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# Developmental toxicity of metaldehyde in the embryos of *Lymnaea stagnalis* (Gastropoda: Pulmonata) co-exposed to the synergist piperonyl butoxide

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1 **Developmental toxicity of metaldehyde in the embryos of**  
2 ***Lymnaea stagnalis* (Gastropoda: Pulmonata) co-exposed**  
3 **to the synergist piperonyl butoxide.**

4  
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18 Abstract

19 Metaldehyde is a tetramer of acetaldehyde and was first introduced as a  
20 molluscicide in 1936, remaining in wide use today for the control of mollusc pests in  
21 agriculture and horticulture. Damage to crops from slugs and snails is a major  
22 problem in many countries associated with relatively warm and wet winters. For  
23 example in the UK it is estimated that over 8 % of the area covered by arable crops  
24 is treated with formulated granular bait pellets containing metaldehyde as the  
25 principle active ingredient. Metaldehyde is hydrophilic ( $\log P = 0.12$ ), water soluble  
26 ( $200 \text{ mg.L}^{-1}$  at  $17^\circ\text{C}$ ) and has been detected in UK surface waters in the  
27 concentration range of typically  $0.2\text{-}0.6 \text{ }\mu\text{g.L}^{-1}$  (maximum  $2.7 \text{ }\mu\text{g.L}^{-1}$ ) during 2008-  
28 2011. In the absence of chronic data on potential hazards to non-target freshwater  
29 molluscs, a laboratory study was conducted to investigate the impact of metaldehyde  
30 on embryonic development in the gastropod *Lymnaea stagnalis* (RENILYS strain)  
31 and using zinc as a positive control. *L. stagnalis* embryos were exposed to  
32 metaldehyde under semi-static conditions at  $20 \pm 1^\circ\text{C}$  and hatching success and  
33 growth (measured as spire height and intraocular distance) examined after 21d.  
34 Exposure concentrations were verified using HPLC and gave 21d<sup>hatching</sup>NOEC and  
35<sup>hatching</sup>LOEC mean measured values of 36 and  $116 \text{ mg MET.L}^{-1}$ , respectively (equal  
36 to the 21d<sup>spire height</sup>NOEC and<sup>spire height</sup>LOEC values). For basic research purposes, a  
37 second group of *L. stagnalis* embryos were co-exposed to metaldehyde and the  
38 pesticide synergist piperonyl butoxide (PBO). Co-exposure to the PBO (measured  
39 concentrations between  $0.47\text{-}0.56 \text{ mg.L}^{-1}$ ) reduced hatching success from 100% to  
40 47% and a 30% reduction in embryo growth (spire height) in snail embryos co-  
41 exposed to metaldehyde at  $34\text{-}36 \text{ mg.L}^{-1}$ ) over 21d. In conclusion, these data  
42 suggest mollusc embryos may have some metabolic detoxification capacity for  
43 metaldehyde and further work is warranted to explore this aspect in order to support  
44 the recent initiative to include molluscs in the OECD test guideline programme.

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46 Keywords: freshwater, pesticide, mollusc, metabolism, OECD

47

## 48 Introduction

49 The control of molluscs and other pests remains a major challenge for food  
50 production in many regions. Crop damage by slug and snail pests is a major  
51 problem in European agriculture and it has been compounded in recent years by the  
52 mild, wet climate and changes in farming practices. It is estimated that over 8% of  
53 the area covered by arable crops in the United Kingdom is treated with the slug  
54 pellets and their active ingredient metaldehyde (Environment Agency, 2009). First  
55 used as a molluscicide in the 1930's (Gimingham and Newton 1937), metaldehyde  
56 (CAS number 108-62-3, molecular weight 176) is a crystalline solid and cyclic  
57 tetramer of acetaldehyde, with a melting point of between 110-120°C. Metaldehyde  
58 is persistent in the aquatic environment and is moderately soluble in water up to 200  
59 mg.L<sup>-1</sup> at 17°C (Bieri 2003; USEPA 2006a; EFSA 2010). Common formulations  
60 include solutions, dusts, pastes, foams, particulates and suspensions or as  
61 formulated granular bait pellets (eg Cekumeta<sup>®</sup>, Deadline<sup>®</sup>, Hardy<sup>®</sup>, Metarex<sup>®</sup> and  
62 Metason<sup>®</sup>) (Zhang et al., 2011). Metaldehyde is also used as a molluscicide in rice  
63 paddies and aquaculture systems in south east Asia (Calumpang et al., 1995;  
64 Coloso et al., 1998). Metaldehyde is also used for some solid fuel camping stoves  
65 and as a fire starter to preheat petrol stoves (Gupta 2012; Zen Stoves 2015). In the  
66 United Kingdom, regulators have raised recent concerns about the relatively high  
67 levels of metaldehyde detected in surface waters. It was first detected in surface  
68 water in autumn 2007 following the development of new mass spectrometric  
69 analytical techniques (Environment Agency 2009). From then until autumn 2012  
70 there was a demonstrable downward trend in the number of occasions where  
71 metaldehyde has been detected in raw and treated water. In 2012, however, the  
72 challenge of a wet and mild summer, which had been the wettest since 1912 (371  
73 mm mean UK average for June to August compared to 320 mm in 2008), followed  
74 by the wettest April in 100 years and above average rainfall in May 2012 (Marshall  
75 2013; Kay and Grayson 2014). These conditions significantly increased slug activity  
76 and production of juvenile populations to levels which jeopardised autumn sowings,  
77 in turn leading to an increase in metaldehyde use during 2012. Metaldehyde is  
78 spread during autumn, due to the wetter weather and crop vulnerability, it can be  
79 found at higher levels in surrounding environments during this time period. The main  
80 mechanism by which metaldehyde enters water is either directly, through point

81 source spillages, via runoff or by-pass flow. Kay and Grayson (2014) reported  
82 concentrations of metaldehyde in the range 0.4 to 0.6  $\mu\text{g.L}^{-1}$  (but sometimes up to  
83 2.7  $\mu\text{g.L}^{-1}$ ) in north east England between 2008 and 2011. Taking the specific  
84 example of the Metarex<sup>®</sup> formulation, the Predicted Exposure Concentrations for  
85 metaldehyde in surface waters ( $\text{PEC}_{\text{sw}}$ ) under FOCUS Step 2 exposure scenario for  
86 Northern Europe ranged from 26.871  $\mu\text{g.L}^{-1}$  and 19.016  $\mu\text{g.L}^{-1}$  after 7 and 42 days,  
87 respectively (EFSA 2010). There is evidence indicating that existing water treatment  
88 processes are inadequate for removing metaldehyde residues from sources of  
89 drinking water. Metaldehyde concentrations up to 8  $\mu\text{g.L}^{-1}$  have been reported in  
90 some UK drinking waters, in contrast to the regulatory limit for pesticide active  
91 ingredients in drinking water of 0.1  $\mu\text{g.L}^{-1}$ ) (Environment Agency 2009). However,  
92 metaldehyde is not effectively removed through adsorption onto activated carbon  
93 and hence there is considerable work to find effective removal methods (Li et al.,  
94 2010; Autin et al., 2012). Unsurprisingly, metaldehyde residues have also been  
95 detected in crops and in soil sampled from various regions (Selim & Seiber 1973;  
96 Zhang & Dai 2006; Zhang et al., 2011).

97 In terms of the hazard profile of metaldehyde, the evidence indicates moderate  
98 mammalian toxicity. Metaldehyde poisoning is characterised by central nervous  
99 system depression and convulsions. Several cases of deliberate or accidental  
100 ingestion by man, pets or domestic animals have been reported (WHO 1996; Jones  
101 & Charlton 1999; Bleakley et al., 2008). Mice receiving an oral dose of 100  $\text{mg.kg}^{-1}$   
102 body weight died within two hours of exposure. Signs of poisoning included sedation,  
103 shivering, whole body tremors, convulsions and death. In cattle, horses and dogs  
104 mild poisoning was evidenced by salivation ataxia and hypernea. Symptoms  
105 observed in severe poisoning included convulsions, sweating, tachycardia and  
106 muscle spasms, with death usually attributed to respiratory failure (WHO 1996).  
107 Metaldehyde induced convulsions in mice were accompanied by a reduction in the  
108 levels of serotonin and noradrenaline in the brain and increased monoamine oxidase  
109 activity (Mills et al., 1992).

110 In molluscs, metaldehyde can act as either a contact or stomach poison. A number  
111 of authors have described metaldehyde's toxic mode of action in molluscs as  
112 causing irreversible damage in the mucous cells of the skin and gut lining. This leads  
113 to excessive mucus production, destruction of the mucus cells, damage to absorptive

114 cells of the heptatopancreas and death (Triebkorn 1989; Triebkorn & Ebert 1989;  
115 Coloso et al., 1998; Triebkorn et al., 1998). In addition to this the quality of mucus  
116 produced is diminished. Furthermore Mills et al. (1990 & 1992) described the  
117 electrophysiological perturbation and feeding disruption associated with the toxic  
118 mode of action of metaldehyde. Experimental analysis indicated that acetaldehyde  
119 was present in the haemolymph of slugs immediately after the end of a metaldehyde  
120 meal (Mills et al., 1990). Comparing the impacts of a pellet formulation on terrestrial  
121 molluscs (slugs) with the freshwater gastropod *Lymnaea stagnalis*, Mills et al. (1990)  
122 reported that a metaldehyde concentration of 5 g.kg<sup>-1</sup> in the pellet reduced the meal  
123 duration and number of bites in slugs by about 70% compared to control. In *L.*  
124 *stagnalis*, however, the same metaldehyde concentration reduced the meal duration  
125 by approximately 25%. Differences in the concentration required to produce a given  
126 effect may be due to differences in the rate of absorption or amount of body contact  
127 with the pellet, or differences in the aversive chemosensory response to  
128 metaldehyde. The study by Mills et al (1990) provides good evidence of  
129 metaldehyde's neurotoxic mode of action and that some of the toxic symptoms in  
130 slugs and other terrestrial molluscs are likely mediated by acetaldehyde. There is  
131 also a growing body of evidence describing the toxicity of metaldehyde to  
132 earthworms and other non-target terrestrial invertebrates (Iglesias et al., 2003;  
133 Langan & Shaw 2006; Edwards et al., 2009; Rae et al., 2009; Gavin et al., 2012;  
134 Cardoso et al., 2015). In terms of aquatic non-target species, the most sensitive  
135 species included in a recent review by EFSA (2010) is the freshwater amphipod  
136 *Gammarus pseudolimnaeus* with a 96h EC50 of 19.3 mg.L<sup>-1</sup>. While Coloso et al.  
137 (1998) reported over 80% snail mortality after 7d in milkfish ponds treated with  
138 metaldehyde at 0.38-1.55 mg.L<sup>-1</sup>, EFSA (2010) reported a 48h EC50 of >200 mg.L<sup>-1</sup>  
139 for the freshwater ramshorn snail *Planorbis corneus*. However, there is a lack of  
140 published experimental data (supported chemical analysis) on the potential longer  
141 terms impacts of metaldehyde on non-target freshwater molluscs. The current work  
142 aims to help address this gap using a recently adopted OECD test species *Lymnaea*  
143 *stagnalis* (Ducrot et al., 2014).

144 From a basic research perspective, relatively little is known about the ability of many  
145 molluscan species to metabolise pesticides. One approach to exploring this  
146 possibility in aquatic species is to use metabolic inhibitors of key detoxification

147 pathways (Ankley et al., 1991; El-Merhibi et al., 2004); Weinstein & Garner 2008).  
148 One such inhibitor is piperonyl butoxide (PBO) (CAS: 51-03-6) which is also a widely  
149 used insecticide synergist (US EPA 2006b). The effectiveness of piperonyl butoxide  
150 as an insecticide synergist lies in its ability to inhibit several isozymes of cytochrome  
151 P450 (CYP450) system (Ankley & Collyard 1995; Feyereisen 2015). Piperonyl  
152 butoxide has also been used as an inhibitor of xenobiotic metabolism in fish where it  
153 inhibits the metabolism of aldrin, methoxychlor and trifluralin (Reinbold & Metcalf  
154 1976). Piperonyl butoxide also reduces the biotransformation 2,8-dichlorodibenzo-p-  
155 dioxin and pentachlorobenzene in goldfish (Sijm et al. 1993). In molluscs, Singh et  
156 al (2005) used piperonyl butoxide or the metabolic inhibitor MGK-264 (also termed  
157 ENT8184) to enhance the impacts of plant-derived molluscicides on reproduction of  
158 *Lymnaea acuminata*. Hence for basic research purposes, in addition to conducting  
159 exposures on metaldehyde *per se* this study also examined the potential for  
160 piperonyl butoxide to modify the developmental toxicity of metaldehyde in the  
161 embryos of *Lymnaea stagnalis*. All experiments included zinc as a positive control  
162 chemical as recommended by UK environmental regulators (Environment Agency  
163 2007).

## 164 **Materials and Methods**

165 **Organism culturing.** A culture of the RENILYS strain of *Lymnaea stagnalis* (kindly  
166 donated by colleagues at INRA, Rennes, France) was established at Plymouth  
167 University in October 2013 and cultured in 30 L aquaria ( $20 \pm 1^\circ\text{C}$  in synthetic fresh  
168 water). The culture water was changed twice weekly and animals fed organic lettuce  
169 *ad libitum* as per the method of Ducrot et al (2014). The aquaria were kept at a  
170 14:10 light dark cycle using full spectrum UV lights.

171 Embryos ('egg masses') were harvested from tanks of adults daily as needed for the  
172 experiments. Adapting the method of Liu et al (2013), individual embryos that had  
173 not developed past the two cell stage were collected by separating each embryo  
174 from the gelatinous mass under low power magnification (10x) and placing them into  
175 culture wells of a 24 well microplate (Thermoscientific, Nunclon Delta Surface).

176 **Embryonic toxicity of zinc over 7d (defining the positive control).** The first  
177 experiment (30 May – 6 June 2014) was conducted to define the optimal  
178 concentration of zinc for use as a positive control in future developmental toxicity

179 experiments using the embryos of the RENILYS strain of *L. stagnalis*. Adapting the  
180 method of Bandow & Weltje (2012), a 7d semi-static toxicity test was conducted with  
181 20 individual embryos each placed into a single well of a 24 well microplate. The test  
182 compound was zinc sulphate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ; CAS number 7446-20-0  
183 purchased from Sigma-Aldrich, Poole UK (purity  $\geq 99\%$ ). Nominal zinc  
184 concentrations used for the 7d study were 0.1, 0.32, 1.0, 3.2, 10 and 32  $\text{mg Zn.L}^{-1}$  in  
185 synthetic freshwater and all control and zinc test solutions were verified by  
186 Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES). Briefly, for  
187 ICP-OES test solution samples were collected and acidified prior to being analysed  
188 using a Thermoscientific iCap 7000 400 with a Burgener PEEK miramist nebuliser  
189 and a cyclone spray chamber. The ICP-OES had an exposure time 2,2; RF product  
190 1150; Viewing height 12; Coolant gas 12; Auxiliary gas flow, 0.5; and additional gas  
191 flow 0). Test solution renewals were conducted every other day and the physico-  
192 chemical parameters of the water checked in parallel microplates. Mean measured  
193 zinc concentrations were calculated based on 4 samples from each treatment over  
194 7d. *L. stagnalis* embryo development was observed daily using an Olympus  
195 stereomicroscope. Nominal and mean measured (in parentheses) zinc  
196 concentrations were 0 (0.003), 0.10 (0.12), 0.32 (0.212), 1.0 (0.66), 3.2 (1.82), 10  
197 (5.93) and 32 (17.34)  $\text{mg Zn.L}^{-1}$ , with an overall mean measured zinc concentration  
198 60.8% of nominal values (based on ICP-OES with a limit of detection of 0.001  $\text{mg}$   
199  $\text{Zn.L}^{-1}$ ). Physico-chemical parameters for the study were: conductivity 866 - 987  
200  $\mu\text{S.cm}^{-1}$ ; dissolved oxygen 60 - 95% saturation; pH 6.9 - 8.1; temperature 20.1 -  
201 21.9°C. Based on this study (Table 1), the 7d EC50 for embryo development was  
202 1.23  $\text{mg Zn.L}^{-1}$  (based on mean measured Zn concentrations) hence a nominal  
203 concentration of 2.0  $\text{mg Zn.L}^{-1}$  was used as a positive control for subsequent  
204 experiments. This compares well with the Environment Agency (2007)  
205 recommendation to use between 0.1 - 10  $\text{mg Zn.L}^{-1}$  for quality assurance purposes  
206 within UK effluent Direct Toxicity Testing programmes using *Daphnia magna*.

207 **Embryonic toxicity of piperonyl butoxide over 14d.** The developmental toxicity of  
208 PBO to *L. stagnalis* embryos was examined over 14 d (from 1-15 July 2014) using  
209 the same static renewal test design as for the zinc exposure study and including a  
210 positive control treatment of 2.0  $\text{mg Zn.L}^{-1}$ . Piperonyl butoxide (CAS number 51-03-  
211 6) was purchased from Sigma-Aldrich (technical grade purity 90%) and solutions

212 were made up in synthetic freshwater and using Analar<sup>®</sup> grade ethanol (0.64 mL.L<sup>-1</sup>)  
213 as a carrier solvent. The experiment was conducted with 20 individual embryos  
214 each placed into a separate well on a 24 well microplate. Nominal PBO  
215 concentrations were chosen based on the papers by Ankley et al (1991) and Ankley  
216 & Collyard (1995) and parallel microplates were run in order to allow analysis of the  
217 PBO concentrations and standard physico-chemical water quality parameters. *L.*  
218 *stagnalis* embryo development was observed daily using an Olympus  
219 stereomicroscope. Also, after 13d embryos were photographed in order to measure  
220 shell spire height and inter-ocular distance as a sign of toxicity (adapting the  
221 zebrafish embryo method described by Loucks & Ahlgren (2012). Test solutions  
222 were changed on alternate days and the physico-chemical parameters of the water  
223 checked in parallel plates. PBO analyses were conducted by using fluorescence  
224 spectrometry based on four sampling points over the 14 d exposure period. Briefly,  
225 the PBO analyses were conducted using a Hitachi F-4500 Fluorescence  
226 spectrophotometer. The system was run using EX start WL 200 nm; Ex end WL 300  
227 nm, EX sampling interval 10.0; EM start WL 300; EM end WL 350; EM sampling  
228 interval 10.0; with a scan speed of 2400 nm.min<sup>-1</sup>; Ex slit 5.0 nm, Em slit 5.0 nm,  
229 PMT voltage 950V, response 0.004s. Using this approach the overall mean  
230 measured PBO concentration was 224% of nominal values (with a limit of detection  
231 of 0.02 mg PBO.L<sup>-1</sup> using fluorescence spectrometry). Physico-chemical parameters  
232 for the study were: dissolved oxygen 69 - 95% saturation; pH 7.5 – 8.0; temperature  
233 18.5 - 22.0°C. Based on the results of this study (Table 2), the embryo 14d <sup>spire</sup>  
234 <sup>height</sup>NOEC and <sup>spire height</sup>LOEC values were 0.43 and 1.03 mg PBO.L<sup>-1</sup>, respectively  
235 and these values were used to aid the design of the following experiment with  
236 metaldehyde ± PBO.

237 **Embryonic toxicity of metaldehyde – 7d range finder.** The impact of metaldehyde  
238 per se on *L. stagnalis* embryonic development was examined using a 7 day static  
239 renewal toxicity test (with 20 individual embryos placed into separate wells of a 24  
240 well microplate. The nominal exposure concentrations of metaldehyde in synthetic  
241 freshwater were 1.0, 3.2, 10, 32 and 100 mg.L<sup>-1</sup>, plus the dilution water control and  
242 positive control (nominally 2.0 mg Zn.L<sup>-1</sup>). Metaldehyde analysis was carried out with  
243 a Shimadzu LC20AD liquid chromatograph, Shimadzu SIL20A HT Autosampler,  
244 Shimadzu SPD20A UV-vis spectrophotometer (Column: Thermo Hypersil-Keystone,

245 ODS, 5  $\mu\text{m}$  150 x 4.6 mm length). Samples of water were added to DNHP  
246 (0.25g.100 mL<sup>-1</sup> of 50% sulphuric acid) reagent. 1 ml of sample and 0.3 ml of DNHP  
247 were added to 1.5 ml capacity vials for the auto sampler. Standards of 1, 2, 5 and 10  
248 mg.L<sup>-1</sup> were used and concentrations higher than 10 mg.L<sup>-1</sup> were diluted 10x in order  
249 to fit within the calibration.

250 **Embryonic toxicity of metaldehyde  $\pm$  PBO over 21d.** In the absence of notable  
251 embryonic mortalities in any metaldehyde exposure group during the 7d range-  
252 finding study, for the 21d experiment (29 July -19 August 2014) the nominal  
253 exposure concentrations of metaldehyde prepared in synthetic freshwater were 1.0,  
254 3.2, 10, 32 and 100 mg.L<sup>-1</sup>, plus the dilution water control and positive control  
255 (nominally 2.0 mg Zn.L<sup>-1</sup>). All metaldehyde exposures were also conducted using a  
256 nominal synergist concentration of 0.5 mg PBO.L<sup>-1</sup> (in ethanol at 0.64 mL.L<sup>-1</sup>). *L.*  
257 *stagnalis* embryo development was observed daily using an Olympus  
258 stereomicroscope. After the initial placement in the wells, each individual was also  
259 photographed using high powered microscopy (Tills et al. 2010), with an Optem  
260 Zoom 70, Allied Vision Technology, Pike f210c camera in order to measure shell  
261 spire height, inter-ocular distance and heart rate as indicators of sublethal toxicity.  
262 The hatching success of each treatment was monitored daily from 14 days post  
263 fertilisation (dpf). Test solutions were changed on alternate days and the physico-  
264 chemical parameters of the water checked in parallel plates. Mean measured zinc  
265 concentrations in the dilution water was 0.002 mg Zn.L<sup>-1</sup> (LOD of 0.001 mg Zn.L<sup>-1</sup>).  
266 The overall mean measured PBO concentrations ranged from 0.47 – 0.56 mg  
267 PBO.L<sup>-1</sup> (with a limit of detection of 0.02 mg PBO.L<sup>-1</sup> using fluorescence  
268 spectrometry). Physico-chemical parameters for the study were: dissolved oxygen  
269 80-99% saturation; pH 7.5 – 8.1; temperature 19.4 - 20.9°C.

270 **Statistical Analyses.** Embryo development data from the initial 7d experiment with  
271 zinc were analysed using SIGMAPLOT 13 from Systat Software Inc<sup>®</sup> to derive the  
272 EC10 and EC50 values and their 95% confidence intervals (based on mean  
273 measured Zn concentrations). The No Observed Effect Concentration  
274 (<sup>development</sup>NOEC) and Lowest Observed Effect Concentration (<sup>development</sup>LOEC) for the  
275 same zinc exposure were calculated by one-way ANOVA using Minitab<sup>®</sup>. For the  
276 14d embryo toxicity test using only PBO, the embryo development, shell spire height

277 and inter-ocular distances were also calculated by one-way ANOVA using Minitab<sup>®</sup>.  
278 It was not possible to calculate the EC10 and EC50 values for this 14d experiment  
279 due to the absence of a full concentration-response curve. Finally, for the 21d  
280 metaldehyde experiment, embryo hatching success at 21d was evaluated using a  
281 series of Kruskal-Wallis rank-based nonparametric tests in Minitab<sup>®</sup> in order to derive  
282 the <sup>hatching</sup>NOEC and <sup>hatching</sup>LOEC for metaldehyde *per se*. The 21d results from the  
283 combined metaldehyde and PBO treatments were also analysed by one-way  
284 ANOVA in Minitab<sup>®</sup> in order to identify statistically significant differences in the  
285 present or absence of the PBO synergist.

286

## 287 **Results**

288 **Embryonic toxicity of zinc over 7d.** The specific purpose of this experiment was  
289 to define a concentration of zinc that would generate a dramatic toxic response in the  
290 embryos of *L. stagnalis* RENILYS (Table 1). After 7d, the <sup>development</sup>EC50 was 2.0267  
291 mg Zn.L<sup>-1</sup> (nominal concentration) which equated to 1.23 mg Zn.L<sup>-1</sup> (based on mean  
292 measured Zn concentrations).

293 **Embryonic toxicity of piperonyl butoxide over 14d.** Exposure of embryos for 14d  
294 up to 2.34 mg PBO.L<sup>-1</sup> (based on mean measured PBO concentrations) generated  
295 no developmental inhibition. In contrast, snail embryos exposed to the positive  
296 control (2.0 mg Zn.L<sup>-1</sup>) had a 90% reduction in normal development (Table 2). The  
297 embryonic shell spire height was inhibited by piperonyl butoxide exposure and gave  
298 14d <sup>spire height</sup>NOEC and <sup>spire height</sup>LOEC values of 0.43 and 1.03 mg PBO.L<sup>-1</sup>,  
299 respectively as mean measured concentrations (one-way ANOVA; P<0.001). In  
300 contrast, the embryonic inter-ocular distance was unaffected by piperonyl butoxide  
301 exposure and gave a 14d <sup>interocular distance</sup>NOEC value of  $\geq 2.34$  mg PBO.L<sup>-1</sup> based on  
302 mean measured concentrations. As a key goal of this experiment was to define a  
303 Maximum Tolerated Concentration (MTC) of piperonyl butoxide that did not cause  
304 developmental toxicity in the snail embryos, the MTC was considered to be  
305 approximately 0.5 mg PBO.L<sup>-1</sup> (Hutchinson et al., 2009).

306 **Embryonic toxicity of metaldehyde  $\pm$  PBO over 21d.** Since there was no  
307 significant snail embryonic mortalities after 7d exposure to metaldehyde up 100

308 mg.L<sup>-1</sup>, this was chosen as the highest exposure concentration for the subsequent  
309 21d experiment in accordance with standard OECD recommendations not to exceed  
310 this value unless there is environmental exposure data to warrant higher test  
311 concentrations. Based on mean measured concentrations of metaldehyde only, this  
312 gave 21d <sup>hatching success</sup>NOEC and <sup>hatching success</sup>LOEC values of 36 and 116 mg.L<sup>-1</sup>,  
313 respectively (one-way ANOVA; P<0.05) (Table 3). Similarly, using mean measured  
314 concentrations of metaldehyde only also gave 21d <sup>spire height</sup>NOEC and <sup>spire height</sup>LOEC  
315 values of 36 and 116 mg.L<sup>-1</sup>, respectively (one-way ANOVA; P<0.05). There were  
316 also statistically significant differences in hatching success between all metaldehyde  
317 treatment in the presence or absence of PBO at a measured concentration of  
318 between 0.47-0.56 mg PBO.L<sup>-1</sup> (P≤0.045). The test concentration of 109 mg MET.L<sup>-1</sup>  
319 and 0.47 mg PBO.L<sup>-1</sup> is statistically significant from the ethanol and dilution water  
320 controls and all other test concentrations (P<0.05). The use of zinc as a positive  
321 control also achieved its aim and no embryos hatched after 21d when exposed to a  
322 nominal concentration of 2.0 mg Zn.L<sup>-1</sup> (Table 3). In terms of the intra-ocular  
323 observations, there were no statistically significant differences between embryos for  
324 any metaldehyde and PBO treatment group whereas there was a significant  
325 difference for the Zn positive control as these embryos failed to develop any  
326 eyespots (P=<0.001). Embryo heart rate data made using video microscopy showed  
327 considerable variability in the dilution water controls (mean values ranging from 59.1  
328 to 75.5 beats.min<sup>-1</sup> measured between 7d to 20d) and the ethanol solvent control  
329 (mean values ranging from 49.5 to 79.8 beats.min<sup>-1</sup> measured between 7d to 20d).  
330 The measured heart rates of embryos exposed to metaldehyde only at 116 mg.L<sup>-1</sup>  
331 had mean values ranging from 31.6 to 65.5 beats.min<sup>-1</sup> between 7d to 20d and for  
332 the 36 mg.L<sup>-1</sup> metaldehyde exposure group had mean values ranging from 31.4 to  
333 77.0 beats.min<sup>-1</sup> between 7d to 20d. For the metaldehyde (33.7 mg.L<sup>-1</sup>) and  
334 piperonyl butoxide (0.47 mg.L<sup>-1</sup>) embryo heart rates ranged from 44.0 to 62.1  
335 beats.min<sup>-1</sup> between 7d to 20d and the range was similar for other metaldehyde and  
336 piperonyl butoxide treatments. Overall there was no clear evidence over time of  
337 metaldehyde or piperonyl butoxide having a consistent effect in heart rate in *L.*  
338 *stagnalis* embryos in this study.

339

340

## 341 Discussion

342 The goals of the project were (1) to generate information on the developmental  
343 toxicity of metaldehyde to non-target freshwater molluscs in order to strengthen the  
344 EFSA (2010) risk assessment for surface waters; and (2) investigate through the use  
345 of a P450 inhibitor whether the embryos of *L. stagnalis* can possibly detoxify  
346 metaldehyde under laboratory conditions. For the first objective, the results of the  
347 21d experiment suggest an overall 21d NOEC value for metaldehyde of 36 mg.L<sup>-1</sup>  
348 based on hatching success and growth (measured as shell spire height) (Table 3).  
349 In comparison, the EFSA (2010) data review cites an acute lethality study using  
350 freshwater ramshorn snails (*Planorbium corneus*) with a 48h EC50 > 200 mg.L<sup>-1</sup>  
351 (Table 4). Given that measured concentrations of metaldehyde in UK freshwater  
352 sites is in the range 0.4 to 0.6 µg.L<sup>-1</sup> (with but sometimes up to 2.7 µg.L<sup>-1</sup> as reported  
353 by Kay and Grayson 2014) this suggests a large margin of safety for non-target  
354 freshwater gastropod populations. This conclusion is also broadly supported by the  
355 EFSA (2010) predictive exposure modelling for one slug bait formulation (Metarex<sup>®</sup>)  
356 which cites Predicted Exposure Concentrations for metaldehyde in surface waters  
357 (PEC<sub>sw</sub>) under a FOCUS Step 2 exposure scenario for Northern Europe of 19.016  
358 µg.L<sup>-1</sup> after 42 days.

359 With regard to the second objective, piperonyl butoxide was successfully used as a  
360 metabolic detoxification inhibitor to suggest that the embryos of *L. stagnalis* can  
361 detoxify metaldehyde under the conditions of the 21d laboratory experiment. The  
362 14d MTC for piperonyl butoxide was successfully defined as nominally 0.5 mg  
363 PBO.L<sup>-1</sup> (14d NOEC of 0.43 mg PBO.L<sup>-1</sup> based on measured values) for *L. stagnalis*  
364 RENILYS embryos (Table 2). The inhibition of *L. stagnalis* embryo growth (as spire  
365 height) at 1.03 and 2.34 mg PBO.L<sup>-1</sup> may be linked to PBO impacts on metabolism  
366 since PBO has the ability to bind to cytochrome P450 (Weinstein & Garner, 2008). In  
367 theory, the resulting reduction in metabolic output could cause a reduction in  
368 organism growth rates such that the snail embryos may have had insufficient energy  
369 to grow and develop normally. The plant growth regulator flurprimidol has also been  
370 reported as reducing growth by blocking the cytochrome P450 system (Rademacher,  
371 2000). More broadly, the piperonyl butoxide data suggest a sensitivity for gastropod  
372 embryos similar to that reported by Ankley et al (1991) for *Ceriodaphnia dubia* (48h  
373 LC50 of 1.0 mg PBO.L<sup>-1</sup>), *Daphnia magna* (48h LC50 of 2.83 mg PBO.L<sup>-1</sup>) and

374 *Daphnia pulex* (48h LC50 of 1.62 mg PBO.L<sup>-1</sup>). As shown in Table 3, snail embryo  
375 hatching success was reduced by very high concentrations of metaldehyde (116  
376 mg.L<sup>-1</sup>). Currently, the embryos were maintained at 20 ± 1°C; however, there was  
377 variation in the hatching time of the different treatments. Embryos in the dilution  
378 water control started hatching at 13 days post-fertilisation (dpf) (10/38) and  
379 continued at a steady rate. However in the 1.0 mg MET.L<sup>-1</sup> exposures, embryo  
380 hatching started at 14 dpf (3/20) which at 3.53, 9.0 and 36 mg MET.L<sup>-1</sup> the start of  
381 embryo hatching was delayed until 15 dpf (18/79) and the 116 mg MET.L<sup>-1</sup> started at  
382 16 dpf (2/40). In comparison, Smirthwaite et al. (2007) investigated the timing  
383 differences in developmental events of several gastropod species and reported that  
384 *L. stagnalis* (strain unspecified) cultured at 20 ± 1°C would typically hatch at 14 dpf.  
385 As shown in Table 3, only the 109 mg MET.L<sup>-1</sup> and 0.47 mg PBO.L<sup>-1</sup> exposure group  
386 totally failed to have any successful hatching after 21d. The metabolism and  
387 detoxification of metaldehyde could be using a substantial amount of energy that the  
388 embryo would usually use for growth and development (Strathmann 1985; Tills et al.,  
389 2010; Munley et al., 2013). However, during this study it was noted that even though  
390 there was a reduction in hatching success, the embryos appeared to develop  
391 normally throughout the 21 days. An explanation for the delay in effects could be the  
392 egg case acting as a barrier to toxicants. *L. stagnalis* embryo development takes  
393 place inside a large gelatinous capsule and therefore this protects against chemicals  
394 such as metaldehyde. Carls and Rice (1988) showed a similar pattern in fish  
395 embryos exposed to hydrocarbons, where there were sub-lethal effects on the  
396 embryos in the absence of mortalities.

397 In conclusion, chronic effects of metaldehyde on embryo development of *L. stagnalis*  
398 under laboratory conditions have been defined (high mg.L<sup>-1</sup> range) and suggest a  
399 low risk to the early life stages of gastropod molluscs relative to reported  
400 environmental exposures (low µg.L<sup>-1</sup> range). As noted by Bandow and Weltje  
401 (2012), the 21d test design could be a very useful supplement to the draft OECD test  
402 guideline to assess reproduction in *L. stagnalis* (Ducrot et al., 2014). Finally  
403 piperonyl butoxide was successfully used to generate evidence that gastropod  
404 embryos may have some P450-based metabolic capacity to detoxify metaldehyde.  
405 Further research is warranted to explore this theme further using a wider range of  
406 agrochemicals and different metabolic inhibitors (Feyereisen 2015).

407 **References**

- 408 Ankley, G.T., Collyard, S.A. (1995). Influence of piperonyl butoxide on the toxicity of  
409 organophosphate insecticides to three species of freshwater benthic  
410 invertebrates. *Comp. Biochem. Physiol.* 110C: 149–155
- 411 Ankley, G.T., Dierkes, J.R., Jensen, D.A., Peterson, G.S. (1991). Piperonyl butoxide  
412 as a tool in aquatic toxicology research with organophosphate insecticides.  
413 *Ectotoxicol. Environ Safety* 21: 266–274
- 414 Autin, O., Hart J., Jarvis P., MacAdam J., Parsons SA., Jefferson B. (2012)  
415 Comparison of UV/H<sub>2</sub>O<sub>2</sub> and UV/TiO<sub>2</sub> for the degradation of metaldehyde:  
416 kinetics and the impact of background organics. *Water Res* 46: 5655-5662
- 417 Bandow, C., Weltje, L. (2012). Development of an embryo toxicity test with the pond  
418 snail *Lymnaea stagnalis* using the model substance tributyltin and common  
419 solvents. *Sci. Total Environ.* 435-436: 90–95
- 420 Bieri, M., (2003). The environmental profile of metaldehyde. In: BCPC Symposium  
421 Proceedings, British Crop Protection Council, pp. 255–262  
422
- 423 Bleakley, C., Ferrie, E., Collum, N., Burke, L. (2008). Self-poisoning with  
424 metaldehyde. *Emergency medicine journal : EMJ*, 25(6), 381–382
- 425 Calumpang, S.M.F., Median, M.J.B., Tejada, A.W., Medina, J.R. (1995).  
426 Environmental impact of two molluscicides: niclosamide and metaldehyde in a  
427 rice paddy ecosystem. *Bull. Environ. Contam. Toxicol.* 55: 494 – 501  
428
- 429 Cardoso, D.N., Santos, M.J.G., Soares, A.M.V.M., Loureiro, S. (2015) Molluscicide  
430 baits impair the life traits of *Folsomia candida* (Collembola): possible hazard to  
431 the population level and soil function. *Chemosphere* 132: 1-7  
432
- 433 Carls, M.G., Rice, S.D. (1988). Sensitivity differences between eggs and larvae of  
434 walleye pollock to hydrocarbons. *Mar Environ Res* 26: 285–297  
435

436 Coloso, R.M., Borlongan, I.G., Blum, R. (1998) Use of metaldehyde as a  
437 molluscicide in semi-commercial and commercial milkfish ponds. *Crop Protection*  
438 17: 669–674  
439

440 Ducrot V., Askem C., Azam D., Brettschneider D., Brown R., Charles S., Coke M.,  
441 Collinet M., Delignette-Muller M., Forfait-Dubuc C., Holbech H., Hutchinson T.H.,  
442 *et al.* 2014. Development and validation of an OECD reproductive toxicity test  
443 guideline with the pond snail *Lymnaea stagnalis* (Mollusca: Gastropoda).  
444 *Regulatory Toxicology & Pharmacology* 70: 605-614  
445

446 Edwards, C.A., Arancon, N.Q., Vasko-Bennett, M., Little, B., Askar, A. (2009) The  
447 relative toxicity of metaldehyde and iron phosphate-based molluscicides to  
448 earthworms. *Crop Protection* 28: 289–294  
449

450 El-Merhibi, A., Kumar, A., Smeaton, T. (2004). Role of piperonyl butoxide in the  
451 toxicity of chlorpyrifos to *Ceriodaphnia dubia* and *Xenopus laevis*. *Ecotoxicol*  
452 *Environ Safety* 57: 202–212

453 Environment Agency (2007). The direct toxicity assessment of aqueous  
454 environmental samples using the juvenile *Daphnia magna* immobilisation test.  
455 *Methods for the Examination of Waters and Associated Materials*. Available from  
456 [https://www.gov.uk/government/uploads/system/uploads/attachment\\_data/file/31](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/316804/daphnia208_1669241.pdf)  
457 [6804/daphnia208\\_1669241.pdf](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/316804/daphnia208_1669241.pdf) (accessed 18 September 2015)  
458

459 Environment Agency (2009) The determination of metaldehyde in waters using  
460 chromatography with mass spectrometric detection. Available from  
461 [https://www.gov.uk/government/uploads/system/uploads/attachment\\_data/file/31](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/316782/Metaldehyde-226b.pdf)  
462 [6782/Metaldehyde-226b.pdf](https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/316782/Metaldehyde-226b.pdf) (accessed 18 September 2015)  
463

464 European Food Safety Authority. 2010. Conclusion on the peer review of the  
465 pesticide risk assessment of the active substance metaldehyde. *EFSA Journal*  
466 8(10): 1856  
467

468 Feyereisen R. (2015) Insect P450 inhibitors and insecticides: challenges and  
469 opportunities. *Pestic Manag Sci* 71: 793-800  
470

471 Gavin, W.E., Mueller-Warrant, G.W., Griffith, S.M., Banowetz, G.M., 2012. Removal  
472 of molluscicidal bait pellets by earthworms and its impact on control of the gray  
473 field slug (*Derocerus reticulatum* Mueller) in western Oregon grass seed fields.  
474 *Crop Protection* 42: 94–101  
475

476 Gimingham, C.T., Newton, H.C.F., 1937. A Poison Bait for Slugs, *The Journal of the*  
477 *Ministry of Agriculture* Vol. XLIV, 3: 242 – 246  
478

479 Gupta, R.C. (2012). *Veterinary Toxicology: Basic and Clinical Principles*. 2nd ed.  
480 London: Elsevier. 624-630  
481

482 Hutchinson T.H., Bögi C., Winter M.J., Owens J.W. 2009. Benefits of the Maximum  
483 Tolerated Dose (MTD) and Maximum Tolerated Concentration (MTC) concept in  
484 aquatic toxicology. *Aquatic Toxicology* 91: 197–202  
485

486 Iglesias, J., Castillejo, J., Castro, R., 2003. The effects of repeated applications of  
487 the molluscicide metaldehyde and the biocontrol nematode *Phasmarhabditis*  
488 *hermaphrodita* on molluscs, earthworms, nematodes, acarids and collembolans:  
489 a two-year study in north-west Spain. *Pest Manag Sci* 59: 1217–1224  
490

491 Jones, A., Charlton, A., 1999. Determination of metaldehyde in suspected cases of  
492 animal poisoning using gas chromatography–ion trap mass spectrometry. *J.*  
493 *Agric. Food Chem* 47: 4675–4677  
494

495 Kay, P., Grayson, R. (2014) Using water industry data to assess the metaldehyde  
496 pollution problem. *Water and Environment Journal* 28: 410-417

497 Langan, A.M., Shaw, E.M. (2006) Responses of the earthworm *Lumbriculus*  
498 *terrestris* (L.) to iron phosphate and metaldehyde slug pellet formulations. *Appl.*  
499 *Soil Ecol.* 34: 184–189  
500

- 501 Li, C., Wu, Y.-L., Yang, T., Zhang, Y. (2010) Determination of metaldehyde in water  
502 by SPE and UPLC-MS-MS. *Chromatographia* 72: 987–991  
503
- 504 Liu, T., Koene, J. M., Dong, X. (2013). Sensitivity of isolated eggs of pond snails : a  
505 new method for toxicity assays and risk assessment. *Environ. Monit Assess* 185:  
506 4183–4190
- 507 Loucks, E., Ahlgren, S. (2012) Assessing teratogenic changes in a zebrafish model  
508 of fetal alcohol exposure. *Journal of Visualized Experiments* 61: 1–6
- 509 Marshal, J. (2013). Briefing paper on , Water UK. Water UK (pp. 1–5)
- 510 Mills, J.D., McCrohan, R.C., Bailey, S.E.R. (1992). Electrophysiological responses to  
511 metaldehyde in neurones of the feeding circuitry of the snail *Lymnaea stagnalis*.  
512 *Pestic Biochem Physiol* 42: 35–42
- 513 Mills, J.D., McCrohan, C.R., Bailey, S.E.R. (1990) Effects of metaldehyde and  
514 acetaldehyde on feeding responses and neuronal activity in the snail *Lymnaea*  
515 *stagnalis*. *Pestic Sci* 28: 89–99
- 516 Munley, K.M., Brix, K.V, Panlilio, J., Deforest, D.K., Grosell, M. (2013). Growth  
517 inhibition in early life-stage tests predicts full life-cycle toxicity effects of lead in  
518 the freshwater pulmonate snail , *Lymnaea stagnalis*. *Aquatic Toxicology*, 128-  
519 129, 60–66
- 520 PAN. (2010) PAN pesticides database, metaldehyde [online].  
521 [http://www.pesticideinfo.org/Detail\\_Chemical.jsp?Rec\\_Id=PC32878](http://www.pesticideinfo.org/Detail_Chemical.jsp?Rec_Id=PC32878) [accessed  
522 12 September 2015].  
523
- 524 Paolo, M., Renzo, B., 1983. Determination of metaldehyde in workroom air. *Bull.*  
525 *Environ. Contam. Toxicol.* 30: 479–484
- 526 Rademacher, W. (2000). Growth retardants: effects on gibberellin biosynthesis and  
527 other metabolic pathways. *Ann Rev Plant Biol* 51: 501–531

- 528 Rae, R.G., Robertson, J.F., Wilson, M.J. (2009) Optimization of biological  
529 (Phasmarhabditis hermaphrodita) and chemical (iron phosphate and  
530 metaldehyde) slug control. Crop Prot 28: 765–773
- 531 Reinbold, K.A., Metcalf, R.L. (1976) Effects of the synergist piperonyl butoxide on  
532 metabolism of pesticides in green sunfish. Pestic Biochem Physiol 6: 401-412
- 533 Selim, S., Seiber, J.N., 1973. Gas chromatographic method for the analysis of  
534 metaldehyde in crop tissues. J. Agric. Food Chem. 21: 430–433
- 535 Shelley, L.K., Balfry, S.K., Ross, P.S., Kennedy, C.J. (2009). Immunotoxicological  
536 effects of a sub-chronic exposure to selected current use pesticides in rainbow  
537 trout (*Oncorhynchus mykiss*). Aquatic Toxicol 92: 95–103
- 538 Sijm, D.T.H.M., Schaap, G., Opperhuizen, A. (1993) The effect of the  
539 biotransformation inhibitor piperonyl butoxide on the bioconcentration of 2,8-  
540 dichlorodibenzo-p-dioxin and pentachlorobenzene in goldfish. Aquatic Toxicol  
541 27: 345–359
- 542 Singh, P., Singh, V.K., Singh, D.K. (2005) Effect of binary combinations of some  
543 plant-derived molluscicides with MGK-264 or piperonyl butoxide on the  
544 reproduction of the snail *Lymnaea acuminata*. Pestic Manag Sci 61: 204-208
- 545 Smirthwaite, J. J., Rundle, S. D., Bininda-Emonds, O. R. P., Spicer, J. I. (2007). An  
546 integrative approach identifies developmental sequence heterochronies in  
547 freshwater basommatophoran snails. Evolution Devel 9: 122–130
- 548 Strathmann, R.R. (1985) Feeding and non-feeding larval development and life  
549 history evolution in marine invertebrates. Ann Rev Ecol Systematics 16: 339-361
- 550 Tills, O., Spicer, J., Rundle, S. (2010) Salinity-induced heterokairy in an upper  
551 estuarine population of the snail *Radix balthica* (Mollusca: Pulmonata). Aquat  
552 Biol 9: 95-105
- 553 Triebkorn, R. (1989). Ultrastructural changes in the digestive tract of *Deroceras*  
554 *reticulatum* (Müller) induced by a carbamate molluscicide and metaldehyde.  
555 Malacologia 31: 141-156  
556

557 Triebkorn, R., Ebert, D. (1989). The importance of mucus production in slugs'  
558 reaction to molluscicides and the impact of molluscicides on the mucus  
559 producing system. In: Slugs and Snails in World Agriculture, ed. I F Henderson,  
560 pp. 373 - 378. BCPC Monograph No. 41.

561 Triebkorn, R., Christensen, K., Heim, I. (1998). Effects of orally and dermally  
562 applied metaldehyde on mucus cells of slugs (*Deroceras reticulatum*) depending  
563 on temperature and duration of exposure. Journal of Molluscan Studies 64: 467-  
564 487

565 US EPA (2006a). Reregistration Eligibility Decision for Metaldehyde. Prevention,  
566 Pesticides and Toxic Substances. Document reference OPP-2005- 0231 (54  
567 pages)

568 US EPA (2006b). Reregistration Eligibility Decision for Piperonyl Butoxide (PBO).  
569 Prevention, Pesticides and Toxic Substances. Document reference EPA 738-R-  
570 06-005 (112 pages)

571 Weinstein, J.E., Garner, T.R. (2008). Piperonyl butoxide enhances the  
572 bioconcentration and photoinduced toxicity of fluoranthene and benzo[a]pyrene  
573 to larvae of the grass shrimp (*Palaemonetes pugio*). Aquatic Toxicol 87: 28-36

574 WHO. (1996). WHO/FAO Data sheets on pesticides. No. 93.

575 Zen stoves (2015) Information on META solid fuel for camping stoves. Available  
576 from <http://zenstoves.net/> [accessed 17 September 2015]

577 Zhang, X.Y., Dai, X.F., (2006) Degradation and determination of residues of  
578 metaldehyde in tobacco and soil. Chinese J. Pestic. Sci. 8: 344–348

579 Zhang, H.-Y., Wang, C., Lu, H.-Z., Guan, W.-B., & Ma, Y.-Q. (2011). Residues and  
580 dissipation dynamics of molluscicide metaldehyde in cabbage and soil. Ecotoxic  
581 Environ Safety 74: 1653–1658

582

583

584 Table 1. Developmental toxicity of the reference chemical zinc sulfate heptahydrate (CAS  
 585 number 7446-20-0) to *Lymnaea stagnalis* RENILYS® embryos under semi-static conditions  
 586 over 7d at 20 ± 2°C.

Time	Response	Embryo development (expressed as mean measured mg Zn.L <sup>-1</sup> ; n=20)			
		EC <sub>10</sub> (± 95% CI)	EC <sub>50</sub> (± 95% CI)	LOEC	NOEC
48 hr	Development (morphology)	6.08 (5.98 – 6.18)	>17.34	>17.34	17.34
96hr	Development (Spinning Behaviour)	1.98 (1.82 – 2.13)	3.78 (3.73 – 3.83)	5.93	1.82
7 day	Development (morphology)	0.53 (0.11 – 1.05)	1.23 (1.18 – 1.31)	1.82	0.66

587

588 Footnote - Nominal and mean measured (in parentheses) zinc concentrations were 0  
 589 (0.003), 0.10 (0.12), 0.32 (0.212), 1.0 (0.66), 3.2 (1.82), 10 (5.93) and 32 (17.34) mg Zn.L<sup>-1</sup>,  
 590 with an overall mean measured zinc concentration 60.8% of nominal values (based on  
 591 Inductively Coupled Plasma - Optical Emission Spectrometry with a limit of detection of  
 592 0.001 mg Zn.L<sup>-1</sup>. Physico-chemical parameters for the study (30 May-6 June 2014) were:  
 593 conductivity 866 - 987 µS.cm<sup>-1</sup>; dissolved oxygen 60 - 95% saturation; pH 6.9 – 8.1;  
 594 temperature 20.1 – 21.9°C.

595

596 Table 2. Developmental toxicity of piperonyl butoxide (PBO) (CAS number 51-03-6) to  
 597 *Lymnaea stagnalis* RENILYS® embryos under semi-static conditions over 14 days at 20 ±  
 598 2°C.

Mean measured exposure concentrations (mg PBO.L <sup>-1</sup> )	Biological responses after 14 days (n=20)		
	% normal development (morphology)	Spire height in µm (mean ± SD)	Interocular distance in µm (mean ± SD)
Dilution water control	95	873 ± 228	220 (n=1)
Ethanol control 0.64 ml.L <sup>-1</sup>	95	857 ± 231	295 (n=1)
Positive control 2.0 mg Zn.L <sup>-1</sup>	10 <sup>aa</sup>	448 ± 187 <sup>aa</sup>	176 ± 28.2
0.018	90	847 ± 312	243 ± 10.6
0.036	95	853 ± 222	183 ± 67.2
0.059	90	859 ± 244	223 ± 37.9
0.225	95	869 ± 225	185 (n=1)
0.43	100	870 ± 104	200 ± 28.3
1.03	100	702 ± 134 <sup>a</sup>	173 ± 24.8
2.34	100	553 ± 108 <sup>aa</sup>	180 ± 21.8
Summary of developmental effects after 14d (measured PBO concentrations):			
development NOEC	≥ 2.34	-	-
development LOEC	> 2.34	-	-
spire height NOEC	-	0.43	-
spire height LOEC	-	1.03	-
interocular distance NOEC	-	-	≥ 2.34
interocular distance LOEC	-	-	> 2.34

599

600 Footnote - Measured zinc concentrations in the ISO reconstituted dilution water was 0.002  
 601 mg Zn.L<sup>-1</sup> (LOD of 0.001 mg Zn.L<sup>-1</sup>). The overall mean measured PBO concentration was  
 602 224% of nominal values (with a limit of detection of 0.02 mg PBO.L<sup>-1</sup> using fluorescence  
 603 spectrometry. Physico-chemical parameters for the study (1-15 July 2014) were: dissolved  
 604 oxygen 69 - 95% saturation; pH 7.5 – 8.0; temperature 18.5 - 22.0°C. ANOVA results  
 605 showing PBO treatments significantly different from the ethanol control shown as <sup>a</sup>(P<0.05)  
 606 and <sup>aa</sup>(P<0.01).

607

608

609 Table 3. Summary of ecotoxicology and analytical chemistry data for freshwater molluscs  
 610 (*Lymnaea stagnalis* RENILYS® strain) exposed to Metaldehyde (ME) (CAS number 9002-91-  
 611 9) and piperonyl butoxide (PBO) (CAS number 51-03-6) using semi-static renewal conditions  
 612 for 21 days at 20 ± 1°C.

Mean measured exposure concentrations (mg ME.L <sup>-1</sup> )	Biological responses after 21 days (n=20)		
	% hatching success	Spire height (µm as mean ± SD)	Interocular distance (µm as mean ± SD)
Dilution Water Control	85	1090 ± 210	238 ± 32.5
Ethanol control 0.64 ml.L <sup>-1</sup>	84	1268 ± 211	262 ± 34.0
Positive control 2.0 mg Zn.L <sup>-1</sup>	0	-	-
<i>Metaldehyde (ME) only treatments:</i>			
1.00	100	1092 ± 135	236 ± 16.4
3.53	100	1091 ± 191	225 ± 19.4
9.00	95	1115 ± 136	232 ± 13.0
36.0	100	1082 ± 188	232 ± 20.9
116	60 <sup>aa</sup>	886 ± 108 <sup>a</sup>	212 ± 20.2
hatching success NOEC	36.0	-	-
hatching success LOEC	116	-	-
spire height NOEC	-	36.0	-
spire height LOEC	-	116	-
interocular distance NOEC	-	-	≥ 116
interocular distance LOEC	-	-	> 116
<i>Metaldehyde (ME) and piperonyl butoxide (PBO) treatments:</i>			
2.36 mg ME.L <sup>-1</sup> + 0.56 mg PBO.L <sup>-1</sup>	40 <sup>b</sup>	924 ± 135	215 ± 24.1
4.46 mg ME.L <sup>-1</sup> + 0.52 mg PBO.L <sup>-1</sup>	25 <sup>b</sup>	833 ± 124	221 ± 18.9
12.4 mg ME.L <sup>-1</sup> + 0.56 mg PBO.L <sup>-1</sup>	47 <sup>b</sup>	894 ± 137	222 ± 27.8
33.7 mg ME.L <sup>-1</sup> + 0.47 mg PBO.L <sup>-1</sup>	47 <sup>b</sup>	879 ± 108	212 ± 18.8
109 mg ME.L <sup>-1</sup> + 0.47 mg PBO.L <sup>-1</sup>	0 <sup>b</sup>	-	-

613

614 Footnote - Measured zinc concentrations in the dilution water was 0.002 mg Zn.L<sup>-1</sup> (LOD of  
 615 0.001 mg Zn.L<sup>-1</sup>). The overall mean measured PBO concentration was 224% of nominal  
 616 values (with a limit of detection of 0.02 mg PBO.L<sup>-1</sup> using fluorescence spectrometry).  
 617 Physico-chemical parameters for the study (29 July-19 August 2014) were: dissolved oxygen  
 618 80-99% saturation; pH 7.5 – 8.1; temperature 19.4 - 20.9°C. ANOVA results showing ME  
 619 only treatments significantly different from the ethanol control shown as <sup>a</sup> (P<0.05) and <sup>aa</sup>  
 620 (P<0.01). T-test results showing significant differences between the ME results for ± PBO  
 621 shown as <sup>b</sup> (P<0.05).

622

623

624 Table 4. Summary of published data on impacts of metaldehyde (ME) on aquatic  
 625 invertebrates.

626

Test species	Life stage	Exposure concentrations verified	Toxic effect (mg ME.L <sup>-1</sup> )	Reference
FRESHWATER STUDIES:				
Algae ( <i>Desmodesmus subspicatus</i> )	LC	Nominal	72h <sup>growth</sup> EC50 > 200	EFSA 2010
Crustacean ( <i>Daphnia magna</i> )	AD	Mean measured	48h <sup>survival</sup> EC50 > 90	EFSA 2010
Crustacean ( <i>Daphnia magna</i> )	LC	Nominal	21d <sup>survival</sup> NOEC = 90 21d <sup>repro</sup> NOEC = 90	EFSA 2010
Crustacean ( <i>Gammarus pseudolimnaeus</i> )	AD	Nominal	96h <sup>survival</sup> EC50 = 19.3	EFSA 2010
Fish ( <i>Oncorhynchus mykiss</i> )	JU	Nominal	96h <sup>survival</sup> LC50 = 75	EFSA 2010
Fish ( <i>Oncorhynchus mykiss</i> )	JU	Nominal	21d <sup>survival</sup> NOEC = 37.5 21d <sup>growth</sup> NOEC = 37.5	EFSA 2010
Mollusc ( <i>Lymnaea stagnalis</i> )	EM	Mean measured	21d <sup>hatching</sup> NOEC = 36 21d <sup>hatching</sup> LOEC = 116 21d <sup>spire height</sup> NOEC = 36 21d <sup>spire height</sup> LOEC = 116	This study
Mollusc ( <i>Planorbarius corneus</i> )	AD	Nominal	48h <sup>survival</sup> EC50 > 200	EFSA 2010
SALTWATER STUDIES: No data available.				
Molluscs ( <i>Cerithidea cingulata</i> ) in brackish aquaculture ponds	Pond study	Nominal	After 7d snail mortality of 86-87% at 0.38-1.55 mg ME.L <sup>-1</sup> compared to 6% in control ponds	Coloso <i>et al.</i> , (1998)

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628 Notes: AD = adult; EC = effective concentration; ECO = ecosystem study; EM = embryo; JU  
 629 = juvenile; LC = life cycle; LOEC = Lowest Observed Effect Concentration; NOEC = No  
 630 Observed Effect Concentration; repro = reproduction. Also, note a 1.0 M solution of  
 631 metaldehyde = 176.212 g.L<sup>-1</sup> and water solubility = 222 mg ME.L<sup>-1</sup> (EFSA 2010).

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