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Early developmental responses of three sea urchin species to tralopyril and its two degradation products

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Abstract

Tralopyril (TLP) is a newly emerged antifouling biocide which rapidly degrades in water. The scientific data on its possible adverse effects to biota is very limited, and even more limited is the ecotoxicity data of its degradation products (DPs). In the present study we investigated the toxicity of TLP and its two main DPs on fertilization and embryogenesis of three sea urchin species: *Clypeaster japonicus*, *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*. The species sensitivity to chemicals was investigated and compared. Additionally, the stability of TLP in test medium was examined.

TLP in test medium degraded into one single degradation product. The degradation was slower at 17 °C (average incubation temperature for winter species *H. pulcherrimus*) than at 20 °C (incubation temperature for the other two species). Both DPs at 100 µg/L did not appear toxic, however, TLP highly affected larval development of all tested species. Sensitivity was similar for *C. japonicus* and *P. depressus*, but higher toxicity was noticed for *H. pulcherrimus*. The increased TLP toxicity on exposed *H. pulcherrimus* embryos could be attributed to the higher TLP stability at lower incubation temperature. Results suggest higher vulnerability to TLP for species spawning in colder seasons. Fertilization test appeared to be less sensitive than the embryotoxicity test.

Keywords: toxicity, antifouling biocide, fertilization, embryotoxicity, sensitivity, environmental fate

Introduction

The most common method to prevent the attachment and growth of fouling organisms to submerged ship surfaces is the application of antifouling paints. These contain biocides which continuously leach into the surrounding water and are toxic to target, and often also to non-target, organisms. The use of antifouling paints therefore represents an unavoidable pollution of aquatic ecosystems. One of the most notorious examples of such pollution with severe consequences was the use of antifouling biocide tributyltin (TBT).

TBT was for its exceptional antifouling properties widely applied in self-polishing polymer ship coatings, but it was later on banned due to its deleterious effects on marine biota (Spence et al. 1990, Tsunemasa and Okamura 2011). New antifouling biocides have emerged, with tralopyril (TLP, Figure 1A) being one of the most recent ones. It is commercialized under the name ECONEA® (Janssen PMP, 2014) and is especially effective against hard biofouling (Oliveira et al., 2014). Because TLP rapidly hydrolyzes in water (Oliveira et al., 2016), it is unlikely it would accumulate in aquatic environments. The chemical structure of TLP was also described in a photocatalytic degradation pathway of an insecticide chlorfenapyr; TLP is there mentioned as one of the first degradation products of chlorfenapyr (Cao et al. 2006; TLP there labeled as a compound B). According to Cao et al. (2006), TLP further degrades into another compound, there named compound C. An assessment report of TLP (Regulation EU, 2014) describes this compound C to be a hydrolysis product of TLP (denominated as CL322,250, in this paper simplified to CL, Figure 1B). Cao et al. (2006) found that CL in the presence of light further degrades into a subsequent compound 4-chlorophenylglycine, from which eventually 4-chlorobenzoic acid (CBA, Figure 1C) is formed.

Exposure of organisms to xenobiotics can lead to toxicity; whether the adverse effects occur and in what extent strongly depends on the type of a chemical and sensitivity of species to that particular chemical (Aldenberg and Jaworska, 2000). Sea urchins as test species have a long history and a high record appearing in ecotoxicity studies due to their high sensitivity to chemicals, especially in their early developmental stages (for instance Kobayashi 1971, 1981, 1990, Manzo et al. 2006, Morroni et al. 2018). They are also one of the key species in marine ecosystems, playing a role as grazers as well as a prey to higher trophic organisms. Several guidelines have been developed for the effect of toxicants on fertilization success, larval development and larval growth (USEPA 1991, USEPA 2002, ASTM 2012). Different sea urchin species are applied in ecotoxicity studies, with *Paracentrotus lividus* and *Arbacia punctulate* seemingly being the most frequently used in research studies. There are evidences that sensitivity of chemicals can vary between different species, even of those belonging to the same genus (see for instance Kobayashi 1981, Rojíčková and Maršálek 1999, Boyd and Williams 2003). We aimed to perform fertilization and embryotoxicity tests

with three sea urchin species, having low records in appearing in ecotoxicity studies - *Clypeaster japonicus*, *Pseudocentrotus depressus* and *Hemicentrotous pulcherrimus*. All of them can be found in waters along the Japanese archipelago, with some having very restricted habitats, appearing in low densities and low population sizes (Agatsuma, 2013), making their populations more vulnerable to toxic chemicals.

It is imperative to know the risks of biocides that enter natural environments. When biocides are not stable in the environment, it is also equally important to assess the risks of its degradation products, as they can exert even higher toxicity than their parent compounds (Sinclair and Boxall, 2003). The information on ecotoxicity of CBA, one of the TLP degradation products (Cao et al., 2006), is severely limited, while the scientific data on ecotoxicity of CL remains virtually non-existent. In addition to our ecotoxicity studies, this paper is unique in providing an insight on the stability of TLP in water and the formation of its degradation product CL.

Materials and methods

Chemicals

Tralopyril (4-bromo-2-(4-chlorophenyl)-5-(trifluoromethyl)-1H-pyrrole-3-carbonitrile, TLP, 98.6 % purity) and its degradation product CL (4-bromo-2-(p-chlorophenyl)-pyrroline-3-carbonitrile-5-carbonic acid, 95 % purity) were generously provided by Janssen PMP. CBA (4-chlorobenzoic acid, 99 % purity) was purchased from Tokyo Chemical Industry, Japan. Acetonitrile (MeCN, HPLC grade), phosphoric acid (analytical grade) and DMSO (spectrophotometric grade) were attained from Wako Pure Chemical Industries, Ltd., Japan. DMSO was employed as a carrier solvent and did not exceed more than 0.1 % in test solutions. DMSO at this concentration was shown safe to the sea urchin embryos (Bellas et al., 2005). Test solutions were prepared using artificial seawater (Marine Art SF-1, Tomita Pharmaceutical Co., Ltd., Japan) with 31.5 ± 0.5 ppt salinity (Atago, master refractometer) and a pH of 8.3 ± 0.5.

81

82 **Chemical analysis**

83 Chemical measurements were performed on a Hitachi semi-micro HPLC system equipped with ODS-MG3
84 column (2 × 100 mm, Nomura chemicals). Mobile phase for TLP and CL consisted of (A) 0.1 % phosphoric
85 acid aqueous solution and (B) MeCN in a 60(A):40(B) ratio. Detection was achieved on a diode array (DAD)
86 and a fluorescence detector (FLD, λ_{ex} = 290 nm, λ_{ex} = 360 nm). To quantify CBA, only DAD was used.
87 Eluents for CBA analysis consisted of (C) 0.02 M KH_2PO_4 and (D) ethanol in 60(C):40(D) proportion. Both
88 methods had an isocratic flow set to 0.2 mL/min and an injection volume of 50 µL.
89

90 **Test organisms**

91 Toxicity tests were performed using gametes of three different sea urchin species: *Clypeaster japonicus*,
92 *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus*. Gametes were provided by WDB
93 Environmental & Biological Research Institute, Japan, except from gametes of *H. pulcherrimus*, which were
94 kindly donated from Tateyama marine laboratory, Marine and coastal research center, Ochanomizu
95 University, Japan. All sea urchin species, from which gametes were attained and brought to our lab, were
96 collected at Boso peninsula, Chiba, Japan, by fisherman's net at 10 m depth (*C. japonicus*), by scuba diving
97 at 10 m depth (*P. depressus*), or were collected by hand at intertidal zone (*H. pulcherrimus*). Toxicity tests
98 were performed at different times, depending on the breeding season of each sea urchin: winter (January –
99 March) for *H. pulcherrimus*, summer (July – August) for *C. japonicus* and autumn (November – December)
100 for *P. depressus*.

101

102 **Toxicity tests**

In order to allow the best comparison of the results between tested species, same test procedures were adopted in each test, unless where stated differently.

Isolated gametes were firstly examined under the microscope (Olympus CKX 31, Japan) and the ability of fertilization was confirmed by egg insemination. Test solutions of TLP, CL and CBA were prepared freshly right before the start of the test due to the fast hydrolysis of TLP. Based on the results of preliminary experiments, concentrations of TLP ranging from 4.5 – 286.1 nM (4.5, 8.9, 17.9, 35.8, 71.5, 143.1 and 286.1 nM) were chosen for toxicity tests; controls and solvent controls were included. Due to the low water solubility of TLP (170 µg/L, 20 °C, Regulation EU, 2014) and low predicted environmental concentrations (PEC_{max} = 0.7 µg/L, Oliveira et al., 2014), the concentrations of its degradation products in natural aquatic environments are also expected to be low, therefore 100 µg/L was chosen as a maximum concentration tested for CL and CBA (638.7 and 307.2 nM, respectively). Test solutions were distributed in 48-micro well plates - each well containing 1 mL of solution. Each treatment consisted of four replicates.

Fertilization toxicity test

Sea urchin fertilization test is a short, yet sensitive test with sea urchin spermatozoa. It was performed according to the USEPA guideline (USEPA, 2002), unless where noted differently. To start the test, a small volume (5 µL) of dense sperm suspension was pipetted into each well containing test solution. Samples were incubated in the dark for 1h at 20 ± 0.5 °C for *C japonicus* and *P. depressus*, and 17 ± 1 °C for *H. pulcherrimus*. After the elapsed time, 10 µL of oocyte suspension, accounting 100 - 200 oocytes, was added to each well. Time was recorded, and the samples were again placed in the incubator. Twenty minutes were given for the fertilization to occur, after which the test was terminated by adding a drop of 10 % formalin into each well. The number of fertilized and unfertilized eggs was counted under the microscope with adjusted 400x magnification. Fertilized eggs can be clearly distinguishable as they exhibit a visible fertilization membrane.

127

128 *Embryotoxicity test*

129 Sea urchin oocytes were fertilized and then introduced to each test well using an electronic repeating pipettor.
130 Each replicate received 100 – 200 fertilized eggs contained in 20 µL solution. Samples were incubated in
131 the dark at 20 ± 0.5 °C and 17 ± 1°C in case of *H. pulcherrimus.* Lower temperature (ranging from 16 –
132 18 °C) was used for *H. pulcherrimus* due to our previous unsuccessful trials at 20 °C. *H. pulcherrimus* is
133 spawning in winter season, when the water temperatures are low (Fujisawa, 1995), which could explain the
134 disturbed embryogenesis at 20 °C. Few research studies performed on *H. pulcherrimus* were carried out at
135 temperatures lower than 20 °C; for example at 18 °C (Kobayashi, 1981) and even at 14 °C (Kurihara et al.,
136 2004).
137 After 48 ± 2 hours of incubation, sea urchin larvae were immobilized by a drop of 10 % formalin.
138 Larval development was investigated under the microscope on 100 individuals per replicate. Larva was
139 considered normal when its arms were fully developed and digestive tract completed. In case of i) malformed
140 skeleton or gut, ii) reduced larval size (more than half of the size of the average normal larvae), or iii)
141 observed pre-pluteus larval stages (fertilized egg, blastula, gastrula, prism), the larva was considered
142 abnormal.
143

144 **Stability study**

145 To understand the kinetics of TLP degradation in the exposure media, TLP at the initial concentration 57.2
146 nM was incubated at 20 and 17 °C in the darkness for total 8 hours and measured in frequent intervals by
147 HPLC. Furthermore, a separate experiment was performed in order to investigate the stability of TLP, CL
148 and CBA during the toxicity tests. The procedure was identical to the toxicity tests with an exception that
149 no animals were included in this study. Solutions of compounds with same initial concentrations as in
150 toxicity tests were prepared with artificial sea water, pre-incubated at 20 °C, distributed in test wells and left

to stand at the room temperature for about 20 minutes, simulating the approximate time required to prepare and distribute the biological sample in toxicity tests. Aliquots for the chemical analysis were taken (representing the start of the experiment, t = 0) and the remaining samples were incubated at 20 °C in the darkness. Aliquots for chemical measurements were then again taken after 80 minutes (simulating the end of the fertilization test) and after 48 hours (simulating the end of the embryotoxicity test). Furthermore,
In both experiments, prior to the analysis of each sample, 10 µL of acetic acid solution was added to 1 mL of aliquot, which decreased its pH to about 3 to prevent the further hydrolysis of TLP.

Data analysis

Results from the ecotoxicity studies were expressed in a comparison to the controls (% control). Statistical analysis was performed using GrapPad Prism 7.02. The comparison between controls and solvent controls from each test was achieved using an unpaired t-test. Concentration response curves were plotted using variable slope model (GrapPad Prism 7.02) and the EC_{50} values were derived. Best-fit values were compared using the extra sum-of-squares F test, where $P < 0.05$ was considered significant.
First order degradation model was applied to plot the degradation curves and to calculate the degradation half-lives.

Results and discussion

Stability of tralopyril in artificial seawater

The degradation of TLP at 20 °C and the formation of CL along the TLP degradation is presented on Figure 2A. The sum of TLP and CL molar concentrations very well matched with the initial TLP concentration (57.2 nM), which is also demonstrated on the graph (SUM). On the Figure 2B, a degradation of TLP is compared between two incubation temperatures used in the toxicity tests; 20 °C (average incubation

174 temperature for *C. japonicus* and *P. depressus*) and 17 °C (average incubation temperature for *H.*
175 *pulcherrimus*). The modeled degradation curves indicate that at 17 °C the degradation of TLP was slower
176 than at 20 °C, with a half-life of 7.4 and 4.0 h, respectively. This is in accordance with the results of Kempen,
177 (2011), who reported a half-life of TLP to be 3 h at 25 °C and 15 h at 10 °C. The results on degradation half-
178 life of TLP also very well match with the ones obtained by Oliveira et al. (2016), where the calculated half-
179 life of TLP in seawater was 6.1 h at 18 °C.

180 The measured concentrations of TLP, CL and CBA at different time points of the stability experiment (t=0,
181 80 min and 48 h), simulating the toxicity test, are presented in Table 1. The method quantification limit for
182 TLP, CL and CBA were 6.0, 9.8 and 140 nM, respectively. The results prove that TLP degrades with time
183 into CL. Already at the start of the test, part of TLP already degraded. With time TLP continued to degrade,
184 and after 48-h incubation period only CL could be detected. The concentrations of CL and CBA at 100 µg/L
185 (307.2 and 638.7 nM, respectively) remained fairly stable along the incubation period.

186 In the degradation process of TLP, no degradation product, other than CL, could be detected by our DAD-
187 FLD system. In a photocatalytic study performed by Cao et al. (2006), CL furtherly degraded into a
188 subsequent product, from which CBA was formed. However, in our experiments of TLP degradation, CL
189 remained fairly stable and CBA could not be detected. This could be due to the absence of light in our
190 experiments, short experimental time, or the concentrations of CBA were too low to be detected with our
191 analytical method.

192

193 **Ecotoxicity tests**

194 The controls and solvent controls in all performed tests did not significantly differ from each other ($P > 0.05$)
195 and were therefore pulled. The ecotoxicity results are for all three species, all tested compounds and
196 all toxicity endpoints collected in Table 1.

197

198 **Effect of tralopyril and its degradation products on the fertilization success**

199 The fertilization success among all controls was above 89.9 %. While no effect of TLP was noticed on
200 gametes of *H. pulcherrimus*, high toxicity was observed on gametes of *C. japonicus*. A concentration -
201 response curve was plotted (Figure 3A) and an EC_{50} of 60.3 nM was derived. TLP posed a slight, yet
202 significant ($P < 0.05$) toxicity on male gametes of *P. depressus* at TLP concentrations above 71.5 nM, but
203 the toxicity did not increase with the increasing TLP concentrations. It appears that the spermatozoa of *C.*
204 *japonicus* to TLP was more sensitive compared to other two species.
205 None of the degradation products at 100 µg/L posed any toxic effect to fertilization success for all species
206 tested (Table 2).
207

208 **Effect of tralopyril and its degradation products on embryogenesis**
209 The average number of normally developed larvae in controls and solvent controls was not lower than
210 88.8 % for all tested species. The results of the effects of TLP to sea urchin embryos are presented on Figure
211 3B. Concentration-response curves demonstrate a high TLP toxicity to embryos for all three species (Table
212 2, Figure 3B). Among all, the highest toxicity was observed for *H. pulcherrimus* (EC_{50} = 14.6 nM). The
213 EC_{50} values for the other two species did not significantly differ ($F_{1,11}$ = 0.01, $p > 0.05$) and their
214 concentration-response curves are nearly overlapping.
215 TLP degradation products CL and CBA at 100 µg/L did not affect the sea urchin embryonic development.
216 In all tests with degradation products, the average percentage of normally developed larvae was no lower
217 than 96.2 % and not higher than 104.6 %, compared to the controls (% control, Table 2).
218 Our results imply that the embryos of *H. pulcherrimus* (incubated at 17 ± 1 °C) were exposed to TLP higher
219 concentrations for a longer time than the embryos of the two species incubated at 20 °C, due to the increased
220 stability of TLP at lower temperatures. This subsequently could have led to a higher toxicity. While the

221 spring and summer spawning species (such as *C. japonicus*) may be more vulnerable to the antifoulants, as
222 the concentrations there are the highest (due to higher boating activities and freshly painted hulls, Martínez
223 et al., 2001), the risk for TLP remains high also for species breeding in winter due to the increased TLP
224 stability. The seawater temperatures during the spawning season of *H. pulcherrimus* can be in northern part
225 of Japan as low as 5 °C (Fujisawa, 1995). TLP stability and subsequently the toxicity risk may be due to
226 that significantly increased. *P. depressus* is spawning in autumn, where the temperatures may not be very
227 low, yet, the populations of *P. depressus* might be vulnerable to pollution due to their low densities and
228 small population sizes (Agatsuma, 2013).
229 TLP was previously examined on embryos of a sea urchin *Paracentrotus lividus* (Oliveira et al., 2014). An
230 EC_{50} of 8.6 nM (3 µg/L) for the effect on larval growth was reported, therefore lower value than that for
231 tested species in our study. This may suggest higher sensitivity of *P. lividus* gametes compared to the
232 gametes of the species tested in our tests. In case of very unstable compounds, such as TLP, the comparison
233 of the toxicity results is problematic. Already the starting concentrations may differ from test to test,
234 depending on the time that passed before the embryos started to be exposed. No information on actual TLP
235 concentrations is provided by Oliveira et al. (2014) in their tests and the degradation products there are not
236 mentioned. In case of TLP, the temperature also plays a remarkable role in degradation kinetics and therefore
237 influences the toxicity. The embryos of *P. lividus* were incubated at 20 °C (Oliveira et al., 2014), therefore
238 at the same temperature than *P. depressus* and *C. japonicus* in our test.

239 Because of a high instability of TLP in water, assessing the toxicity of its degradation products is urgent. So
240 far, there is no scientific data on toxicity of CL to biota, however a regulation document for tralopyril
241 (Regulation EU, 2014) reports significantly lower toxicities for CL compared to TLP when tested on
242 rainbow trout (96 h acute lethality test), zebra fish (35 days larval survival), eastern oyster (96 h test), mysid
243 shrimp (28 day test), marine algae (96 day test) and freshwater plant (7 day test). These results are however
244 not equipped with a description of the tests. 4-chlorobenzoic acid (CBA) has never been assessed on sea

urchins before and generally very limited data can be found in the literature. Ecotoxicological data reports the EC_{50}s of CBA on several species at concentrations tested far higher than in our tests (100 µg/L): EC_{50} = 6400 µg/L for luminescent bacteria *Vibrio fisheri* (Zhao et al., 1998), IC_{50} = 150 000 – 749 000 µg/L, depending on water pH, for water flea *Daphnia magna* (Zhao et al., 1998) and EC_{50} = 17 300 µg/L for alga *Raphidocelis subcapitata* (Lee and Chen, 2009).

Conclusions

The stability study of TLP in test media showed that TLP degrades in artificial seawater slower at lower temperatures. TLP degradation products did not appear to be toxic in any of our tests, but a very high toxicity of TLP was observed on exposed embryos for all three sea urchin species. Nearly the same EC_{50}s were obtained for *C. japonicus* and *P. depressus*. Higher toxicity was observed for *H. pulcherrimus*, which could be associated with a slower TLP degradation at the lower incubation temperatures applied in this test. The risk of TLP adverse effects may be therefore higher for the species spawning in winter, as the exposure to higher TLP concentrations can be prolonged. Differences in toxicities can also be dependent on the species tested. This was demonstrated in our fertilization toxicity tests with sea urchin male gametes, where TLP showed different toxicities depending on the sea urchin species. Fertilization test was in all cases less sensitive that the embryotoxicity test.

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268

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330

331 TABLES

332 Table 1: Measured concentrations of TLP and its degradation products CL and CBA in a stability study.
333 Concentrations were measured at different time points, representing the start of the toxicity tests (t=0), the
334 end of the fertilization toxicity test (t=80 min) and the end of the embryotoxicity test (t=48 h). Incubation
335 was carried out at 20 °C in the darkness.

Initial nominal concentration (nM)	Measured concentration (nM)					
Compound	t=0		t=80 min		t=48 h	
TLP	TLP	CL	TLP	CL	TLP	CL
17.9	8.7	<9.8	<6.0	<9.8	<6.0	16.4
35.8	21.9	<9.8	7.4	18.6	<6.0	32.8
71.5	45.7	11.2	22.8	36.0	<6.0	64.9
143.1	95.9	23.5	48.7	76.0	<6.0	133.6
286.1	202.0	41.4	96.6	167.2	<6.0	288.1
CL	start of the test		end of FT test		end of ET test	
307.2	285.7		273.5		272.2	
CBA	start of the test		end of FT test		end of ET test	
638.7	743.3		720		704.4	

336 Abbreviations: TLP = tralopyril, CL = main degradation product of TLP, CBA = 4-chlorobenzoic acid,
337 degradation product of TLP. For chemical structures of compounds refer to Figure 1.

338

339 Table 2: Toxicity data for tralopyril and its two degradation products CL and CBA in fertilization toxicity
340 test and embryotoxicity tests for three sea urchin species: *P. depressus*, *C. japonicus* and *H. pulcherrimus.*

	Test species		
	C. japonicus	***P. depressus***	***H. pulcherrimus***
TLP - EC_{50} (nM)			
Fertilization	60.3 (58.0 – 62.6)	> 286	> 286
Embryotoxicity	41.1 (39.8 – 42.8)	39.3 (34. 7 – 43.9)	14.6 (11.7 – 17.5)
CL - inhibition (% control) at 307.2 nM			
Fertilization	0.4 ± 0.9	10 ± 1.4	1.0 ± 1.8
Embryotoxicity	- 4.6 ± 2.7	0.6 ± 3.3	3.8 ± 5.7
CBA - inhibition (% control) at 638.7 nM			
Fertilization	- 2.0 ± 0.9	0.9 ± 3.8	1.0 ± 1.8
Embryotoxicity	- 1.8 ± 1.2	1.1 ± 4.9	3.8 ± 5.7

341 Abbreviations: TLP = tralopyril, CL = main degradation product of TLP, CBA = 4-chlorobenzoic acid,
342 degradation product of TLP. The interval in parentheses represents a 95 % confidence interval of
343 calculated EC_{50}. ± values represent the standard deviation (n=4). Concentrations are based on nominal
344 values.

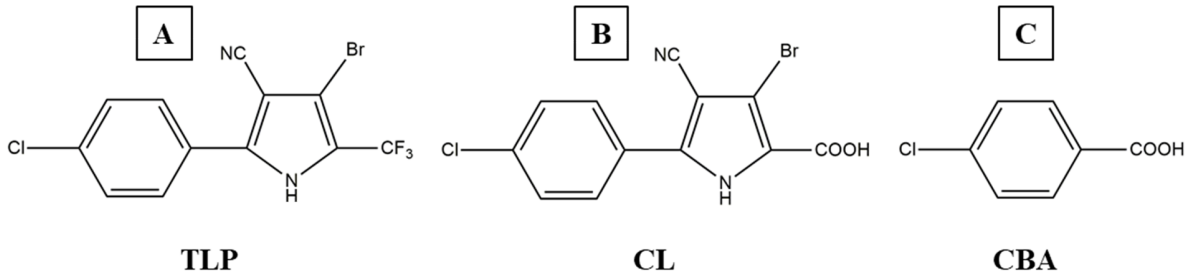
345

346 FIGURES

347

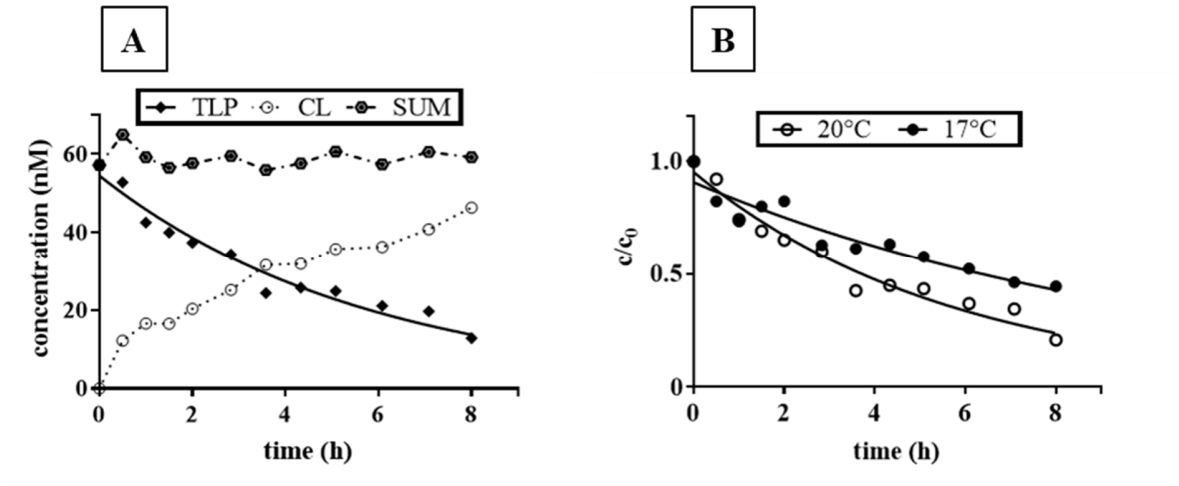
348 Figure 1

349



350

351 Figure 2



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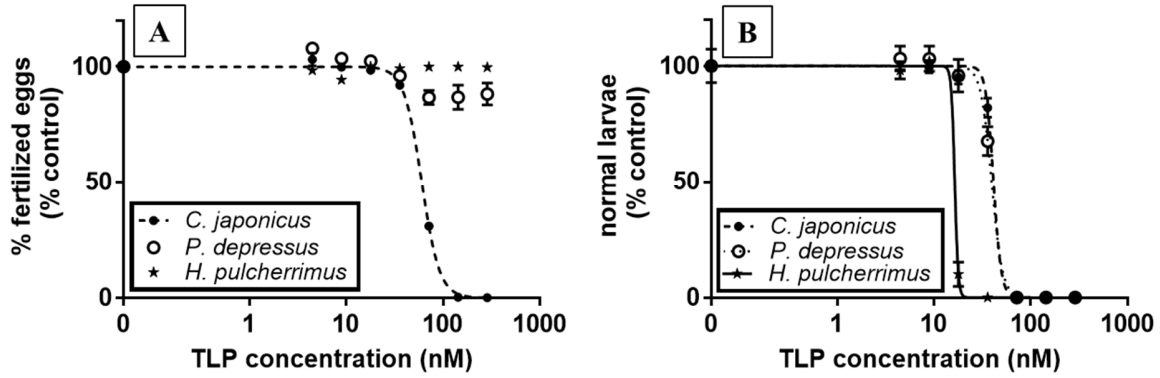
354 Figure 3:



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356

357 FIGURE CAPTIONS

358 Figure 1. Chemical structures of antifoulant tralopyril (A, TLP) and its two main transformation products:
359 CL (B, called CL322,250 by the registrant) and 4-chlorobenzoic acid (C, CBA).

360

361 Figure 2 A: Degradation of tralopyril (TLP) and formation of its degradation product CL along the
362 incubation at 20 °C. SUM represents the sum of molar concentrations of both compounds. B: Degradation
363 curves of TLP incubated at 20 °C and 17 °C.

364

365 Figure 3: Early developmental responses of three sea urchin species (*C. japonicus, P. depressus* and *H.*
366 *pulcherrimus*) exposed to tralopyril in a fertilization test (A) and embryotoxicity test (B). Concentrations
367 are based on nominal values. Error bars represent the SEM (n = 4) and cannot be noticed when the error is
368 very small.