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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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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 $mg.L^{-1}$ at 17°C) and has been detected in UK surface waters in the
27 concentration range of typically 0.2-0.6 $\mu g.L^{-1}$ (maximum 2.7 $\mu g.L^{-1}$) 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 embryionic 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 ± 1°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 ^{hatching}NOEC and
35 ^{hatching}LOEC mean measured values of 36 and 116 mg $MET.L^{-1}$, respectively (equal
36 to the 21d $^{spire\ height}$NOEC and $^{spire\ height}$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 0.47-0.56 $mg.L^{-1}$) 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 34-36 $mg.L^{-1}$) 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.

45

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 $mg.L^{-1}$ 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®, Deadline®, Hardy®, Metarex® and
62 Metason®) (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

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

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

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

114 cells of the heptatopancreas and death (Triebskorn 1989; Triebskorn & Ebert 1989;
115 Coloso et al., 1998; Triebskorn 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 $g.kg^{-1}$ 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 $mg.L^{-1}$. 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 $mg.L^{-1}$, EFSA (2010) reported a 48h EC50 of >200 $mg.L^{-1}$
139 for the freshwater ramshorn snail *Planobarius 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 ± 1ºC 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 ($ZnSO_4.7H_2O$; CAS number 7446-20-0
183 purchased from Sigma-Aldrich, Poole UK (purity ≥99%). Nominal zinc
184 concentrations used for the 7d study were 0.1, 0.32, 1.0, 3.2, 10 and 32 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) 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 mg
199 $Zn.L^{-1}$. Physico-chemical parameters for the study were: conductivity 866 - 987
200 $\mu S.cm^{-1}$; 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 mg $Zn.L^{-1}$ (based on mean measured Zn concentrations) hence a nominal
203 concentration of 2.0 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 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 mg $Zn.L^{-1}$. Piperonyl butoxide (CAS number 51-03-
211 6) was purchased from Sigma-Aldrich (technical grade purity 90%) and solutions

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

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

ODS, 5 µm 150 x 4.6 mm length). Samples of water were added to DNHP (0.25g.100 mL^{-1} of 50% sulphuric acid) reagent. 1 ml of sample and 0.3 ml of DNHP were added to 1.5 ml capacity vials for the auto sampler. Standards of 1, 2, 5 and 10 $mg.L^{-1}$ were used and concentrations higher than 10 $mg.L^{-1}$ were diluted 10x in order to fit within the calibration.

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

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

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

Results

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

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

Embryonic toxicity of metaldehyde ± PBO over 21d. Since there was no significant snail embryonic mortalities after 7d exposure to metaldehyde up 100

mg.L^{-1}, this was chosen as the highest exposure concentration for the subsequent 21d experiment in accordance with standard OECD recommendations not to exceed this value unless there is environmental exposure data to warrant higher test concentrations. Based on mean measured concentrations of metaldehyde only, this gave 21d $^{\text{hatching success}}$NOEC and $^{\text{hatching success}}$LOEC values of 36 and 116 mg.L^{-1}, respectively (one-way ANOVA; $P<0.05$) (Table 3). Similarly, using mean measured concentrations of metaldehyde only also gave 21d $^{\text{spire height}}$NOEC and $^{\text{spire height}}$LOEC values of 36 and 116 mg.L^{-1}, respectively (one-way ANOVA; $P<0.05$). There were also statistically significant differences in hatching success between all metaldehyde treatment in the presence or absence of PBO at a measured concentration of between 0.47-0.56 mg PBO.L^{-1} ($P\leq0.045$). The test concentration of 109 mg MET.L^{-1} and 0.47 mg PBO.L^{-1} is statistically significant from the ethanol and dilution water controls and all other test concentrations ($P<0.05$). The use of zinc as a positive control also achieved its aim and no embryos hatched after 21d when exposed to a nominal concentration of 2.0 mg Zn.L^{-1} (Table 3). In terms of the intra-ocular observations, there were no statistically significant differences between embryos for any metaldehyde and PBO treatment group whereas there was a significant difference for the Zn positive control as these embryos failed to develop any eyespots ($P=<0.001$). Embryo heart rate data made using video microscopy showed considerable variability in the dilution water controls (mean values ranging from 59.1 to 75.5 beats.min^{-1} measured between 7d to 20d) and the ethanol solvent control (mean values ranging from 49.5 to 79.8 beats.min^{-1} measured between 7d to 20d). The measured heart rates of embryos exposed to metaldehyde only at 116 mg.L^{-1} had mean values ranging from 31.6 to 65.5 beats.min^{-1} between 7d to 20d and for the 36 mg.L^{-1} metaldehyde exposure group had mean values ranging from 31.4 to 77.0 beats.min^{-1} between 7d to 20d. For the metaldehyde (33.7 mg.L^{-1}) and piperonyl butoxide (0.47 mg.L^{-1}) embryo heart rates ranged from 44.0 to 62.1 beats.min^{-1} between 7d to 20d and the range was similar for other metaldehyde and piperonyl butoxide treatments. Overall there was no clear evidence over time of metaldehyde or piperonyl butoxide having a consistent effect in heart rate in *L. stagnalis* embryos in this study.

Discussion

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

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

374 *Daphnia pulex* (48h LC50 of 1.62 mg PBO.L^{-1}). As shown in Table 3, snail embryo
375 hatching success was reduced by very high concentrations of metaldehyde (116
376 mg.L^{-1}). 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^{-1} exposures, embryo
380 hatching started at 14 dpf (3/20) which at 3.53, 9.0 and 36 mg MET.L^{-1} the start of
381 embryo hatching was delayed until 15 dpf (18/79) and the 116 mg MET.L^{-1} 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^{-1} and 0.47 mg PBO.L^{-1} 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^{-1} range) and suggest a
399 low risk to the early life stages of gastropod molluscs relative to reported
400 environmental exposures (low µg.L^{-1} 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).

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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 $Zn.L^{-1}$; *n=20*)			
		EC_{10} (± 95% CI)	EC_{50} (± 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 $Zn.L^{-1}$,
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 $Zn.L^{-1}$. Physico-chemical parameters for the study (30 May-6 June 2014) were:
593 conductivity 866 - 987 $\mu S.cm^{-1}$; 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^{-1})	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^{-1}	95	857 ± 231	295 (*n=1*)
Positive control 2.0 mg Zn.L^{-1}	10^{aa}	448 ± 187^{aa}	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^{a}	173 ± 24.8
2.34	100	553 ± 108^{aa}	180 ± 21.8
Summary of developmental effects after 14d (measured PBO concentrations):			
^{development}NOEC ^{development}LOEC	≥ 2.34 > 2.34	- -	- -
$^{spire\ height}$NOEC $^{spire\ height}$LOEC	- -	0.43 1.03	- -
$^{interocular\ distance}$NOEC $^{interocular\ distance}$LOEC	- -	- -	≥ 2.34 > 2.34

599

600 Footnote - Measured zinc concentrations in the ISO reconstituted dilution water was 0.002
601 mg Zn.L^{-1} (LOD of 0.001 mg Zn.L^{-1}). The overall mean measured PBO concentration was
602 224% of nominal values (with a limit of detection of 0.02 mg PBO.L^{-1} 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 ^{a}($P<0.05$)
606 and ^{aa}($P<0.01$).

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^{-1})	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^{-1}	84	1268 ± 211	262 ± 34.0
Positive control 2.0 mg Zn.L^{-1}	0	-	-
Metaldehyde (ME) only treatments:			
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^{aa}	886 ± 108^{a}	212 ± 20.2
$^{\text{hatching success}}$NOEC	36.0	-	-
$^{\text{hatching success}}$LOEC	116	-	-
$^{\text{spire height}}$NOEC	-	36.0	-
$^{\text{spire height}}$LOEC	-	116	-
$^{\text{interocular distance}}$NOEC	-	-	≥ 116
$^{\text{interocular distance}}$LOEC	-	-	> 116
Metaldehyde (ME) and piperonyl butoxide (PBO) treatments:			
2.36 mg ME.L^{-1} + 0.56 mg PBO.L^{-1}	40^{b}	924 ± 135	215 ± 24.1
4.46 mg ME.L^{-1} + 0.52 mg PBO.L^{-1}	25^{b}	833 ± 124	221 ± 18.9
12.4 mg ME.L^{-1} + 0.56 mg PBO.L^{-1}	47^{b}	894 ± 137	222 ± 27.8
33.7 mg ME.L^{-1} + 0.47 mg PBO.L^{-1}	47^{b}	879 ± 108	212 ± 18.8
109 mg ME.L^{-1} + 0.47 mg PBO.L^{-1}	0^{b}	-	-

613

614 Footnote - Measured zinc concentrations in the dilution water was 0.002 mg Zn.L^{-1} (LOD of
615 0.001 mg Zn.L^{-1}). The overall mean measured PBO concentration was 224% of nominal
616 values (with a limit of detection of 0.02 mg PBO.L^{-1} 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 ^{a} ($P<0.05$) and ^{aa}
620 ($P<0.01$). T-test results showing significant differences between the ME results for ± PBO
621 shown as ^{b} ($P<0.05$).

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

627

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^{-1} and water solubility = 222 mg ME.L^{-1} (EFSA 2010).

632