filter-feeding chordates belonging to the Subphylum Urochordata, recognized as the sister group  
62 of vertebrates (Delsuc et al., 2006). Their zygotes divide 13 to 15 times in 18 hours to give rise to

63 a transparent vertebrate-like tadpole larvae (Yamada and Nishida, 2014). The ascidian larva  
64 possesses both central and peripheral nervous system (CNS and PNS) (reviewed in Hudson, 2016;  
65 Dumollard et al., 2017; Gomes et al., 2019a). The CNS of ascidian larvae is composed of a sensory  
66 vesicle and a motor ganglion homologous, respectively, to the vertebrate diencephalon and  
67 hindbrain (Holland and Holland, 1999). The sensory vesicle within the CNS contains two easily  
68 identifiable dark pigmented sensory organs (PSO), the otolith (Ot) and the ocellus (Oc). The PNS  
69 consists of the papillary neurons in palps, the epidermal sensory neurons, and the bipolar tail  
70 neurons (Hudson, 2016). A prominent feature of ascidian larva are the adhesive organs, or palps,  
71 which are part of the PNS (Takamura, 1998). Hence, a number of morphological features can be  
72 assessed in the transparent ascidian larva to discriminate between non specific teratogenicity (i.e.  
73 problems in general morphogenesis of the larva) and specific neurodevelopmental toxicity (i.e.  
74 problems in the development of PNS and CNS structures). Ascidian neurodevelopment might be  
75 especially sensitive to NR-targeting EDCs as several NRs are expressed in neurogenic domains of  
76 the ascidian embryo (reviewed in Gomes et al., 2019a).

77 High-content analysis is a technique that involves scoring of multiple endpoints on a single image  
78 which can be achieved by automated or semi-automated image analysis and is now commonly  
79 applied to drug discovery screening using biological systems (Esner et al., 2018). The aim of this  
80 study was to analyse the teratogenic effects of different classes of compounds (EDCs, NR ligands,  
81 genotoxic and cytotoxic compounds) by quantifying larval malformations of the ascidian *Phallusia*  
82 *mammillata*. We have hypothesized that by performing high-content analysis of morphological  
83 malformations in *Phallusia* tadpole larvae, we will determine phenotypic signatures of different  
84 types of compounds, such as a toxicant (sodium azide), genotoxicants (TBT, etoposide, mitomycin  
85 C), known/suspected EDCs (lindane, atrazine, chlordane, chlorpyrifos, BPA, estradiol benzoate)

86 and nuclear receptors (NRs) ligands. Etoposide, TBT, and mitomycin were used as reference  
87 genotoxic compounds (Kirkland et al., 2016). The pesticides analyzed in the current study (lindane,  
88 atrazine, chlordane, chlorpyrifos), as well as BPA, are often referred to as EDCs (Mnif et al., 2011);  
89 however, their mechanism of action in marine invertebrates is unknown. Our previous work  
90 showed that several NRs (RAR, PXR/VDR $\alpha$ , PPAR, ROR, and ERR) are expressed in neurogenic  
91 regions of *Phallusia mammillata* embryos (Gomes et al., 2019a, Gomes et al., 2019b). We  
92 analyzed known agonists/antagonists of these NRs, such as UVI3003 (RXR antagonist), all-trans  
93 retinoic acid (RAR agonist), rifampicin and SR12813 (PXR agonists), SR1078 (ROR agonist),  
94 BADGE (PPAR agonist), diethylstilbestrol, and 4-hydrotamoxifen (ERR antagonists).

95 To complement our morphometric analysis of embryonic development we also performed a  
96 genotoxicity assay. Genotoxicity is the loss of DNA structural or functional integrity. There are  
97 several protocols for analysing DNA damage induced by genotoxic compounds (OECD test N.  
98 487; OECD test N. 473; OECD test N. 489) and some of them have been successfully applied to  
99 marine invertebrates (Dixon et al., 2002; Saotome et al., 1999;). However, it is not clear which  
100 phenotypic signature is associated with DNA damage and genotoxicity in the embryo. The ascidian  
101 embryo is an interesting model for scoring genotoxicity, since the spindle assembly checkpoint is  
102 not active at early stages of development (Chenevert et al., 2020). Thus, even upon DNA damage  
103 the embryo will continue dividing without pausing to repair DNA damage, resulting in overt DNA  
104 aberrations such as micronuclei, multinucleated cells or DNA bridges.

105 We describe here methods for ascidian embryonic cultures and provide different morphological  
106 endpoints of the ascidian larva that can be scored to discriminate larval phenotypes. Such high  
107 content analysis of ascidian larval phenotypes can be coupled with DNA staining to correlate  
108 embryonic phenotypes with the level of genotoxicity. We show a first screen of 19 molecules using

109 these protocols and found that genotoxicants affect all morphological endpoints whereas EDCs  
110 and NR ligands affect mostly neural endpoints.

## 111 2. Materials & Methods

### 112 2.1. Animals

113 *Phallusia mammillata* were collected at Sète (Etang de Tau, Mediterranean coast, France) and kept  
114 in the aquaria of the “Centre de Ressources Biologiques” (CRB) of the Institut de la Mer à  
115 Villefranche (IMEV) which is an EMBRC-France certified service (see [https://www.embrc-](https://www.embrc-france.fr/fr/nos-services/fourniture-de-ressources-biologiques/organismes-modeles/ascidie-phallusia-mammillata)  
116 [france.fr/fr/nos-services/fourniture-de-ressources-biologiques/organismes-modeles/ascidie-](https://www.embrc-france.fr/fr/nos-services/fourniture-de-ressources-biologiques/organismes-modeles/ascidie-phallusia-mammillata)  
117 [phallusia-mammillata](https://www.embrc-france.fr/fr/nos-services/fourniture-de-ressources-biologiques/organismes-modeles/ascidie-phallusia-mammillata)). Animals were maintained in the aquaria on open system with running  
118 seawater (pH  $8.2 \pm 0.05$ , salinity  $40 \pm 0.5$  ppt) at 16°C for at least two weeks before use. Animals  
119 were fed 3 to 5 times per week with commercial microalgae concentrates (2-3 mL per aquarium  
120 of 60 L), Shellfish diet 1800® and Isochrysis 1800® (Instant Algae®, Reed Mariculture Inc.,  
121 Campbell, CA, USA, 95008) purchased from a french distributor. Ascidian eggs were collected  
122 by oviduct dissection. In order to ensure proper penetration of the studied compounds into eggs,  
123 they were dechorionated with 0.1% Trypsin (Sigma-Aldrich, T9201) in filtered sea water (FSW)  
124 for 60 min at 20°C, then transferred to fresh sea water and stored at 18°C until required. Sperm  
125 was collected by syringe aspiration from a sperm duct and was activated with high pH 9.2 sea  
126 water for 10–20 min, then used to fertilize dechorionated eggs (Gomes et al., 2019b; see full  
127 protocols on [http://lbdv.obs-vlfr.fr/en/research/research\\_](http://lbdv.obs-vlfr.fr/en/research/research_groups/ascidian-biocell-group/projets/fertilization-and-meiosis/protocols.html)  
128 [group/projets/fertilization-and-meiosis/protocols.html](http://lbdv.obs-vlfr.fr/en/research/research_groups/ascidian-biocell-group/projets/fertilization-and-meiosis/protocols.html)). The embryos were reared in dark  
129 conditions to avoid photodegradation of chemicals.

### 130 2.2. Tested Chemicals

131 Sodium azide (CAS Number: 26628-22-8; purity:  $\geq 99\%$ ), tributyltin (CAS Number 1461-22-9;  $\geq$   
132 96%), etoposide (CAS Number 33419-42-0; reference standard), atrazine (CAS Number 1912-24-



133 9; analytical standard), lindane (CAS Number 58-89-9; analytical standard), chlordane (CAS  
134 Number 5103-71-9; analytical standard), chlorpyrifos (CAS Number 2921-88-2; analytical  
135 standard), SR12813 (CAS Number 126411-39-0;  $\geq 98\%$ ), diethylstilbestrol (CAS Number 56-53-  
136 1;  $\geq 99\%$ ), 4-hydroxytamoxifen (CAS Number 68392-35-8; analytical standard), rifampicin (CAS  
137 Number 13292-46-1;  $\geq 97\%$  (HPLC)), mitomycin C (CAS Number 50-07-7; reference standard),  
138 bisphenol A (CAS Number 80-05-7;  $\geq 99\%$ ), estradiol benzoate (CAS Number 50-50-0;  $\geq 97\%$   
139 (HPLC)), ATRA (CAS Number 302-79-4;  $\geq 98\%$ ), BMS493 (CAS Number 215030-90-3;  $\geq 98\%$ ),  
140 and UVI3003 (CAS Number 847239-17-2;  $\geq 98\%$ ) were purchased from the Sigma-Aldrich Co.  
141 (St Louis, MO, USA). BADGE (CAS Number 1675-54-3;  $\geq 95\%$ ) and SR1078 (CAS Number  
142 1246525-60-9;  $\geq 98\%$  (HPLC)) were purchased from Tocris Bioscience (Bristol, UK). Table 1  
143 shows tested compounds and concentrations used in the current study. We have performed toxicity  
144 testing to determine the dose that will produce serious toxicological effects, therefore the  
145 concentrations used in the current study were mostly higher than environmentally relevant.

### 146 *2.3. Experimental design*

147 All compounds were resuspended in DMSO for a stock concentration. Immediately before the  
148 treatment, stock solutions were diluted with DMSO to a 10 000x working concentration. Finally,  
149 prediluted stocks were added to the embryo culture medium (FSW, 5 mM TAPS, 0.5 mM EDTA,  
150 pH 8, salinity  $40 \pm 0.5$  ppt) at 1:10 000 ratio giving the required concentration of a compound in  
151 the medium and 0.01% DMSO (v/v). The same amount of DMSO (0.01% in embryo culture  
152 medium) was used as a vehicle control. All solutions were controlled to be at pH 8 and prechilled  
153 to 18°C prior to embryo exposure. Concentrations of compounds were selected based on  
154 preliminary experiments. We have chosen the concentrations which induced malformations, but

155 were not lethal (i.e. which did not induce 100% undevelopped embryos) or the highest soluble  
156 concentration.

157 Dechorionated eggs (approximately 1000 eggs) were mixed with activated sperm ( $10^6$ /ml diluted  
158 in FSW) in the embryo culture medium for fertilization (Sardet et al., 2011; Gomes et al., 2019b).

159 After fertilization, embryos were washed twice with FSW to remove excess of sperm and to avoid  
160 polyspermy. Washed embryos at 1-cell stage were then transferred to 12-well plates, each well is  
161 coated with GF (gelatin/formaldehyde, see Sardet et al., 2011), at a concentration of 100  
162 embryos/well. Each well contained either vehicle control or a tested concentration of compound  
163 in a total of 3 mL of FSW. Plates with embryos were kept in dark humid chambers to avoid  
164 photodegradation of xenobiotics at 18°C. Embryos were left to develop to gastrula or neurula stage  
165 (7 – 9 hpf at 18°C) and ~30 embryos were collected for genotoxicity analysis (Supplementary Fig.  
166 1). The rest of embryos was left to develop to swimming larval stage (stage 26, 22 hpf at 18°C;  
167 see Gomes et al., 2019b). Embryos were exposed to tested compounds from 1-cell stage till  
168 fixation.

169 When embryos in the control reached swimming larval stage, all cultures were fixed with 0.4%  
170 formaldehyde to stop motility. Fixed embryos were transferred to a chamber slide and imaged with  
171 a Zeiss Axiovert200 inverted microscope at 10X magnification. For each culture, a minimum of  
172 30 embryos were analyzed per treatment (**N = 30 technical replicates**). Each experimental  
173 condition was repeated at least 3 times (**n = 3 biological replicates**).

#### 174 ***2.4. Morphological analysis of phenotypes***

175 Analysis of the phenotypes at larval stage was performed with Toxicosis, a software developed in  
176 our laboratory with a proprietary code and filed by the CNRS at the APP (Agency for the

177 Protection of Programs) on July 13th 2018 under the reference  
178 IDDN.FR.001.330013.000.S.P.2018.000.10000. The analyzed endpoints are summarized in  
179 Supplementary Figure 2 (B – H). We analyzed the total area of PSO (Oc+Ot area) and the distance  
180 between Oc and Ot (Oc/Ot distance) in order to describe development of the central nervous  
181 system. The presence/absence of palps was used as a marker of peripheral nervous system  
182 development. The trunk length to width ratio (trunk L/W) and tail length reflect general  
183 morphogenesis of the embryo.

184 The experiments were performed during years 2015 – 2019 in different seasons using wild animals  
185 from Sète (Etang de Tau, Mediterranean coast, France). Thus variations in natural animal  
186 population in addition to seasonal rearing conditions contributed to variations in morphological  
187 parameters of control embryos (Table 2). **In order to eliminate the effect of external factors we**  
188 **have compared and normalized each endpoint with the corresponding value in the control group**  
189 **(0.01% DMSO) done on the same day.** Statistical analysis was performed on the raw (not-  
190 normalized) data using the Kruskal–Wallis test. Analyses were performed at a significance level  
191 of 0.01 (for Oc/Ot area, Oc/Ot distance, trunk L/W ratio and tail length) or 0.05 (for % embryos  
192 with palps) using STATISTICA v. 13.0 software for Windows. Raw measurements and  
193 normalized values are presented in Tables 2 – 5.

194 In addition, normal, malformed or undevelopped embryos were scored manually. **An embryo was**  
195 **considered malformed if the embryo presented signs of antero-posterior elongation (indicating it**  
196 **had undergone gastrulation) or if tadpoles had a crooked tail, an absence of tail, an absence of**  
197 **PSO, or a brain protrusion were observed. Embryos were considered undevelopped if they failed to**  
198 **reach neurula stage (no sign of anteroposterior elongation or gastrulation).** We calculated the  
199 percentage of: 1) normal embryos [number of normal embryos \*100 / (number of normal +

200 malformed + undeveloped)]; 2) malformed [number of malformed embryos \*100 / (number of  
201 normal + malformed + undeveloped)]; 3) undeveloped [number of undeveloped embryos \*100 /  
202 (number of normal + malformed + undeveloped)].

### 203 **2.5. Genotoxicity assay**

204 Embryos from gastrula to neurula stage were fixed with a fixation solution (4% paraformaldehyde,  
205 0.5 M NaCl in PBS) for 1h at 20°C on a shaker. Then the fixative was removed and embryos were  
206 washed twice with PBS. After washing, the samples were incubated in PBS containing 0.1% Triton  
207 X-100 and 3% bovine serum albumin (PBSB) for 1h at 20°C on a shaker. The embryos were  
208 stained with 1 µg/ml Hoechst in PBSB for 1-2h at 20°C on a shaker. **The embryos were either**  
209 **stained with Hoecsht only or double stained with Hoecsht and 4 ng/ml phalloidin in PBSB to**  
210 **observe both DNA and membrane. Double staining was done to ascertain the presence of DNA**  
211 **bridges between two cells and/or multiple nuclei within the same cell.** Finally, the embryos were  
212 washed twice with PBSB and transferred on a glass slide. The DNA aberrations (multinucleated  
213 cells, micronuclei or DNA bridges) were imaged using a confocal Leica SP8 fitted with 40×/1.1NA  
214 water objective lens and scored manually. **The number of embryos scored in each treatment is**  
215 **shown in Table 6. Embryos were exposed to etoposide 10 µM as a positive control for DNA**  
216 **damage. The relationships between % embryos with DNA aberrations, normal, malformed and**  
217 **undeveloped embryos were quantified according to the Spearman's correlation tests. The t-test**  
218 **was used to establish significance of the correlation between pairs of parameters at a significance**  
219 **level of 0.05.**

220

221

## 222 3. RESULTS

### 223 3.1. *Morphometric analysis of larval development in untreated embryos*

224 Figure 1 and Table 2 show changes in the morphology of ascidian embryos, scored quantitatively  
225 for PSO development (Oc/Ot area, Oc/Ot distance), trunk and tail morphogenesis (trunk L/W ratio,  
226 tail length) and qualitatively for palps development (presence of palps). Such morphological  
227 changes occur from stage 22 (late tailbud) to stage 26 (hatching larva, see  
228 <https://www.bpni.bio.keio.ac.jp/chordate/faba/1.4/top.html> for staging) (Fig. 1 B-F). At 18 hour-  
229 post-fertilization (hpf; stage 22), late tailbud embryos start to pigment their PSO (only one  
230 pigmented area =  $72 \pm 24 \mu\text{m}^2$ ) and no palp is apparent at the anterior tip of the trunk (Fig. 1B).  
231 At stage 23, two distinct pigment cells are visible, the otolith (Ot) and the ocellus (Oc) (forming  
232 the PSO of an area of  $253 \pm 64 \mu\text{m}^2$ ; Oc/Ot distance =  $18 \pm 8 \mu\text{m}$ ) and palps are first visible (Fig.  
233 1C). At stage 24, the pigment cells are separated from each other (the otolith positioned anteriorly  
234 and the ocellus posteriorly; Oc/Ot distance =  $23 \pm 6 \mu\text{m}$ ), and the trunk starts to elongate (Trunk  
235 L/W =  $1.8 \pm 0.3$ ; Fig. 1D). From this stage and up to stage 26, the ocellus and otolith continue to  
236 spread and move apart from each other, while the trunk continues to elongate (Fig. 1E, 1F). The  
237 tail length only increases until stage 23 and then remains constant (tail length is  $\sim 500 \mu\text{m}$ , Table  
238 2). The stage 26 was selected for further analysis of phenotypes induced by exposure to xenobiotics  
239 as it is the developmental stage of hatching. At this stage PSO area (mentioned Oc+Ot area in  
240 Table 2 and radar charts), PSO distance and tail length reach maximum, palps are fully developed.  
241 Later stages are characterized by further trunk elongation, and tail shortening (data not shown, see  
242 <https://www.bpni.bio.keio.ac.jp/chordate/faba/1.4/top.html>). Therefore in further analysis the  
243 cultures were fixed and analysed when controls reached stage 26.

244

### 245        **3.2. Phenotypes induced by general toxicants**

246        When *Phallusia* embryos are exposed to toxic compounds, deviations from normal embryonic  
247        development (i.e. malformations) can be observed as alterations in the quantified endpoints (PSO  
248        area and distance, trunk L/W ratio, tail length), compared to untreated control larvae. Images of  
249        larval phenotypes are presented in Supplementary Fig. 3. We first analysed the phenotypes induced  
250        by exposure to a cytotoxic compound, sodium azide (SA), and to three genotoxicants, etoposide,  
251        mitomycin and tributyltin (TBT) (Kirkland et al., 2016) (Table 3, Supplementary figure 3). All  
252        four tested chemicals significantly affected all the assessed endpoints, resulting in an embryo  
253        resembling a mid tailbud stage embryo (st 22-23, Fig. 1A-D; Supplementary Fig. 3).

254        Concerning the cytotoxicant, the maximum dose of SA tested (2.5 mM) decreased Oc/Ot distance  
255        by 81%, number of embryos with palps by 95%, tail length by 42% and trunk L/W ratio by 43%  
256        (Table 3). Exposure to 2.5 mM SA induced 86±14% of malformed, 12±12% normal, and 2±2  
257        undevelopped embryos (Fig. 2). The lowest observed effective concentrations (LOEC) in our study  
258        was 1 mM for SA (Tables 1 and 3).

259        Mitomycin significantly reduced Oc/Ot distance, trunk L/W ratio, and tail length at 10 µM (LOEC)  
260        and the percentage of embryos with palps was also reduced at this dose (though  $P > 0.01$ ) (Table  
261        3). At 60 µM, mitomycin strongly affected all endpoints and induced malformations in 91±5% of  
262        embryos (Table 3; Supplementary Fig. 3). Exposure to etoposide at concentrations 50 – 75 nM  
263        affected PSO area (by 14% at 75 nM), Oc/Ot distance (by 42% at 75 nM), and trunk L/W ratio (by  
264        14% at 75 nM), but had no effect on tail length or on the percentage of embryos with palps (Table  
265        3). At a higher dose of 100 nM, etoposide had an effect similar to SA and mitomycin (60 µM),  
266        affecting all studied endpoints (malformation rate 79±16%, Fig. 2). Similarly, TBT at 10 – 20 nM

267 induced a significant reduction of PSO area (by 40% at 10 nM TBT), of Oc/Ot distance (by 52%  
268 at 10 nM TBT), and trunk L/W ratio (by 27% at 10 nM TBT) (Table 3). Exposure to a high dose  
269 of TBT (50 nM) also led to severe malformations in general morphology of the larvae, with  $82\pm 6\%$   
270 of malformed embryos (Fig. 2; Supplementary Fig. 3).

### 271 ***3.3. Phenotypes induced by known/suspected EDCs***

272 Lindane exposure resulted in significant malformations in developing ascidian embryos at 10  $\mu\text{M}$ .  
273 This pesticide affected primarily the Oc/Ot distance (62% of control value) and trunk L/W ratio  
274 (76% of control value) of ascidian larvae (Table 4, Supplementary Fig. 4). When the concentration  
275 of compound was increased to 20  $\mu\text{M}$ , the PSO area decreased to 20% of control value and distance  
276 between PSO was 36% of that in control. At 20  $\mu\text{M}$  lindane  $53.3\pm 9\%$  of embryos were malformed  
277 (Fig. 3).

278 Similar malformations were observed in atrazine-exposed embryos (Table 4, Supplementary Fig.  
279 4). Our results show that exposure to 25 – 70  $\mu\text{M}$  atrazine induce a small but significant decrease  
280 in Oc/Ot distance (to 69% of the control value at 70  $\mu\text{M}$ ) and trunk L/W ratio (87% of the control  
281 value at 70  $\mu\text{M}$ ) without affecting other parameters. Only  $12\pm 7\%$  embryos were malformed and  
282  $83\pm 7\%$  were normal at 70  $\mu\text{M}$  of atrazine (Fig. 3).

283 Bisphenol A (BPA), a xenoestrogen, had a very specific effect on ascidian embryos. All tested  
284 concentrations of BPA (5 – 10  $\mu\text{M}$ ) specifically and significantly reduced PSO pigmentation (PSO  
285 area) and Oc/Ot distance (Table 4, Supplementary Fig. 4). The rate of malformed embryos reached  
286  $53\pm 8\%$  after exposure to 10  $\mu\text{M}$  BPA with  $39\pm 6\%$  normal embryos (Fig. 3).

287 Chlordane provoked phenotypes similar to lindane, with PSO area (86% of control) and Oc/Ot  
288 distance (73 % of control) affected at concentrations of 10  $\mu\text{M}$  (Table 4, Supplementary Fig. 4).

289 The highest tested concentration (50  $\mu$ M) also decreased the trunk L/W ratio (92% of control), but  
290 tail length and palps formation were not significantly affected. Exposure to 50  $\mu$ M chlordane  
291 resulted in 38 $\pm$ 6% malformed embryos and 59 $\pm$ 7% normal embryos (Fig. 3).

292 A slightly different phenotype was observed after exposure of *Phallusia* embryos to chlorpyrifos  
293 (Table 4, Supplementary Fig. 4). Low concentrations 10 – 25  $\mu$ M affected only Oc+Ot area (85%  
294 of the control value) and trunk L/W ratio (89% of the control value). The highest tested  
295 concentration (50  $\mu$ M) induced significant reduction of PSO area (69% of control), Oc/Ot distance  
296 (52 % of control), trunk L/W ratio (75% of the control value) and tail length (67% of control), thus  
297 affecting also the morphogenesis of the tadpole and not only neural development. Chlorpyrifos at  
298 50  $\mu$ M also induced malformations in 13 $\pm$ 7% of exposed embryos (Fig. 3).

299 We have also analyzed the effect of  $\beta$ -Estradiol 3-benzoate (E2B), an ER ligand used in veterinary  
300 medicine, on *Phallusia* embryo development. Exposure to E2B did not affect any studied endpoint  
301 (Table 4, Fig. 3, Supplementary Fig. 4).

302

### 303 **3.4. Phenotypes induced by NR-agonists/antagonists**

304 We then analysed the phenotypes induced by compounds known to specifically bind a vertebrate  
305 NR. The RAR agonist all-trans retinoic acid (ATRA) and the RAR antagonist BMS493 both had  
306 a strong inhibitory effect on palp development (Table 5, Supplementary Fig. 5). Exposure to 0.1  
307  $\mu$ M ATRA significantly decreased the number of embryos with palps (36% of the control value)  
308 without affecting other endpoints. Higher concentrations of ATRA also led to decreased trunk L/W  
309 ratio (85% of the control value at 1  $\mu$ M). A similar phenotype was observed with the RAR inverse



310 agonist, BMS493, which primarily affected palp development at 3  $\mu$ M (15% of the control value)  
311 and slightly reduced PSO area (87% of the control value) and the tail length (93% of the control).

312 An RXR antagonist, UVI3003, at 1  $\mu$ M also significantly affected palps formation (40% of the  
313 control value), and reduced PSO area (86% of the control value) (Table 5, Supplementary Fig. 5).  
314 Exposure to 2  $\mu$ M of UVI3003 also led to tail shortening (63% of the control value) and trunk  
315 rounding (92% of the control value).

316 In our study, 4-OHT affected embryo development at concentrations of 5–10  $\mu$ M (Table 5,  
317 Supplementary Fig. 5). At 5  $\mu$ M 4-OHT reduced mainly the Oc/Ot distance (51 % of control) and  
318 the trunk L/W ratio to a lesser extent (83% of the control). At 10  $\mu$ M tail length was slightly but  
319 significantly affected (to 88% of the corresponding control value). In contrast, DES affected  
320 *Phallusia* larval development already at 1  $\mu$ M by reducing PSO area (to 84%), Oc/Ot distance (to  
321 64%) and trunk L/W ratio (to 80% of the corresponding control value; Table 5, Supplementary  
322 Fig. 5). At 2  $\mu$ M all studied endpoints were significantly affected, showing high toxicity of DES  
323 to developing ascidian embryos.

324 Rifampicin exposure significantly affected only Oc/Ot distance (23% of control at 100  $\mu$ M) and  
325 trunk L/W ratio (70% of control at 100  $\mu$ M; Table 5, Supplementary Fig. 5). Another PXR agonist  
326 SR12813 (which was not tested against ascidian PXR) also decreased the distance between PSOs  
327 (55% of control at 3  $\mu$ M) but affected other endpoints such as PSO area (80% of control at 3  $\mu$ M),  
328 and trunk elongation (84% of control at 3  $\mu$ M) (Table 5, Supplementary Fig. 5). The highest tested  
329 concentration (7.5  $\mu$ M) significantly reduced all studied parameters, thus showing non-specific  
330 toxicity.

331 Embryo exposure to the ROR $\alpha$ / $\gamma$  agonist, SR1078, reduced PSO area (80% of the control value)  
332 and trunk L/W ratio (93% of the control value) at 2  $\mu$ M (Table 5, Supplementary Fig. 5). At 5  $\mu$ M  
333 of SR1078, all studied endpoints except the rate of embryos with palps were significantly reduced,  
334 showing nonspecific toxicity of the compound.

335 Exposure of *Phallusia* embryos to BADGE, a PPAR antagonist, induced a phenotype similar to  
336 lindane and atrazine (Table 5, Supplementary Fig. 5). At 1  $\mu$ M BADGE only the trunk L/W ratio  
337 was significantly affected (87% of control value). Higher concentrations of BADGE (2 – 5  $\mu$ M)  
338 further reduced the trunk L/W ratio (to 74% at 5  $\mu$ M) and affected distance between PSO (to 57%  
339 at 5  $\mu$ M) without affecting the other endpoints.

340

### 341 ***3.5. Measurement of the genotoxicity induced in ascidian embryos***

342 High concentrations of SA and reference genotoxicants (mitomycin, etoposide, TBT) affect all  
343 endpoints in the tadpole larvae (Table 3, Fig. 2), resulting in arrest of development before stage  
344 26. We assessed genotoxicity of these drugs by staining embryo DNA and scoring DNA  
345 aberrations. Table 6 and Supplementary Fig. 6 show the scores of DNA aberrations induced by the  
346 toxics, after imaging Hoechst-stained *Phallusia* embryos at gastrula and neurula stages.

347 In the control cultures, embryos showed well aligned nuclei of constant size and tightly packed  
348 nuclear DNA in all 50 embryos (Supplementary Fig. 6A). Control cultures also showed more than  
349 80% of normally developed larvae at 22 hpf (stage 26), without major malformations (Fig. 2).  
350 Following exposure to 2.5 mM SA we observed appearance of few micronuclei and DNA  
351 aberrations in 70% embryos, thus showing that such a dose of SA is genotoxic to ascidian embryos  
352 (Table 6; Supplementary Fig. 6B).

353 Similarly to SA-exposed embryos, micronuclei and multinucleated cells were observed in TBT-  
354 exposed embryos at 50 nM (Table 6, Supplementary Fig. 6C). In contrast, the embryos exposed to  
355 the reference genotoxic compound etoposide (100 nM) exhibited severe DNA damage with  
356 formation of DNA bridges in some cells (Table 6, Supplementary Fig. 6D). Exposed embryos were  
357 able to develop to tadpole, but showed high rate of malformations (Fig. 2). The higher dose of  
358 etoposide (10  $\mu$ M) also led to formation of DNA bridges, but in the whole embryo (Table 6,  
359 Supplementary Fig. 6E). Such embryos developed normally till gastrula stage and did not show  
360 any disorganization of cell pattern, the embryo development was blocked shortly after gastrulation  
361 (100% undeveloped embryos at 22hpf; Fig. 2). Another reference genotoxic compound,  
362 mitomycin C (60  $\mu$ M), induced formation of DNA bridges in every embryos (Table 6,  
363 Supplementary Fig. 6F), but only in parts of the ectoderm (data not shown). Such embryos were  
364 still able to develop to the tadpole with 91 $\pm$ 5% of malformed and only 8 $\pm$ 7% of undeveloped  
365 embryos (Fig. 2).

366 We have also assessed whether high **concentrations** of EDCs (lindane, atrazine, BPA, chlordane,  
367 and chlorpyrifos) were genotoxic to ascidian embryos. Most of the pesticides induced little or no  
368 DNA damage in *Phallusia* embryos (Table 6). Exposure to 20  $\mu$ M lindane led to appearance of  
369 few micronuclei per gastrula without affecting the pattern of cellular divisions (Supplementary  
370 Fig. 6G). This low level of DNA damage is associated with 53.3 $\pm$ 9% of malformed embryos (Fig.  
371 3). In presence of 70  $\mu$ M atrazine no DNA aberrations was observed and only 12 $\pm$ 7% embryos  
372 were malformed (Table 6; Fig. 3). Exposure to 50  $\mu$ M chlordane resulted in appearance of rare  
373 micronuclei (Table 6; Supplementary Fig. 6H) and only 38 $\pm$ 6% malformed embryos (Fig. 3).  
374 Chlorpyrifos at 50  $\mu$ M also had low genotoxic effect (Table 6) and induced malformations in

375 13±7% of exposed embryos (Fig. 3). In the culture treated with 10 µM BPA only 14 out of 119  
376 embryos (11%) were scored as having DNA aberrations (Table 6).

377 We have also analyzed correlation between the percentage of embryos showing DNA aberrations,  
378 with the percentage of normal, malformed and undeveloped embryos (Table 7). According to  
379 Spearman's test, the presence of DNA aberrations in *Phallusia* embryos exhibited a strong  
380 negative correlation with the percentage of normal embryos in the culture ( $r = -0.93$ ,  $p < 0.05$ ) and  
381 showed not significant positive correlations with malformed ( $r = 0.5$ ,  $p > 0.05$ ) and undeveloped  
382 embryos ( $r = 0.35$ ,  $p > 0.05$ ).

383

#### 384 4. DISCUSSION

385 We present here a test for developmental toxicity based on larval phenotypes of *Phallusia*  
386 *mammillata* embryos. Previous toxicological studies using ascidian embryos lacked standardized  
387 endpoints and protocols for quantification of larval malformations (Dumollard et al., 2017). The  
388 teratogenic potential of the tested xenobiotics was assessed by high content analysis of  
389 neurodevelopmental endpoints such as pigment sensory organs (PSO) area, distance between  
390 ocellus and otolith, rate of embryos with palps, as well as general morphogenesis endpoints such  
391 as trunk length/width ratio and tail length. We implemented serial embryonic cultures, imaged and  
392 analysed them by scoring quantitative and qualitative endpoints. Transparency of *Phallusia* larvae  
393 allows assessment of neurodevelopmental disorders without additional staining. This feature  
394 distinguish our model from existing tests on marine invertebrate embryos (Sarkar et al., 2006). Our  
395 assay also affords to determine the genotoxic potential of tested compounds in the same cohort of  
396 embryos, to correlate genotoxicity with teratogenicity.

397 Results obtained in the current study show that exposure to toxicants may either induce a  
398 phenotype restricted to one or two endpoints or result in **malformations resembling a** delay in  
399 embryonic development (when all endpoints are affected, Supplementary Fig. 3-5). Such delay in  
400 embryonic development in response to stress or DNA damage is well described in the literature  
401 (Häder et al., 2011; Chiarelli et al., 2019). Therefore **phenotypes that show 4 or 5 affected**  
402 **endpoints were considered either non-specific or indicative of DNA damage.** The distance between  
403 Oc and Ot is the most **sensitive** endpoint as it was affected by most treatments. The reduction of  
404 Oc/Ot distance can be used to determine LOEC values of the compounds. **In contrast, specific**  
405 **phenotypes, such as the embryos without palps but with unaffected PSO or tail length, could be**  
406 **characteristic of some toxicants.** For example, the rate of embryos with palps is mostly affected

407 upon exposure to RAR/RXR ligands and the absence of palps may thus be a good indicator of  
408 alterations in the RAR/RXR pathway. Conversely, tail length was the least affected parameter  
409 demonstrating that tail extension is prevented only at high level of toxicity or in cases of  
410 genotoxicity.

411

#### 412 **4.1. Phenotypes of general toxicants and genotoxicants**

413 **Etoposide, mitomycin, and TBT have been shown to induce DNA damage in numerous test**  
414 **systems (reviewed in Kirkland et al., 2016).** Our present protocol couples the scoring of DNA  
415 aberrations with the morphometric analysis of larval phenotypes, in order to estimate the impact  
416 of genotoxicity on teratogenicity. DNA problems can quickly accumulate in ascidian embryos as  
417 the early embryo proliferates without an active spindle assembly checkpoint (Chenevert et al.,  
418 2020). Therefore, at gastrula and neurula stages DNA aberrations can be easily observed with  
419 nuclei staining (in our study with Hoechst). Previous studies on sea urchin (Saotome et al., 1999)  
420 and zebrafish (Le Bihanic et al., 2016) showed that mitomycin induced micronuclei formation at  
421 concentrations 5 µg/mL and 0.03 µg/mL respectively. In our study, we observed genotoxicity of  
422 mitomycin only at 60 µM (20 µg/mL). The longer exposure time in both sea urchin and zebrafish  
423 protocols may explain their higher sensitivity. Nevertheless, our approach allowed us to see several  
424 types of DNA damage (micronuclei, multinucleated cells, DNA bridges) and correlate them with  
425 embryonic phenotypes in a shorter time.

426 DNA damage was associated with a significant reduction of all studied endpoints. Reduction of  
427 all endpoints by SA, TBT, etoposide and mitomycin resulted in phenotypes **resembling** untreated  
428 control embryos at stages 22-23. Interestingly, TBT and SA which are known to have cytotoxic

429 effects (Oyanagi et al., 2015; Weyermann et al., 2005) induced formation of multinucleated cells  
430 and micronuclei, whereas clastogenic genotoxicants (mitomycin C and etoposide) led to formation  
431 of DNA bridges. Formation of DNA bridges in the whole embryo at gastrula stage led to complete  
432 arrest in development (see etoposide 10  $\mu$ M, Fig. 2).

433 TBT has been shown to affect embryo development in invertebrates (Alzieu, 2000; Gallo, Tosti,  
434 2013; Matthiessen, 2019) and to slow down development rate in vertebrates (Bentivegna and  
435 Piatkowski, 1998). Bellas et al (2005) found that TBT affects embryo development of the ascidian  
436 *Ciona intestinalis* with EC<sub>50</sub> at 7.1  $\mu$ g/l (22 nM); our study revealed significant changes in PSO  
437 parameters at a similar range of concentrations (10 – 50 nM TBT). Even though no DNA  
438 aberrations were observed at 10 nM TBT (data not shown), the teratogenicity of such a dose of  
439 TBT was detected as a reduction in PSO area and trunk L/W ratio. Higher concentrations of TBT  
440 (50 nM) induced micronuclei and multinucleated cells, demonstrating DNA damage associated  
441 with reduction of the ratio of normal embryos in the culture.

442 Previous study have shown that 5 mM SA inhibited myoplasmic reorganization and establishment  
443 of anteroposterior axis in embryos of *Ciona intestinalis* (Ishii et al., 2014). In our study, embryos  
444 of *Phallusia* were significantly affected by 1 – 2.5 mM SA and DNA damage was observed after  
445 exposure to 2.5 mM SA. The phenotypes observed in *Ciona* embryos exposed to 5 mM SA must  
446 then be due to the strong genotoxic effect of SA at this dose.

447 Altogether, our observations show that mild DNA damage is reflected in micronuclei at the  
448 gastrula/neurula stage and such DNA damage is permissive for development up to stage 23 (TBT,  
449 SA). Ascidian embryos can also tolerate appearance of DNA bridges in the epidermis (mitomycin)  
450 or in parts of the embryo (etoposide 100 nM). However, extensive DNA damage caused by

451 accumulation of double strand breaks and formation of DNA bridges throughout the embryo  
452 prevents development past stage 10 (gastrula). There was a strong negative correlation between  
453 DNA damage and the percentage of normal embryos in the culture. Our study thus allows to  
454 speculate that a high percentage of undeveloped embryos is indicative of strong genotoxicity of a  
455 tested compound. In contrast observing embryos arrested at stage 22 (and before PSO  
456 pigmentation) would indicate medium genotoxicity of the tested compounds. Nevertheless, further  
457 studies are needed to confirm this finding.

458 The highest tested concentrations of xenobiotics (20  $\mu$ M lindane, 50  $\mu$ M chlordane, and 50  $\mu$ M  
459 chlorpyrifos, 10  $\mu$ M BPA) induced formation of micronuclei and the percentage of embryos with  
460 DNA problems correlated positively with the percentage of embryos with malformations (Tables  
461 6-7, Fig. 3). However, these concentrations of pesticides induced reduction of at least three studied  
462 morphological parameters, but did not lead to the same phenotypes like genotoxicants. Our  
463 previous studies showed severe genotoxicity of BPA at 40  $\mu$ M (Gomes et al., 2019b); however, a  
464 small number of embryos with micronuclei was detected already at 10  $\mu$ M (Table 6). In contrast,  
465 atrazine did not induce any detectable DNA damage, and only two endpoints were affected.

466 Taken together these results suggest that high concentrations of EDCs may induce DNA damage,  
467 which severely affects embryo development. However, most of these xenobiotics (lindane,  
468 atrazine, chlordane, chlorpyrifos, and BPA) induced embryonic phenotypes at lower  
469 concentrations than the ones inducing mild DNA damage, indicating that their main mechanism  
470 of action is not related to genotoxicity.

471

## 472 **4.2. Phenotypes of EDCs and NRs ligands**



473 We compared the phenotypic signature of EDCs with that of known NR ligands because a major  
474 mode of action (MoA) of EDCs is via modification of NR activity. The presence of palps was  
475 sensitive to RXR and RAR inhibition and activation, but because other NR ligands had overlapping  
476 phenotypes it was not possible to find other specific phenotypic signatures.

477 Previous studies have shown that the retinoic acid (RA) signaling pathway regulates axial  
478 patterning and neurogenesis in the developing central and peripheral nervous systems of chordates  
479 including ascidians (Fujiwara and Kawamura, 2003; Nagatomo et al., 2003; Zieger et al., 2018).  
480 RA signal is mediated by an RA-binding heterodimeric transcription factor consisting of RAR and  
481 RXR (Mangelsdorf and Evans, 1995). Both receptors are expressed in neurogenic domains of  
482 ascidian embryos (Nagatomo et al., 2003, Gomes et al., 2019a), thus their ligands can most likely  
483 affect ascidian larval brain formation (Nagatomo et al., 2003). In the current study we analyzed  
484 the effect of the RAR agonist all-trans retinoic acid (ATRA), of the RAR antagonist BMS493 and  
485 of the RXR antagonist UVI3003, on *Phallusia* embryos. All compounds completely blocked  
486 formation of the adhesive palps and slightly but consistently increased the distance between PSO.  
487 These results are in agreement with previous studies (Dumollard et al., 2017; Nagatomo et al.,  
488 2003) showing similar phenotypes in ascidian embryos treated with ATRA. The similar  
489 phenotypes induced by activation and inactivation of RAR can be explained by a downregulation  
490 of RA metabolism in ATRA-exposed tailbud embryo (Nagatomo et al., 2003) as found in the  
491 mouse (Lee et al., 2012) but this hypothesis should be confirmed in ascidians.

492 The RAR-orphan receptor (ROR) is ubiquitously expressed from the 16-cell to the tailbud stage  
493 (Gomes et al., 2019a). In the tadpole, ROR expression is observed in the anterior region adjacent  
494 to the brain, suggested to give rise to the future oral siphon in the adult (Gomes et al., 2019a;  
495 Tolkin and Christiaen, 2016; Veeman et al., 2010). The ROR agonist SR1078 slightly reduced

496 PSO area and trunk L/W ratio at 2  $\mu$ M, whereas higher concentration affected almost all studied  
497 endpoints (except tail length), indicating non-specific toxicity. This suggests that ROR might  
498 participate in PSO development but this should be confirmed by specific ROR knockdown.

499 It is known that the vertebrate pregnane X receptor (PXR) is a member of the NR super-family  
500 which regulates transcription of genes involved in the metabolism and excretion of endogenous  
501 and exogenous toxic compounds (Fidler et al., 2012). PXR transcriptional targets in mammals  
502 include the cytochrome P450 (CYP) 2C and 3A enzymes, and transporters such as multidrug  
503 resistant protein (MDR, ABCB1) (Reschly et al., 2007). Those proteins in turn regulate, transport  
504 and metabolize xenobiotics, steroid hormones, and vitamins. In ascidians, such as *Ciona*  
505 *intestinalis* and *Phallusia mammillata*, genome encodes an orthologue of the vertebrate PXR and  
506 vitamin D receptor (VDR), denoted VDR/PXR (Yagi et al., 2003). Our previous study indicate  
507 that, in *Phallusia*, VDR/PXR is expressed in the brain region of embryos and larvae (Gomes et al.,  
508 2019a). As predicted, the current study shows significant changes in PSO development and trunk  
509 elongation after exposure to lindane and chlordane (PXR ligands according to Lemaire et al.,  
510 2004), and PXR agonists (rifampicin and SR12813). Functional inactivation of PXR in ascidians  
511 is now required to confirm the role of PXR in PSO development and trunk elongation.

512 Ascidians possess a single copy of the PPAR gene (Yagi et al., 2003), which is expressed in few  
513 cells within the ascidian brain, in the vicinity of the PSO (Gomes et al., 2019a). Interestingly, a  
514 synthetic ligand of PPAR $\gamma$ , BADGE (Wright et al., 2000), showed significant effect on PSO  
515 development at 1 – 5  $\mu$ M. Similar **cocentrations** of BADGE also caused neurodevelopmental  
516 toxicity in an amphibian (*Rhinella arenarum*, EC<sub>50</sub> = 0.5  $\mu$ M) (Hutler et al., 2016).

517 The majority of EDCs act primarily via steroid receptors (UNEP/WHO, 2013) but ascidians lack  
518 steroid binding nuclear receptors such as ER or AR (Yagi et al., 2003). As expected, estradiol  
519 benzoate (which binds only ER) was not toxic to ascidian embryos. The xenoestrogens DES,  
520 4OHT and BPA all inhibited PSO development potentially via inhibition of the estrogen related  
521 receptor (ERR) which is expressed in the PSO of the ascidian larva (Gomes et al., 2019b). Indeed,  
522 even though ERR is an orphan NR, synthetic molecules such as DES and 4-OHT can directly bind  
523 ascidian (Park et al., 2009) and vertebrate ERRs (Coward et al., 2001, Gibert et al., 2011), while  
524 BPA binds vertebrate ERR $\gamma$  (Tohmé et al., 2014). Furthermore, ERR plays important roles in the  
525 development of the vertebrate brain and sensory organs (Hermans-Borgmeyer et al., 2000; Lim et  
526 al., 2015; Chen and Nathans, 2007). Together, our observations suggest that ERR is involved in  
527 ascidian larval brain formation (see also Gomes et al., 2019b). The xenobiotics targeting ERR  
528 (such as BPA, DES or 4OHT) are predicted to exert neurodevelopmental toxicity in ascidians.  
529 However, since PSO development was affected also by other EDCs and other NR ligands, further  
530 studies should clarify the role of ERR in PSO development.

531

## 532 **5. CONCLUSION**

533 Overall we conclude that *Phallusia mammillata* is a suitable and sensitive model for toxicity  
534 screening. The ability to easily assess neural development in the transparent *Phallusia* larva also  
535 offers to assess the impact on neurodevelopment of sublethal **concentrations** of toxicants shedding  
536 light on potential mechanisms of teratogenicity or mode of actions of toxicants. The proposed  
537 embryo/larval assay allows a fast and quantitative evaluation of teratogenic effects of xenobiotics  
538 on ascidian embryo and a correlation with the extent of genotoxicity.

539 Analysis of embryo phenotypes induced by different compounds indicate that severe  
540 malformations resembling developmental delay are associated with genotoxicity. In contrast,  
541 compounds like EDCs and NR ligands are impacting mostly neurodevelopmental endpoints. This  
542 study sets the stage for quantitative analysis of embryo phenotypes that could be used to study not  
543 only the teratogenicity of NR-targeting EDCs, but also the potential effects of pollutants that can  
544 be dissolved in sea water.

545 Functional studies are needed to characterize *Phallusia* embryonic phenotypes observed after  
546 activation or inhibition of NRs, and consequently compare these phenotypes with phenotypes  
547 induced by a library of compounds, as a first step for performing predictive toxicology on marine  
548 invertebrate organisms.

549

#### 550 **Declaration of interest**

551 We declare that we have no financial or non-financial competing interests.

552

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**Table 1.** List of chemicals used for toxicity screening and concentrations analyzed in the current study, listed in the order presented on figures 2 - 4. The LOEC is the lowest tested concentration (experimental) that is significantly different from the control as determined by one or several endpoints (Kruskal-Wallis test,  $P < 0.05$ ). Last column mention the affected endpoint at LOEC.

<b>Chemical name</b>	<b>Doses assessed in the current study</b>	<b>LOEC<sup>1</sup></b>	<b>Affected endpoints at LOEC</b>
<b>Sodium azide</b>	0.1 – 2.5mM	1 mM	PSO <sup>2</sup> area, PSO distance, Trunk L/W ratio <sup>3</sup> , Tail length
<b>Tributyltin</b>	10 – 50 nM	10 nM	PSO area, PSO distance, Trunk L/W ratio
<b>Etoposide</b>	50 nM – 10 $\mu$ M	50 nM	PSO area, PSO distance, Trunk L/W ratio
<b>Mitomycin C</b>	10 – 60 $\mu$ M	10 $\mu$ M	PSO distance, Trunk L/W ratio, Tail length
<b><math>\gamma</math>-BHC (Lindane)</b>	2.5 – 20 $\mu$ M	10 $\mu$ M	PSO distance, Trunk L/W ratio
<b>Atrazine</b>	1 – 70 $\mu$ M	50 $\mu$ M	PSO distance, Trunk L/W ratio
<b>Bisphenol A (BPA)</b>	1 – 50 $\mu$ M	1 $\mu$ M (Gomes et al., 2019b)	PSO area, PSO distance
<b>cis/trans-Chlordane</b>	10 - 50 $\mu$ M	10 $\mu$ M	PSO area, PSO distance
<b>Chlorpyrifos</b>	10 – 100 $\mu$ M	10 $\mu$ M	PSO area
<b>Estradiol benzoate (E2B)</b>	5 – 10 $\mu$ M	ND <sup>4</sup>	ND <sup>4</sup>

<b>ATRA (all trans retinoic acid)</b>	0.01 – 5 µM	0.1 µM	% embryos with palps, Trunk L/W ratio
<b>BMS493</b>	1 – 5 µM	3 µM	PSO area, % embryos with palps, Tail length
<b>UVI3003</b>	0.1 – 2 µM	1 µM	PSO area, % embryos with palps
<b>4-Hydroxytamoxifen (4HT)</b>	1 – 50 µM	5 µM	PSO distance
<b>Diethylstilbestrol (DES)</b>	0.1 – 25 µM	1 µM	PSO area, PSO distance, Trunk L/W ratio
<b>Rifampicin</b>	10 – 100 µM	50 µM	PSO distance, Trunk L/W ratio
<b>SR 1078</b>	0.1 – 5 µM	2 µM	PSO area
<b>BADGE</b>	1 – 100 µM	1 µM	Trunk L/W ratio
<b>SR 12813</b>	1.5 – 10 µM	3 µM	PSO area, PSO distance, Trunk L/W ratio

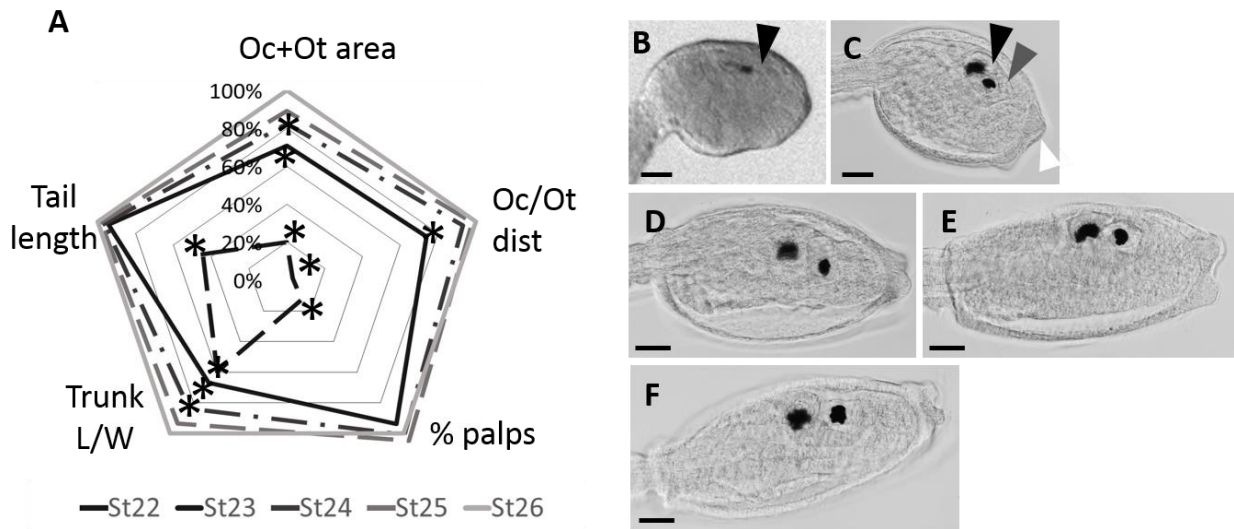
<sup>1</sup> LOEC - the lowest tested concentration (experimental) that is significantly different from the control as determined by one or several endpoints (Kruskal-Wallis test,  $P < 0.05$ ).

<sup>2</sup> PSO – pigmented sensory organs

<sup>3</sup> Trunk L/W ratio – trunk length / width ratio

<sup>4</sup>ND – not determined

**Figure 1.** Changes of morphological parameters during embryonic development (between stage 22 and stage 26). **A.** The measured endpoints are summarized in the radar chart depicting normalized values (normalized to stage 26 values): ocellus (Oc) + otolith (Ot) area ( $\mu\text{m}^2$ ); Oc/Ot distance ( $\mu\text{m}$ ); percentage of embryos with palps (%); trunk L/W (length/width) ratio; tail length ( $\mu\text{m}$ ). Measurement of all parameters was performed for min N=50 embryos. Asterisks indicate significant difference compared to the control (Kruskal-Wallis test,  $P < 0.05$ ). **B.** Trunk region at 18 hpf (stage 22). Black arrow shows formation of ocellus (Oc). **C.** Trunk region at 19 hpf (stage 23). Black arrow shows formation of ocellus (Oc), dark grey arrow shows formation of otolith (Ot) and separation of two pigmented sensory organs (PSO) at stage 23. White arrow indicates formation of palps at stage 23 **D.** Trunk region at 20 hpf (stage 24). **E.** Trunk region at 21 hpf (stage 25). **F.** Trunk region at 22 hpf (stage 26). Scale bars correspond to 20  $\mu\text{m}$



**Table 2.** Changes of morphological parameters during embryonic development. (between stage 22 and stage 26). The mean values  $\pm$  SD are presented. N is the number of embryos assessed at each stage. The line “St26 controls-2015-209” shows average values of morphological parameters from all cultures performed in this study.

Stage of embryo development	N	PSO area, $\mu\text{m}^2$	PSO distance, $\mu\text{m}$	Palps, % <sup>1</sup>	Trunk L/W ratio <sup>2</sup>	Tail length, $\mu\text{m}$
Stage 22	49	72 $\pm$ 24	0,8 $\pm$ 3	12	1,3 $\pm$ 0,1	237 $\pm$ 24
Stage 23	47	253 $\pm$ 64	18 $\pm$ 8	89	1,4 $\pm$ 0,1	506 $\pm$ 37
Stage 24	49	294 $\pm$ 47	23 $\pm$ 6	96	1,8 $\pm$ 0,3	516 $\pm$ 173
Stage 25	46	317 $\pm$ 41	24 $\pm$ 4	100	1,9 $\pm$ 0,2	527 $\pm$ 146
Stage 26	75	320 $\pm$ 47	24 $\pm$ 7	100	2 $\pm$ 0,2	535 $\pm$ 116
Stage 26, controls 2015-2019	4903	292 $\pm$ 142	20 $\pm$ 11	92 $\pm$ 3.2	2.6 $\pm$ 1.5	483 $\pm$ 162

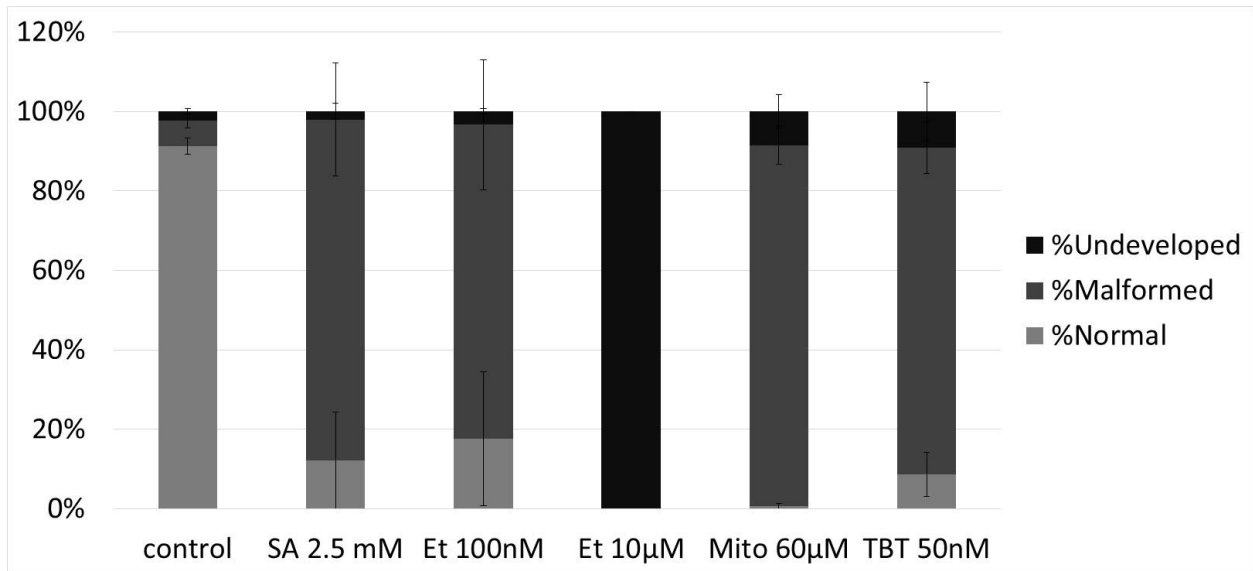
<sup>1</sup>Palps, % – the percentage embryos with palps;

<sup>2</sup>Trunk L/W ratio – trunk length / width ratio

**Table 3.** Morphometric analysis of phenotypes observed in *Phallusia mammillata* larvae exposed to toxicants. N shows the number of embryos analyzed. The table summarize the following endpoints: ocellus (Oc) + otolith (Ot) area ( $\mu\text{m}^2$ ); Oc/Ot distance ( $\mu\text{m}$ ); percentage of embryos with palps (%); trunk L/W (length/width) ratio; tail length ( $\mu\text{m}$ ). All measurements are performed at 22 hpf. Data presented as means  $\pm$  S.E and as % to the control. Asterisks indicate significant difference compared to the control (Kruskal-Wallis test,  $P < 0.05$ ). SA indicate sodium azide ; TBT – tributyltin.

Treatment	N	Oc/Ot area, $\mu\text{m}^2$ , mean $\pm$ S.E.	Oc/Ot area, % to the control	Oc/Ot distance, $\mu\text{m}$ , mean $\pm$ S.E.	Oc/Ot distance % to the control	% palps, means $\pm$ S.E.	Palps% to the control	Trunk L/W ratio, means $\pm$ S.E.	Trunk L/W % to the control	Tail length, $\mu\text{m}$ , means $\pm$ S.E.	Tail length % to the control
control	322	362.9 $\pm$ 8.1 -		19.7 $\pm$ 0.6 -		91.7 $\pm$ 3.2 -		2.3 $\pm$ 0.03 -		550.1 $\pm$ 4.6 -	
SA 100uM	317	355.3 $\pm$ 8.6	93%	19.4 $\pm$ 0.6	95%	90.1 $\pm$ 2.9	100%	2.3 $\pm$ 0.03	94%	531.1 $\pm$ 4.7	97%
SA 1mM	151	<b>268 <math>\pm</math> 16.6 *</b>	<b>58%</b>	<b>10.1 <math>\pm</math> 0.9 *</b>	<b>37%</b>	35.6 $\pm$ 31.5	37%	<b>1.7 <math>\pm</math> 0.03 *</b>	<b>67%</b>	<b>425.3 <math>\pm</math> 18.6 *</b>	<b>66%</b>
SA 2.5mM	115	<b>300.2 <math>\pm</math> 19.2 *</b>	<b>56%</b>	<b>4.1 <math>\pm</math> 0.9 *</b>	<b>19%</b>	<b>3.8 <math>\pm</math> 3.8 *</b>	<b>6%</b>	<b>1.2 <math>\pm</math> 0.01 *</b>	<b>57%</b>	<b>339.2 <math>\pm</math> 11.2 *</b>	<b>58%</b>
control	120	314.1 $\pm$ 12.4 -		21.7 $\pm$ 1.1 -		93.7 $\pm$ 3.7 -		2.2 $\pm$ 0.04 -		510.9 $\pm$ 12.2 -	
TBT 10nM	106	<b>186.8 <math>\pm</math> 13.5 *</b>	<b>60%</b>	<b>10.5 <math>\pm</math> 1.2 *</b>	<b>48%</b>	66.1 $\pm$ 14.5	72%	<b>1.6 <math>\pm</math> 0.05 *</b>	<b>73%</b>	477.7 $\pm$ 14.1	99%
TBT 20nM	133	<b>164.3 <math>\pm</math> 12.1 *</b>	<b>47%</b>	<b>9.4 <math>\pm</math> 1 *</b>	<b>38%</b>	46.4 $\pm$ 17.3	50%	<b>1.6 <math>\pm</math> 0.05 *</b>	<b>70%</b>	443.6 $\pm$ 13.3	79%
TBT 50nM	122	<b>40.8 <math>\pm</math> 7.5 *</b>	<b>10%</b>	<b>1.2 <math>\pm</math> 0.5 *</b>	<b>4%</b>	<b>13.3 <math>\pm</math> 12.3 *</b>	<b>14%</b>	<b>1.3 <math>\pm</math> 0.03 *</b>	<b>54%</b>	<b>302.3 <math>\pm</math> 13.3 *</b>	<b>49%</b>
control	211	298.1 $\pm$ 6.5 -		25.6 $\pm$ 0.6 -		98.8 $\pm$ 1.2 -		2.2 $\pm$ 0.02 -		433.8 $\pm$ 18.8 -	
Etoposide 50nM	168	<b>252.4 <math>\pm</math> 6.3 *</b>	<b>95%</b>	<b>21.3 <math>\pm</math> 0.8 *</b>	<b>80%</b>	98.8 $\pm$ 0.6	100%	<b>2 <math>\pm</math> 0.02 *</b>	<b>92%</b>	428.9 $\pm$ 20.3	97%
Etoposide 75nM	116	<b>221.3 <math>\pm</math> 7.4 *</b>	<b>86%</b>	<b>14.6 <math>\pm</math> 1.1 *</b>	<b>58%</b>	70.9 $\pm$ 16.9	73%	<b>1.7 <math>\pm</math> 0.03 *</b>	<b>86%</b>	463.7 $\pm$ 19.2	92%
Etoposide 100nM	139	<b>120.8 <math>\pm</math> 7.7 *</b>	<b>44%</b>	<b>4.2 <math>\pm</math> 0.7 *</b>	<b>18%</b>	<b>20.1 <math>\pm</math> 18.7 *</b>	<b>21%</b>	<b>1.3 <math>\pm</math> 0.02 *</b>	<b>63%</b>	<b>253.2 <math>\pm</math> 16.1 *</b>	<b>56%</b>
control	410	366.3 $\pm$ 5.4 -		20.6 $\pm$ 0.5 -		93.6 $\pm$ 1.5 -		2.4 $\pm$ 0.01 -		443.9 $\pm$ 1.5 -	
Mitomycin C 10 $\mu\text{M}$	361	340.8 $\pm$ 6.7	95%	<b>15.7 <math>\pm</math> 0.7 *</b>	<b>81%</b>	56.5 $\pm$ 11.7	41%	<b>1.5 <math>\pm</math> 0.01 *</b>	<b>56%</b>	<b>317.4 <math>\pm</math> 4.7 *</b>	<b>93%</b>
Mitomycin C 40 $\mu\text{M}$	284	285.6 $\pm$ 10.3	96%	<b>10.3 <math>\pm</math> 1 *</b>	<b>44%</b>	<b>12.4 <math>\pm</math> 6.2 *</b>	<b>15%</b>	<b>1.3 <math>\pm</math> 0.01 *</b>	<b>46%</b>	<b>234.4 <math>\pm</math> 5.8 *</b>	<b>71%</b>
Mitomycin C 60 $\mu\text{M}$	165	<b>226 <math>\pm</math> 13.4 *</b>	<b>62%</b>	<b>4.4 <math>\pm</math> 0.9 *</b>	<b>32%</b>	<b>0.3 <math>\pm</math> 0.3 *</b>	<b>0%</b>	<b>1.2 <math>\pm</math> 0.01 *</b>	<b>44%</b>	<b>421.5 <math>\pm</math> 2.6 *</b>	<b>59%</b>

**Figure 2.** The percentage of normal (light grey), malformed (dark grey) and undeveloped (black) embryos in the sodium azide (SA) 2.5 mM, etoposide (Et) 100 nM and 10  $\mu$ M, mitomycin 60  $\mu$ M, tributyltin (TBT) 50 nM. Data are presented as means  $\pm$  SEM.

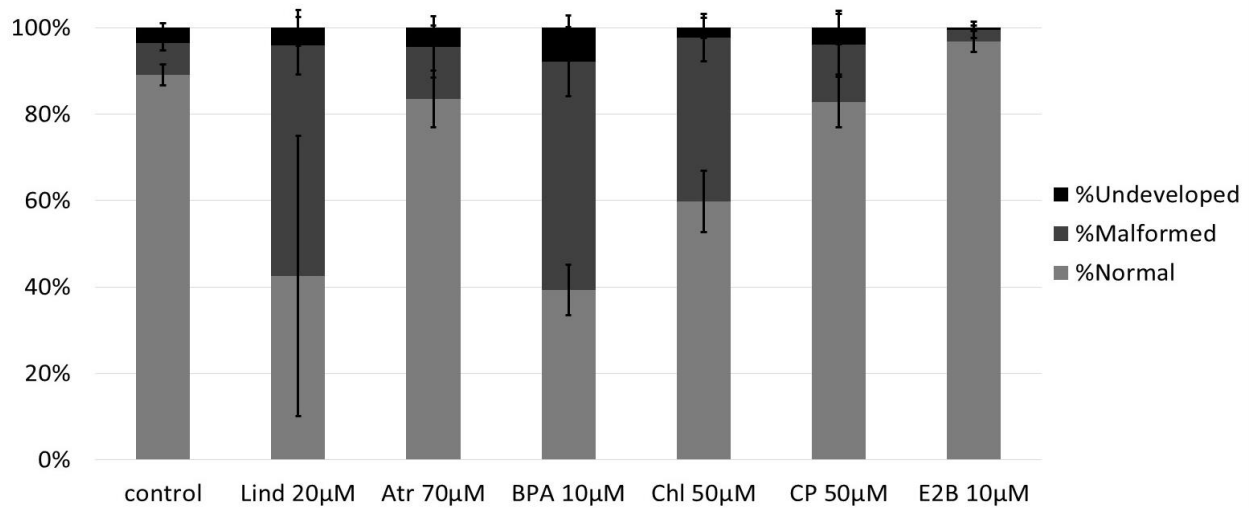




**Table 4.** Morphometric analysis of phenotypes observed in *Phallusia mammillata* larvae exposed to EDCs. N shows the number of embryos analyzed. The table summarize the following endpoints: ocellus (Oc) + otolith (Ot) area ( $\mu\text{m}^2$ ); Oc/Ot distance ( $\mu\text{m}$ ); percentage of embryos with palps (%); trunk L/W (length/width) ratio; tail length ( $\mu\text{m}$ ). All measurements are performed at 22 hpf. Data presented as means  $\pm$  S.E and as % to the control. Asterisks indicate significant difference compared to the control (Kruskal-Wallis test,  $P < 0.05$ ). BPA indicate bisphenol A ; E2B – estradiol benzoate.

Treatment	N	Oc/Ot area, $\mu\text{m}^2$ , mean $\pm$ S.E.	Oc/Ot area % to the control	Oc/Ot distance, $\mu\text{m}$ , mean $\pm$ S.E.	Oc/Ot distance % to the control	% palps, means $\pm$ S.E.	Palps% to the control	Trunk L/W ratio, means $\pm$ S.E.	Trunk L/W % to the control	Tail length, $\mu\text{m}$ , means $\pm$ S.E.	Tail length % to the control
control	112	321.1 $\pm$ 14.8-		20.3 $\pm$ 1.2-		80.1 $\pm$ 1.6-		2.2 $\pm$ 0.04-		499.5 $\pm$ 12.5-	
Lindane 10 $\mu\text{M}$	136	286.7 $\pm$ 12.2	92%	<b>12.4 <math>\pm</math> 1.1 *</b>	<b>62%</b>	68 $\pm$ 4.5	85%	<b>1.7 <math>\pm</math> 0.02 *</b>	<b>77%</b>	489 $\pm$ 8	95%
Lindane 15 $\mu\text{M}$	139	<b>224.4 <math>\pm</math> 12.2 *</b>	<b>71%</b>	<b>8.8 <math>\pm</math> 1 *</b>	<b>44%</b>	60.6 $\pm$ 11.1	75%	<b>1.5 <math>\pm</math> 0.02 *</b>	<b>70%</b>	476.2 $\pm$ 5.4	92%
Lindane 20 $\mu\text{M}$	56	<b>245.2 <math>\pm</math> 16.7 *</b>	<b>83%</b>	<b>8.2 <math>\pm</math> 1.3 *</b>	<b>36%</b>	52.2 $\pm$ 17.2	66%	<b>1.6 <math>\pm</math> 0.04 *</b>	<b>75%</b>	443.9 $\pm$ 19.4	85%
control	193	293.5 $\pm$ 11.2-		17.1 $\pm$ 0.9-		77.1 $\pm$ 7.4-		1.9 $\pm$ 0.03-		520.1 $\pm$ 11-	
Atrazine 25 $\mu\text{M}$	172	262.4 $\pm$ 10.8	87%	15.2 $\pm$ 1	96%	78.9 $\pm$ 8.5	101%	1.8 $\pm$ 0.03	94%	525 $\pm$ 9.5	101%
Atrazine 50 $\mu\text{M}$	160	274.1 $\pm$ 9.8	95%	<b>14.1 <math>\pm</math> 1 *</b>	<b>83%</b>	75.1 $\pm$ 6.4	102%	<b>1.7 <math>\pm</math> 0.03 *</b>	<b>92%</b>	500.4 $\pm$ 10.1	99%
Atrazine 70 $\mu\text{M}$	112	277.5 $\pm$ 11.7	87%	<b>13.2 <math>\pm</math> 1.2 *</b>	<b>69%</b>	85.7 $\pm$ 10.7	108%	<b>1.8 <math>\pm</math> 0.03 *</b>	<b>87%</b>	554.5 $\pm$ 7.2	101%
control	387	294.4 $\pm$ 6.8-		20.4-		84.1 $\pm$ 5.3-		2.1 $\pm$ 0.02-		535.7 $\pm$ 5.9-	
BPA 5 $\mu\text{M}$	165	<b>124.8 <math>\pm</math> 5.7 *</b>	<b>50%</b>	<b>7.6 <math>\pm</math> 0.8 *</b>	<b>38%</b>	95.9 $\pm$ 1.4	98%	2.3 $\pm$ 0.03	96%	574.1 $\pm$ 6.1	101%
BPA 10 $\mu\text{M}$	343	<b>91.6 <math>\pm</math> 5.3 *</b>	<b>35%</b>	<b>4.4 <math>\pm</math> 0.5 *</b>	<b>26%</b>	63.7 $\pm$ 10	75%	<b>1.8 <math>\pm</math> 0.02 *</b>	<b>83%</b>	486.1 $\pm$ 6.1	90%
control	195	285.6 $\pm$ 10.5-		17.0-		78 $\pm$ 5.3-		1.7 $\pm$ 0.03-		521.9 $\pm$ 8.3-	
Chlordane 10 $\mu\text{M}$	131	<b>208.9 <math>\pm</math> 11.9 *</b>	<b>86%</b>	<b>11.7 <math>\pm</math> 1.1 *</b>	<b>73%</b>	52.2 $\pm$ 7.9	72%	<b>1.6 <math>\pm</math> 0.03 *</b>	<b>94%</b>	476.6 $\pm$ 10.6	93%
Chlordane 25 $\mu\text{M}$	101	<b>199.9 <math>\pm</math> 11.9 *</b>	<b>84%</b>	<b>10.5 <math>\pm</math> 1.2 *</b>	<b>64%</b>	69.9 $\pm$ 10.1	95%	<b>1.6 <math>\pm</math> 0.03 *</b>	<b>96%</b>	490.7 $\pm$ 10.6	93%
Chlordane 50 $\mu\text{M}$	105	<b>175 <math>\pm</math> 11.5 *</b>	<b>65%</b>	<b>6.6 <math>\pm</math> 1.1 *</b>	<b>25%</b>	65 $\pm$ 12.3	85%	<b>1.5 <math>\pm</math> 0.02 *</b>	<b>92%</b>	450.6 $\pm$ 13.1	86%
control	281	336 $\pm$ 6.2-		21.2 $\pm$ 0.6-		91.1 $\pm$ 3.1-		2.4 $\pm$ 0.03-		562.3 $\pm$ 4-	
Chlorpyrifos 10 $\mu\text{M}$	274	<b>285.6 <math>\pm</math> 6.3 *</b>	<b>85%</b>	18.1 $\pm$ 0.7	76%	77.9 $\pm$ 8	85%	<b>2.2 <math>\pm</math> 0.04 *</b>	<b>89%</b>	523.8 $\pm$ 7.2	90%
Chlorpyrifos 25 $\mu\text{M}$	152	<b>292.3 <math>\pm</math> 6.6 *</b>	<b>83%</b>	21.7 $\pm$ 0.9	84%	85.9 $\pm$ 8.3	90%	2.4 $\pm$ 0.04	94%	544.8 $\pm$ 8.5	91%
Chlorpyrifos 50 $\mu\text{M}$	216	<b>245.1 <math>\pm</math> 7.2 *</b>	<b>70%</b>	<b>13.8 <math>\pm</math> 0.8 *</b>	<b>52%</b>	69 $\pm$ 13.3	80%	<b>2.1 <math>\pm</math> 0.03 *</b>	<b>76%</b>	<b>464 <math>\pm</math> 8.5 *</b>	<b>66%</b>
control	193	379.5 $\pm$ 9.5-		24.9 $\pm$ 0.7-		92.8 $\pm$ 4.5-		2.6 $\pm$ 0.03-		461.1 $\pm$ 22.5-	
E2B 5 $\mu\text{M}$	159	427.6 $\pm$ 4.4	96%	20.7 $\pm$ 0.7	85%	98.5 $\pm$ 1.5	101%	2.3 $\pm$ 0.01	95%	416.1 $\pm$ 22.1	94%
E2B 10 $\mu\text{M}$	165	353.8 $\pm$ 8.6	95%	20.8 $\pm$ 0.9	94%	93.7 $\pm$ 5.6	101%	2.4 $\pm$ 0.03	96%	482.6 $\pm$ 22.8	104%

**Figure 3.** The percentage of normal (light grey), malformed (dark grey) and undeveloped (black) embryos in the lindane (Lind) 20  $\mu$ M, atrazine (Atr) 70  $\mu$ M, bisphenol A (BPA) 10  $\mu$ M, chlordane (Chl) 50  $\mu$ M, chlorpyrifos (CP) 50  $\mu$ M, estradiol benzoate (E2B) 10  $\mu$ M. Data are presented as means  $\pm$  SEM.



**Table 5.** Morphometric analysis of phenotypes observed in *Phallusia mammillata* larvae exposed to agonists/antagonists of nuclear receptors (NR). N shows the number of embryos analyzed. The table summarize the following endpoints: ocellus (Oc) + otolith (Ot) area ( $\mu\text{m}^2$ ); Oc/Ot distance ( $\mu\text{m}$ ); percentage of embryos with palps (%); trunk L/W (length/width) ratio; tail length ( $\mu\text{m}$ ). All measurements are performed at 22 hpf. Data presented as means  $\pm$  S.E and as % to the control. Asterisks indicate significant difference compared to the control (Kruskal-Wallis test,  $P < 0.05$ ). ATRA indicate all trans retinoic acid ; 4-OHT – 4-Hydroxytamoxifen ; DES – diethylstilbestrol ; BADGE – Bisphenol A diglycidyl ether.

Treatment	N	Oc/Ot area, $\mu\text{m}^2$ , mean $\pm$ S.E.	Oc/Ot area % to the control	Oc/Ot distance, $\mu\text{m}$ , mean $\pm$ S.E.	Oc/Ot distance % to the control	% palps, means $\pm$ S.E.	Palps % to the control	Trunk L/W ratio, means $\pm$ S.E.	Trunk L/W % to the control	Tail length, $\mu\text{m}$ , means $\pm$ S.E.	Tail length % to the control
control	468	140.8 $\pm$ 3.1-		14.1 $\pm$ 0.4-		77.1 $\pm$ 2.1-		1.7 $\pm$ 0.02-		416.1 $\pm$ 1.7-	
ATRA 0.1 $\mu\text{M}$	385	130.8 $\pm$ 4.5	104%	14 $\pm$ 0.6	120%	32.7 $\pm$ 6.3 *	36%	1.4 $\pm$ 0.01 *	84%	404.5 $\pm$ 2.4	97%
ATRA 0.5 $\mu\text{M}$	358	114.7 $\pm$ 4.1 *	103%	11.7 $\pm$ 0.6	122%	15.9 $\pm$ 6.3 *	23%	1.4 $\pm$ 0.01 *	89%	398.5 $\pm$ 2.2	98%
ATRA 1 $\mu\text{M}$	135	162.9 $\pm$ 9.9	114%	15.5 $\pm$ 1.2	113%	4.3 $\pm$ 2.5 *	6%	1.4 $\pm$ 0.02 *	85%	410.2 $\pm$ 4.9	98%
control	276	137.9 $\pm$ 3.6-		13.8 $\pm$ 0.5-		73.5 $\pm$ 3.8-		1.5 $\pm$ 0.02-		400 $\pm$ 2.3-	
BMS493 1 $\mu\text{M}$	241	127.9 $\pm$ 4.3	103%	12.3 $\pm$ 0.6	101%	54 $\pm$ 5	74%	1.6 $\pm$ 0.02	99%	395.1 $\pm$ 2.6	97%
BMS493 3 $\mu\text{M}$	100	108.6 $\pm$ 5.9 *	87%	11.3 $\pm$ 1.1	110%	19.8 $\pm$ 7.7 *	16%	1.4 $\pm$ 0.02	100%	366.8 $\pm$ 8.2 *	94%
BMS493 5 $\mu\text{M}$	60	105.7 $\pm$ 6.2 *	70%	11.1 $\pm$ 1.2	94%	10.6 $\pm$ 6.6 *	6%	1.4 $\pm$ 0.02 *	90%	359.9 $\pm$ 6.6 *	87%
control	139	129.5 $\pm$ 4.9-		13.6 $\pm$ 0.7-		80.4 $\pm$ 5.1-		1.4 $\pm$ 0.02-		356.8 $\pm$ 10.7-	
UVI3003 0,1 $\mu\text{M}$	94	130.2 $\pm$ 6.1	94%	15 $\pm$ 1.1	125%	48 $\pm$ 3.2	53%	1.5 $\pm$ 0.03	95%	330.2 $\pm$ 14.3	96%
UVI3003 1 $\mu\text{M}$	141	108 $\pm$ 4.9 *	86%	10.3 $\pm$ 0.8	88%	22.7 $\pm$ 8.5 *	40%	1.5 $\pm$ 0.02	97%	318.2 $\pm$ 12.7	100%
UVI3003 2 $\mu\text{M}$	158	102.3 $\pm$ 4.5 *	75%	10.3 $\pm$ 1.1	82%	8.3 $\pm$ 3.3 *	23%	1.4 $\pm$ 0.02 *	92%	237.3 $\pm$ 11.5 *	63%
control	375	322 $\pm$ 6-		22.9 $\pm$ 0.5-		95.4 $\pm$ 3.6-		2.6 $\pm$ 0.03-		478 $\pm$ 14-	
A-OHT 2.5 $\mu\text{M}$	128	391.1 $\pm$ 7.2 *	103%	20.6 $\pm$ 1.1	96%	95.6 $\pm$ 4.4	108%	2.2 $\pm$ 0.03	93%	471.5 $\pm$ 23.4	100%
4-OHT 5 $\mu\text{M}$	269	290.7 $\pm$ 9.7	88%	12.8 $\pm$ 0.9 *	51%	72.8 $\pm$ 4.9 *	79%	2.2 $\pm$ 0.04 *	83%	416.8 $\pm$ 17 *	88%
4-OHT 10 $\mu\text{M}$	112	365.7 $\pm$ 12.6	100%	13.5 $\pm$ 1.3 *	60%	78.4 $\pm$ 10.5	87%	1.9 $\pm$ 0.03 *	77%	413.9 $\pm$ 25.1 *	88%
control	550	326.2 $\pm$ 5.4-		19.7 $\pm$ 0.5-		90.4 $\pm$ 4.3-		2.3 $\pm$ 0.02-		483.7 $\pm$ 10.5-	
DES 1 $\mu\text{M}$	464	285.4 $\pm$ 7.1 *	84%	12.9 $\pm$ 0.6 *	64%	76.9 $\pm$ 7.8	85%	1.9 $\pm$ 0.02 *	80%	408.4 $\pm$ 10	87%
DES 2 $\mu\text{M}$	125	155.5 $\pm$ 11.7 *	48%	8.9 $\pm$ 1 *	44%	49.7 $\pm$ 11.6	52%	1.6 $\pm$ 0.03 *	69%	377.6 $\pm$ 17.3 *	73%
control	81	280.8 $\pm$ 18.9-		15.1 $\pm$ 1.5-		74.1 $\pm$ 5.4-		1.8 $\pm$ 0.07-		396.7 $\pm$ 31.3-	
Rifampicin 50 $\mu\text{M}$	97	308.6 $\pm$ 18.9	100%	9.9 $\pm$ 1.2 *	53%	55.4 $\pm$ 19.9	94%	1.6 $\pm$ 0.05 *	73%	328.2 $\pm$ 20.6	73%
Rifampicin 100 $\mu\text{M}$	72	236 $\pm$ 18.8	94%	3.1 $\pm$ 1 *	23%	42.3 $\pm$ 18.7	72%	1.3 $\pm$ 0.04 *	70%	266.3 $\pm$ 27.5	75%
control	306	274.5 $\pm$ 7.2-		21.8 $\pm$ 0.7-		81.1 $\pm$ 8.5-		2.1 $\pm$ 0.02-		434.1 $\pm$ 17-	
SR1078 1 $\mu\text{M}$	320	262.5 $\pm$ 5.6	99%	19.7 $\pm$ 0.7	81%	85.8 $\pm$ 5	109%	2.1 $\pm$ 0.02	99%	467.4 $\pm$ 13.4	104%
SR1078 2 $\mu\text{M}$	223	255.7 $\pm$ 5.4 *	90%	22.4 $\pm$ 0.7	89%	92.8 $\pm$ 2.3	98%	2.1 $\pm$ 0.02 *	93%	447 $\pm$ 15.8	94%
SR1078 5 $\mu\text{M}$	248	159.1 $\pm$ 7.1 *	63%	7.8 $\pm$ 0.7 *	34%	51.2 $\pm$ 9.9	65%	1.6 $\pm$ 0.02 *	76%	374.8 $\pm$ 14 *	83%
control	131	322.3 $\pm$ 12.7-		21.3 $\pm$ 1.1-		82.8 $\pm$ 5.9-		2.2 $\pm$ 0.03-		447.5 $\pm$ 22.8-	
SR12813 3 $\mu\text{M}$	137	251.7 $\pm$ 10.3 *	80%	13.9 $\pm$ 1.1 *	55%	77.4 $\pm$ 8.9	91%	2 $\pm$ 0.02 *	84%	451.4 $\pm$ 18.5	101%
SR12813 5 $\mu\text{M}$	225	185.4 $\pm$ 8.2 *	63%	7.5 $\pm$ 0.8 *	32%	54.1 $\pm$ 14.4	63%	1.7 $\pm$ 0.02 *	72%	415.7 $\pm$ 14.2	93%
SR12813 7.5 $\mu\text{M}$	115	168.6 $\pm$ 12.2 *	39%	13.3 $\pm$ 1.3 *	33%	17.8 $\pm$ 11.2 *	41%	1.8 $\pm$ 0.03 *	70%	376.9 $\pm$ 24	84%
control	223	340.4 $\pm$ 10.2-		20.4 $\pm$ 0.8-		85.6 $\pm$ 9.5-		2.3 $\pm$ 0.03-		419 $\pm$ 28.1-	
BADGE 1 $\mu\text{M}$	113	375 $\pm$ 9.2	104%	18.8 $\pm$ 1.1	94%	85.2 $\pm$ 7.6	104%	2 $\pm$ 0.03 *	87%	325.7 $\pm$ 26.5	86%
BADGE 2 $\mu\text{M}$	215	338.5 $\pm$ 7.6	101%	13.1 $\pm$ 0.8 *	62%	84.9 $\pm$ 4.8	102%	1.8 $\pm$ 0.02 *	81%	378.2 $\pm$ 17	102%
BADGE 5 $\mu\text{M}$	198	342.1 $\pm$ 6.6	92%	13.5 $\pm$ 0.8 *	57%	87 $\pm$ 0.8	92%	1.9 $\pm$ 0.02 *	75%	354.5 $\pm$ 17.6	92%

**Table 6.** Genotoxicity of toxicants and EDCs in developing embryos of *Phallusia mammillata* (7 – 9 hpf). The number of embryos with DNA aberrations was estimated from images of embryos either at gastrula or early neurula stage, where DNA was stained with Hoechst (Supplementary Fig. 4). The percentage of embryos with DNA aberrations in each culture is shown in the last column.

<b>Tested compound</b>	<b>Number of embryos with DNA aberrations / total number of embryos imaged</b>	<b>The percentage of embryos with DNA aberrations (%)</b>
<b>0.01% DMSO</b>	0/50	0
<b>Sodium azide 2.5mM</b>	21/30	70
<b>Mitomycin 60µM</b>	32/32	100
<b>Etoposide 100nM</b>	40/59	68
<b>Etoposide 10µM</b>	30/30	100
<b>TBT<sup>1</sup> 50nM</b>	18/25	72
<b>Lindane 20µM</b>	10/20	50
<b>Atrazine 70µM</b>	0/17	0
<b>Chlordane 50µM</b>	5/15	33
<b>Chlorpyrifos 50µM</b>	5/18	27
<b>BPA<sup>2</sup> 10µM</b>	14/119	11

<sup>1</sup>TBT – tributyltin

<sup>2</sup>BPA – bisphenol A

**Table 7.** Correlation coefficients between % embryos with DNA aberrations, normal, malformed and undeveloped. The t-test is used to establish significance of the correlation between pairs of parameters. Asterisks indicate  $p < 0.05$  according to the Spearman's test.

	DNA aberrations (%)	Normal (%)	Malformed (%)	Undeveloped (%)
DNA aberrations (%)	1.00	<b>-0.93*</b>	0.50	0.35
Normal (%)	<b>-0.93*</b>	1.00	-0.48	-0.48
Malformed (%)	0.50	-0.48	1.00	-0.12
Undeveloped (%)	0.35	-0.48	-0.12	1.00

**Declaration of interests**

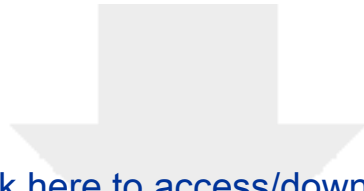
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Authors contributions:

**Ievgeniia Gazo**: performed experiments; participated in data analysis; prepared a draft manuscript. **Isa D. L. Gomes**: performed cultures with BPA; participated in draft preparation. **Thierry Savy**: developed Toxicosis software. **Nadine Peyrieras**: participated in conceptualization of Toxicosis software. **Lydia Besnardeau**: assisted with experiments. **Celine Hebras**: assisted with experiments. **Sameh Benaicha**: data analysis. **Manon Brunet**: performed cultures with ATRA, BMS, UVI. **Olena Shaliutina**: performed cultures with mitomycin. **Alex McDougall**: coordinated the study. **Rémi Dumollard**: coordinated the study, participated in data analysis, edited and rewrote draft





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