

# 1 Sensitivity of the early life stages of mayfly to fine sediment and 2 orthophosphate levels

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## 13 **Abstract:**

14 The ecological effects of interacting stressors within lotic ecosystems have been widely  
15 acknowledged. In particular, the ecological effects of elevated fine sediment inputs and phosphate  
16 have been identified as key factors influencing faunal community structure and composition.  
17 However, while knowledge regarding adult and larval life stage responses to environmental stressors  
18 has grown, there has been very limited research on their eggs. In this study, the eggs of the mayfly  
19 *Serratella ignita* (Ephemeroellidae: Ephemeroptera) were collected and incubated in laboratory  
20 aquaria to hatching under differing concentrations of inert suspended sediment (SS) and  
21 orthophosphate (OP), individually and in combination. Results indicate that SS and OP have greater  
22 effects on egg hatching in combination than when either were considered in isolation. SS displayed a  
23 greater effect on egg survival than OP in isolation or when OP was added to elevated SS treatments.  
24 Egg mortality in control treatments was around 6% compared to 45% in treatments with 25 mg l<sup>-1</sup> SS  
25 and 52% in 0.3 mg l<sup>-1</sup> OP treatments. Even relatively modest levels of each stressor (10 mg l<sup>-1</sup> SS; 0.1  
26 mg l<sup>-1</sup> OP), below national legal thresholds, had significant effects on egg survival to hatching. The  
27 results support calls for legal levels of SS to be reassessed and suggest that more research is required  
28 to assess the impacts of pollution on invertebrate egg development given their different sensitivity  
29 and exposure pathways compared to other life stages.

30 **Capsule:** This study is the first to demonstrate that the survival of mayfly eggs to hatching is  
31 significantly reduced by low levels of widespread environmental pollutants in rivers.

32

33

34 **1. Introduction**

35 Freshwater organisms are currently subjected by multiple, simultaneous and interacting pressures,  
36 due to the co-occurrence of effects associated with climate and land-use change (Tockner et al.  
37 2010; Mantyka-Pringle et al. 2014; Jackson et al. 2016; Sebatier et al. 2016). A meta-analysis of  
38 research from marine ecosystems has shown that the effect of multiple stressors on aquatic  
39 organisms are complex and most frequently synergistic or additive, with the effects being greater or  
40 equal when stressors are combined (Przeslawski et al. 2015). In contrast, a recent meta-analysis of  
41 freshwater ecosystems reported that the majority of interactions were antagonistic, with effects  
42 lower than expected for individual stressors (Jackson et al. 2016); although there has been less  
43 research on lotic systems. The uncertainty surrounding the ecological response to multiple, co-  
44 occurring stressors can lead to unexpected ecological responses (Christensen et al., 2006;  
45 Lindenmayer et al., 2010; Dehedin et al., 2013). For example, Piggott et al. (2012) identified that the  
46 negative impacts of fine sediment on invertebrate and algal diversity were greater when water  
47 temperature was increased. Holmstrup et al. (2010) reviewed the impacts of multiple stressors on  
48 individual organisms, rather than the entire community, and reported that the majority of studies  
49 (including temperature, desiccation and chemicals) resulted in synergistic effects.

50 Aquatic communities are adapted to hydrological regime variability and associated fluxes of solutes  
51 and fine sediment (organic and inorganic) derived from the catchment. Lotic ecosystems require  
52 sediment inputs to maintain habitat heterogeneity and facilitate nutrient fluxes, but excessive  
53 loadings can have negative effects on river ecosystem functioning (Wood and Armitage, 1997; Jones  
54 et al. 2012). The US Environmental Protection Agency identified fine sediment deposition as the  
55 number one source of stream impairment and habitat degradation nationwide (USEPA, 2000; Evans-  
56 White et al. 2013). Fine sediment may degrade aquatic faunal communities and directly affect  
57 individual organisms due to burial, scour or abrasion of soft tissues, clogging of respiration structures  
58 (gills of invertebrates and fish), as well as reducing habitat quality and increased emigration from  
59 degraded habitats (e.g. Billota and Brazier, 2008; Béjar et al. 2017). In addition, fine sediments can  
60 reduce habitat availability by covering coarser sediments, filling interstices and modifying  
61 biogeochemical conditions by reducing dissolved oxygen concentrations whilst leading to elevation  
62 of the concentrations of pollutants within the substrate (Kemp *et al.*, 2011; Jones *et al.*, 2012;  
63 Descloux et al. 2014; Mathers et al. 2017). The majority of research centred on fine sediment  
64 deposition on aquatic organisms has focused on invertebrate larval community composition or adult  
65 life stages (Roy *et al.*, 2003; Extence *et al.*, 2013; Bona et al. 2016). For example, the detrimental  
66 effects of fine sediment on freshwater mussel population has been examined in detail given that  
67 many species are national or internationally endangered and have important functional roles in

68 rivers (Denic & Geist, 2015; Lummer et al. 2016). However, with the exception of salmonid fish (e.g.  
69 Grieg et al. 2005; Jensen et al. 2009; Sternecker & Geist 2010; Chapman et al. 2014), few studies  
70 have considered the effects of enhanced fine sediment loading on the egg / embryonic life stages of  
71 aquatic fauna.

72 The effects of elevated phosphorus concentrations on aquatic environments, particularly the  
73 proliferation of nuisance phytoplankton and both epiphytic and benthic algae has been widely  
74 documented (Mainstone and Parr, 2002; Evans-White et al. 2013; Azevedo et al. 2015) and  
75 represents a significant threat to water quality and environmental integrity, internationally (Nijboer  
76 and Verdonschot, 2004; Smith and Schindler, 2009; Javie et al. 2015). It is well established that  
77 nutrient enrichment (eutrophication) has resulted in the reduction of macroinvertebrate community  
78 richness through the extirpation of sensitive taxa, particularly within the insect orders  
79 Ephemeroptera, Plecoptera and Trichoptera (Ortiz and Puig, 2007; Friberg *et. al.* 2010; Bini et al.  
80 2014). Orthophosphate (OP) or 'soluble reactive phosphorus' is bioavailable to freshwater organisms  
81 and the exceedance of the OP standard has been identified as the single largest cause of water  
82 bodies not achieving 'good ecological status' in the UK, under the European Union Water Framework  
83 Directive (WFD) (Environment Agency 2012). Phosphorous concentrations have increased in many  
84 regions, often linked to human and animal waste; for example, concentrations of Total Dissolved  
85 Phosphorous increased by 2000% between 1970 and 2000 in northern Chinese Rivers (Strokal et al.  
86 2016). Phosphorous can be particularly problematic because ecological recovery does not  
87 necessarily follow a reduction of concentrations in the environment due to lag times in ecological  
88 responses, complex indirect impacts of elevated phosphorous on aquatic communities, and the  
89 effects of associated stressors (Javie et al. 2013). In addition, phosphorous can be bound to sediment  
90 and remobilised at a later date when phosphorous inputs into the system may be negligible (Meng  
91 et al. 2014; Wood et al. 2015; Emelko et al. 2016). Internationally, elevated nutrient and sediment  
92 loads are a management priority and are acknowledged to be the primary contributing factor to over  
93 40% of US waters being in poor biological condition (Evans-White et al. 2013).

94 Some pollutants may have potentially greater effects on early life stages of aquatic biota as they are  
95 typically the least mobile and therefore the most vulnerable to disturbance events (Clements and  
96 Newman, 2002; Przeslawski et al. 2015). Despite a substantial literature on fish eggs and  
97 sedimentation (e.g. see Kemp et al. 2011), relationships between aquatic invertebrates (e.g. Denic  
98 and Geist, 2015) and especially their egg survival and environmental stressors are almost completely  
99 lacking (but see Gleason et al. 2003; Kefford et al. 2010). Therefore, this study focuses on the effects  
100 of increasing suspended sediment (SS) and OP concentrations individually and in combination on the  
101 survival and hatching success of the eggs of a widespread and ecologically important aquatic insect

102 larvae, *Serratella ignita* (Ephemerellidae: Ephemeroptera) under experimental conditions. This was  
103 achieved by investigating whether:

- 104 1. elevated SS concentration impaired egg survival and hatching.
- 105 2. elevated OP concentration impaired egg survival and hatching.
- 106 3. higher concentrations of SS and/or OP had greater effects on egg survival and hatching than  
107 lower concentrations.
- 108 4. SS and OP in combination effect hatching / survival to a greater degree than in isolation.

109

## 110 **2. Methodology:**

### 111 **2.1. Target Organism**

112 The Blue-Winged Olive Mayfly (*Serratella ignita* (Poda, 1761): Ephemeroptera: Ephemerellidae) is  
113 one of the most common Ephemeroptera species in the British Isles and is present across most of  
114 Europe, including the Mediterranean region. Typically nymphs are found in unpolluted, fast flowing  
115 systems, emerging between June and September, with nymphs present in the river from March to  
116 September (Elliot & Humpesch, 2010; Macadam and Bennett, 2010), although this varies depending  
117 on thermal regime and flow permanence (Lopez-Rodriguez et al. 2009). Their life cycle typically  
118 includes a long overwintering period in the egg stage. Females of *S. ignita* produce a ball of eggs  
119 attached to the posterior underside of the abdomen. The animal descends to the water surface,  
120 releasing the egg mass which sinks and becomes anchored to the substrate via fibrous attachments  
121 (Gaino & Bongiovanni, 1992). *S. ignita* is ecologically important because of its widespread  
122 distribution and high abundance, which makes it significant for supporting fisheries. However,  
123 numbers have declined in a number of UK rivers over the past 20 years, particularly chalk streams  
124 (Bennett & Gilchrist, 2010). *S. ignita* larvae are known to be sensitive to fine sediment loading and  
125 OP concentration, with investigations linking losses of *S. ignita* to enhanced fine sediment loading  
126 effects in European rivers (Everall, 2010; Larsen et al., 2011; Minutoli et al. 2013).

127

### 128 **2.2. Experimental Set-Up and Overview**

129 Experiments were undertaken in experimental, laboratory chambers (Figure 1). A total of 24  
130 chambers were run in parallel for the duration of the experimental period, each representing a  
131 different treatment. Each experimental chamber housed 3 glass laboratory slides, which acted as a  
132 substrate for *S. ignita* eggs. Each slide contained approximately 30 egg masses which were left to

133 develop on the slides for 8 months under either control or experimental treatment conditions. The  
134 experimental chambers consisted of plastic funnels with a slide mount lodged above the outflow.  
135 The 3 glass slides were held vertically at 45 degrees and in parallel in the slide mount and remained  
136 submerged for the duration of the experiment in the 20 mm diameter circular container.

137 The water used in experiments was aerated and had pre-determined concentrations of OP and SS. It  
138 was held in 25 l reservoirs, elevated above the experimental chamber and allowed to flow through  
139 the system under gravity from a tap, which limited the flow rate to 0.65 ml min<sup>-1</sup>. Water drained  
140 through the experimental chamber and out through a pipe to a drain (Figure 1).

141

### 142 **2.3. *Serratella ignita* egg collection and laboratory acclimation**

143 Hundreds of swarming gravid adult *Serratella ignita* were collected from above the water surface of  
144 the River Manifold in Staffordshire, UK (53°09'49.15''N; 001°51'35.70''W) in August 2015. Adult *S.*  
145 *ignita* were carefully transferred in ventilated plastic aquaria (40 x 25 cm) fitted with temporary  
146 cardboard floors to the laboratory where they were placed on top of white plastic trays (38 x 22 x 5  
147 cm). Each tray bottom was lined with 36 sterilised glass slides and covered by 2 cm of aerated water  
148 from the sample site. The temporary cardboard floors of the adult mayfly aquaria were removed  
149 allowing gravid female *S. ignita* to access the river water surface in the glass slide lined trays to lay  
150 their eggs.

151 Over 24 hours, the gravid female *S. ignita* laid their egg masses on to the water surface whereupon  
152 they sank and became attached to the glass slides lining the trays. Spent *S. ignita* spinners and body  
153 parts (Supplementary Material A) were carefully removed from the egg slides using sterilised steel  
154 forceps, paying attention not to disturb the deposited egg masses. The egg mass covered slides were  
155 left *in situ* for another 24 hours to allow egg mass adhesion to the glass slides after which they were  
156 transferred to slide holders in the treatment chambers (Supplementary Material B) using sterilised  
157 forceps. Prior to transfer, each slide had the number of egg masses per slide recorded in indelible ink  
158 on the slide. All 24 experimental chambers had been running for 20 days with a discharge flow of  
159 0.65 ml min<sup>-1</sup> of carbon-limestone filtered tap water prior to slide introduction.

160 All of the egg mass slides were left to acclimate in flow through chambers supplied with de-  
161 chlorinated, filtered tap water for a further 24 hours prior to the commencement of experiments  
162 with treatment exposures. The acclimation and treatment bioassays were subject to ambient  
163 outdoor air temperatures, humidity and light regime during the experimental period between  
164 August 2015 and March 2016.

165

#### 166 **2.4. Bioassay design and egg monitoring**

167 A summary of the bioassay treatments used in the experiment are presented in Table 1. Every  
168 month of the bioassay the 3 slides in each treatment were placed under a microscope for a few  
169 minutes in a wet mount containing the appropriate bioassay test solution for observation. The slides  
170 were examined for any egg mass loss, egg mass emergence, and secondary biological growth e.g.  
171 fungal hyphomycetes. Egg mass emergence was considered when approximately >90% of the viable  
172 eggs in the mass had hatched. Egg mass loss was considered to have occurred if a similar proportion  
173 of the individual eggs within the mass had died or if the egg mass had fallen from the slide,  
174 identification of which was aided by marks left on the slide by displaced egg masses (Supplementary  
175 material C). Secondary fungal hyphomycetes growth was clearly identifiable under a microscope. In  
176 subsequent analysis, the status of egg masses was aggregated across the 3 slides.

177 After 3 months the slides in each treatment were carefully checked monthly for individual egg  
178 mortalities, within egg masses. It was not possible to count every egg within all egg masses. Instead,  
179 an egg mass was randomly selected from each of the 3 slides in each experimental chamber and the  
180 state of 200 eggs were counted under a microscope. Eggs were recorded as being either healthy or  
181 dead, where dead eggs were readily identified because they turned an opaque white, as reported  
182 previously by Yeo and Dechoretz (1973). Any egg mass chosen for egg mortality observation had an  
183 indelible dot placed on the reverse side of the slide so that it was not chosen again for observation.  
184 In subsequent analysis, egg mortality was aggregated across the 3 slides in each experimental  
185 chamber, giving a total sample of 600 individual eggs for each treatment.

186

#### 187 **2.5. Chemical dosing and testing**

188 The base and experimental control water during the acclimation and bioassay testing period was tap  
189 water run at 0.65 ml min<sup>-1</sup> through filters housing a mix ratio of 5:1 6 mm limestone chippings:  
190 granular activated carbon to reduce background levels of OP in tap water and remove any trace  
191 impurities. Test compounds were made up from the base water with the required dose additions of  
192 Sigma Aldrich 1000 mg l<sup>-1</sup> Orthophosphate and 1000 mg l<sup>-1</sup> Total Suspended Solids (inert silica  
193 particles, diameter 5 – 100 µm) calibration standards.

194 Water temperature in experimental test chambers was recorded daily in the control and once a  
195 month in other treatments. Daily water temperature mirrored ambient air temperature

196 (Supplementary material D). Dissolved oxygen and pH were also recorded once a month across all  
197 the treatments. Water samples were taken from all of the bioassay test chambers once a month  
198 across the 8 month experimental period using an overflow valve fitted into the treatment rigs  
199 (Figure 1). All samples for chemical analysis were sent to the UKAS Accredited National Laboratory  
200 Service for monthly analyses of Total Nitrogen, Ammoniacal Nitrogen, Nitrite, Alkalinity (to pH 4.5 as  
201 CaCO<sub>3</sub>), Orthophosphate, pH, Suspended Solids (at 105 °C), Boron, Calcium, Iron, Lithium,  
202 Magnesium, Manganese, Sodium, Water hardness (Total as CaCO<sub>3</sub>), Arsenic, Selenium, Cadmium,  
203 Copper, Lead, Mercury, Nickel and Zinc (Supplementary material E). The test treatments were also  
204 tested monthly for respective actual dosed SS and OP levels.

205 Mean physical-chemical properties for each of the bioassay chambers from 8 monthly samples  
206 across the study period are presented in Table 2. Individual monthly measurements of physical-  
207 chemical conditions are presented in Supplementary material D and clearly indicate that background  
208 water quality was stable with low level of trace chemicals in the control and test diluent water  
209 during the tests (Supplementary material E). With the exception of the test variables, there were no  
210 significant differences found between diluent physical-chemical conditions in the bioassays across  
211 the 8 month experiment ( $p < 0.01$ ; ANOVA). The measured concentrations of SS and OP in water  
212 samples also display good spatial and temporal stability with the dosed concentration. In control  
213 experiments, OP concentrations were  $\sim 0.04 \text{ mg l}^{-1}$  despite  $\sim 96\text{-}98\%$  phosphate removal from the  
214 baseline diluent tap water, similar to the performance of phosphorous removal of other workers  
215 using this type of filter (e.g. Hussain *et. al.*, 2011).

216

## 217 **2.6. Statistical analysis**

218 A regression modelling approach was used to examine the impact of concentration gradients of OP  
219 and SS on egg mortality. The number of dead eggs recorded over time, from a sub-sample of 600  
220 eggs, was recorded for different concentrations and combinations of OP or SS. Regression models  
221 were developed for the association between the total number of dead eggs and the concentration of  
222 OP and/or SS for different exposure periods. In addition, regression models were developed for the  
223 association between the percentage of egg masses that emerged at the end of the experiment and  
224 OP and SS concentrations, and combinations to examine additive effects.

225

## 226 **3. Results:**



227 **3.1. Egg mortality within bioassays**

228 The number of dead eggs recorded within egg masses increased as the concentration of OP and SS  
229 increased above control levels. Egg mortality in control experiments were consistent between  
230 treatments and remained low, averaging 5.8% of sampled eggs across all treatments and ranging  
231 from 27 to 42 eggs out of 600. As concentration of SS and OP increased, there were substantial  
232 increases in egg mortality, representing a 972% increase under the highest OP levels and 1261%  
233 increase under the highest SS concentration over control levels. Mortality increased exponentially  
234 with SS and OP concentration when dosed individually (Figure 2a; b), with significant regression  
235 models developed between OP or SS concentration and mortality after 71 days of exposure (SS  $p <$   
236  $0.01$ ,  $R^2 = 0.88$ ; OP  $p < 0.01$ ,  $R^2 = 0.99$ ). After 183 days of exposure, exponential relationships  
237 between OP or SS concentration remained significant ( $p < 0.01$  in both cases) with high explanatory  
238 power (98% of variance in both cases) (Table 3).

239 When SS and OP were dosed in combination, mortality increased over equivalent concentrations in  
240 isolation (Figure 2c; d). The increase in egg mortality when  $0.07 \text{ mg l}^{-1}$  OP was added was small, but  
241 consistent and the relationship remained exponential. In contrast, the addition of  $10 \text{ mg l}^{-1}$  of SS to  
242 OP concentrations resulted in a marked increase in mortality and a change in the relationship  
243 between egg mortality and OP concentration from exponential to linear (Table 3).

244 In control runs and low doses of SS and OP ( $<5 \text{ mg l}^{-1}$  and  $< 0.1 \text{ mg l}^{-1}$ , respectively), egg mortality did  
245 not increase over time, but remained around 6% of sampled eggs (Figure 3a; b). Although mortality  
246 increased when SS was elevated to above  $10 \text{ mg l}^{-1}$ , egg mortality did not increase substantially over  
247 time, only increasing by about 10% of the sampled eggs over the duration of experiments ( $10 \text{ mg l}^{-1}$   
248  $10\%$  to  $20\%$ ;  $15 \text{ mg l}^{-1}$   $19\%$  to  $29\%$ ). When SS was above  $20 \text{ mg l}^{-1}$ , mortality increased through time  
249 linearly, from 45% to 80% of sampled eggs in the case of the highest dose (Figure 3a). For OP,  
250 mortality consistently increased through time for all treatments except the control; however this  
251 was limited to less than 6% for all treatments, except the highest two concentrations (Figure 3b).  
252 Similar patterns were observed when OP and SS were dosed in combination, with egg mortality  
253 increasing through time with the rate of mortality increasing as dosage increased. When SS was  
254 added to OP treatments, egg mortality increased faster and to a higher percentage of sampled eggs  
255 in comparison to when OP was added to SS treatments (Figure 3c, d).

256

257 **3.2. Egg mass emergence in bioassays**

258 The number of egg masses that emerged decreased exponentially as concentration of OP or SS  
259 increased (OP  $R^2 = 0.998$ ; SS  $R^2 = 0.963$ ;  $p < 0.01$  in both cases). OP effects were discernible from  
260 control treatments at  $0.1 \text{ mg l}^{-1}$  and from SS controls at  $10 \text{ mg l}^{-1}$  (Figure 4a, b). The emergence of  
261 egg masses exposed to OP declined substantially when  $10 \text{ mg l}^{-1}$  of suspended sediment was added  
262 to treatments, supporting the findings of individual egg counts (Figure 4a). An exponential  
263 relationship between egg emergence and OP persisted with the addition of SS ( $p < 0.01$ ;  $R^2 = 0.998$ )  
264 but with greater egg mass emergence at concentrations of OP  $0.1 \text{ mg l}^{-1}$  and above. In contrast,  
265 when  $0.07 \text{ mg l}^{-1}$  of OP was added to SS treatments, there was no clear difference between egg mass  
266 emergence with and without OP (Figure 4b).

267 The 3 separate slides within each treatment indicated very high consistency in results observed. The  
268 largest difference in egg mass emergence between the 3 slides within each treatment was 27% for  
269 those subjected to  $0.3 \text{ mg l}^{-1}$  OP plus  $10 \text{ mg l}^{-1}$  SS (Figure 5).

270

#### 271 **4. Discussion:**

##### 272 **4.1. Effects of multiple stressors**

273 When low levels of SS were added to OP treatments, the mortality rate of eggs increased markedly,  
274 indicating that SS and OP had a greater impact on *S. ignita* when combined than when present  
275 individually. However, when low levels of OP were added to SS treatments, there was no discernible  
276 effect on mortality rates above SS in isolation, suggesting SS had a greater effect on egg and egg  
277 mass survival than OP.

278 Studies focusing on multiple stressors have consistently reported fine sediment to be a more  
279 pervasive stressor to the abundance of individual invertebrate species (Wagenhoff *et. al.*, 2011) and  
280 invertebrate communities (Piggot *et al.* 2015; ; Elbrecht *et al.* 2016) than enhanced nutrient  
281 concentrations, indicating that priority should be given to minimising fine sediment over nutrient  
282 inputs. However, contrasting results have been found in some cases where chemical composition of  
283 fine sediment was more important than sediment quantity in controlling invertebrate community  
284 composition (von Bertrub *et al.*, 2013). For example, Andersen *et al.* (2006) found uncontaminated  
285 sediments had no effect on the survival of several invertebrate species (*Hyalella Azteca* [amphipoda;  
286 Hyalellidae]; *Proclouon* sp. [Ephemeroptera; Baetidae]; *Chironomus dilutes* [Diptera; Chironomidae]).  
287 In the current experiments, suspended sediment was inert silica particles, clearly demonstrating that  
288 it is the deposition of sediment, rather than associated chemicals, that effected *S. ignita* egg

289 development. A potential explanation for the difference between studies is that the species  
290 examined by Andersen et al. (2006) were characteristic of slow flowing lowland streams or marginal  
291 habitats dominated by macrophytes, where fine sediment concentrations and accumulations were  
292 naturally high. This contrasts with *S. ignita*, which is typical of moderate flowing streams with coarse  
293 substrates and where SS concentration is likely to be much lower than lowland reaches. As such, *S.*  
294 *ignita* is adapted to environments with naturally lower concentrations of fines and as a result fine  
295 sediment potentially acts as a stressor at lower concentrations than for many species adapted to  
296 slow flowing habitats (Elliot and Humpesch, 2010). Consequently, it is likely that the relative  
297 significance of SS and associated contaminants will depend on the receptor species and their  
298 association with specific habitats.

299 Each female *S. ignita* produces many eggs and as a result the effect of the elevated egg mortality on  
300 the viability of populations is difficult to assess. For example, it is possible that if hatching success  
301 was high in rivers, density dependent processes may result in many early instar larvae perishing,  
302 reducing the population level effects of egg mortality due to anthropogenic stressors. Therefore the  
303 results here do not necessary imply population level impacts. In addition, *S. ignita* is a common  
304 species in the UK and Europe and often occurs in high abundance. However, *S. ignita* larvae have  
305 been shown to be highly sensitive to sedimentation and their presence is used in national biological  
306 metrics to indicate reduced fine sediment pressure (Extence et al. 2011). In addition, the proportion  
307 of individuals commonly surviving through to reproduction is not known and there is both anecdotal  
308 and documented evidence that the abundance of *S. ignita* has declined over the past 20 years in  
309 some English rivers (Bennett & Gilchrist, 2010).

310

#### 311 **4.2. The effect of suspended sediment on eggs**

312 The results of this study indicate that the egg stage of *Serratella ignita* is susceptible to sustained  
313 high levels of SS concentration during their 8 month developmental period. Concentration  
314 dependent mortality of *S. ignita* eggs was evident at annual mean equivalent concentrations of 10 -  
315 25 mg l<sup>-1</sup> but levels of fine sediment < 10 mg l<sup>-1</sup> displayed no markedly higher egg mortality than the  
316 control treatments. The cause of egg mortality is hypothesised to be reduced oxygen transfer due to  
317 sediment coating egg surfaces. Additionally, the build-up of fine sediment over time caused some of  
318 the egg masses to be dislodged from the slides after 6 months of exposure in the 20 and 25 mg l<sup>-1</sup>  
319 treatments. These egg masses were eroded and lost within the dosing rig sumps and, therefore, it is

320 not clear if the individual eggs were still viable; however, in watercourses dislodgement exposing egg  
321 masses to scour damage, burial and predation would be highly disadvantageous.

322 Other experiments examining the effect of fine sediment covering on invertebrate eggs have  
323 reported reduced survival and hatching for *Chironomus cloacalis* (Diptera; Chironomidae), *Physa*  
324 *acuta* (Gastropod; Physidae) and *Gyraulus tasmanica* (Gastropod; Planorbidae) (Kefford et al. (2010).  
325 In control treatments without fine sediment, 100% of viable eggs of all three species hatched, but  
326 this was reduced when buried with clay (kaolin) or sand; although, the direct effects of suspended  
327 sediment were limited. Similarly, Gleason et al. (2003) found that burial to 0.5 cm caused a 99.7%  
328 reduction in the emergence of invertebrate eggs from wetlands. The impact of SS on invertebrates is  
329 complex because of associated contaminants; for example, the source of sediment has been shown  
330 to be important for salmonid embryo development, primarily because the organic matter content of  
331 sediment consumes oxygen as it degrades, potentially reducing oxygen availability to developing  
332 embryos (Sear et al. 2014). In addition, the influence of other stressors confound ecological  
333 response; for example, Doretto et al. (2017) found that high availability of coarse particulate organic  
334 matter mitigated the negative effects of fine sediment, which clogged interstitial spaces in artificial  
335 substrates in the Po River, Italy.

336

337 Fish eggs are negatively effected by fine sediment (Kemp et al. 2011). Much of the research on fish  
338 embryo development and fine sediment has focused on the clogging of interstitial spaces in  
339 salmonid fish redds and associated reduction of interstitial flow volume and velocity (Jensen et al.  
340 2009; Chapman et al. 2014). However, deposition of clay particles directly onto salmon (*Salma salar*)  
341 eggs has been shown to reduce oxygen exchange across the egg membrane and increase mortality  
342 (Greig et al. 2005). This mechanism is also hypothesised to be responsible for mayfly egg mortality in  
343 these experiments. Research has also demonstrate that salmonid fish egg development can be  
344 effected by sedimentation by the prevention of the expulsion of metabolic wastes from the egg  
345 chorion (Chapman 1988; Bennett et al. 2003). Concentrations of nitrates and ammonia may  
346 significantly affect salmonid egg development (e.g. Sternecker et al. 2013) and Reynolds & Guillaume  
347 (1998) found phosphate concentrations of 0.5 mg l<sup>-1</sup> resulted in earlier emergence of European  
348 Bitterling (*Rhodeus sericeus*) embryos from eggs deposited within the gills of freshwater mussels.  
349 However, little research has investigated the link between elevated phosphorous concentration and  
350 fish embryo development.

351

#### 352 **4.3. Phosphorous effects on egg development**

353 Mortality of *S. ignita* eggs was evident at annual mean equivalent concentrations of 0.1 - 0.3 mg l<sup>-1</sup>  
354 OP but levels of biologically available phosphorous < 0.1 mg l<sup>-1</sup> resulted in no higher egg mortality  
355 than in control treatments. The cause of egg mortality in the highest dose of 0.3 mg l<sup>-1</sup> appeared to  
356 be related to the growth of aquatic fungal filaments smothering the egg masses after 1 month of  
357 exposure. The adhesive, mucous coating of mayfly eggs has been postulated to protect the egg from  
358 bacterial and fungal attack (Gaino *et. al.*, 2009), although any protection appeared to have been lost  
359 as a result of elevated OP stimulating microbial growth, resulting from the high availability of  
360 phosphorous. From light microscopy examination of fungal smothered eggs both undetermined  
361 aquatic hyphomycete species and *Fusarium aquaeductum* were identified coating the egg surfaces.  
362 Aquatic hyphomycetes growing on fish eggs have been found to be pathogenic (Wedekend *et. al.*,  
363 2010) and *Fusarium* species have been documented parasitizing eggs of the Penaeid prawn  
364 *Marsupenaeus japonicus* (Momoyama, 1987).

365

366 Egg mortality in treatments with lower OP levels, where there was no evidence of fungal growth,  
367 suggested other direct impacts of elevated biologically available phosphorous levels. Chronic  
368 exposure to sub-lethal concentrations of phosphates have been reported to have negative effects on  
369 early stages of aquatic fauna; for example, abnormal embryonic development in sea urchin  
370 (*Lytechinus variegatus*) (Bottger and McClintock, 2002). In addition, the cells of more complex  
371 organisms have also shown impaired gene expression (Rutherford *et. al.*, 2006) and cell membrane  
372 scrambling (Voelkl *et. al.*, 2014) with increasing extracellular phosphate concentrations. Therefore, it  
373 is hypothesised that egg mortality in the range of continuous OP exposures of 0.1 – 0.2 mg l<sup>-1</sup> may  
374 have been due to direct physiological and genotoxic impacts.

#### 375 **4.4. Concentrations of OP and SS in rivers**

376 The ability of an organism to survive exposure to a stressor is dependent upon the concentration of  
377 the parameter and duration of exposure (Tabak and Gibbs, 1991; Zhao and Newman, 2006; Cope *et*  
378 *al.* 2008). The total duration of exposure to a concentration of suspended solids is acknowledged to  
379 be a key variable determining its effect on aquatic biota (Billota and Brazier, 2008). For example,  
380 Maturana *et al.* (2014) found that continuous, chronic exposure of sediment had a greater  
381 detrimental impact on salmonid embryos than instantaneous pulses of sediment. The exposure  
382 conditions here were not directly comparable to natural conditions within a river, where pulsed and  
383 intermittent exposure of organisms to sediments and nutrients are common (Alabaster and Lloyd,  
384 1980; Davies and Bothwell, 2012; Outram *et al.* 2014). However, SS and OP levels used in these

385 experiments represent relatively modest concentrations for many English rivers, typically below  
386 WFD specified thresholds.

387 The Environment Agency (EA), the statutory environmental regulator in England, recorded 32549  
388 spot measurements of OP across England in 2015 and 22% of those were above 0.3 mg l<sup>-1</sup>, the  
389 highest concentration used in these experiments. Monthly spot measures were made at 1812  
390 locations across England in 2015, where at least 9 measurements were made throughout the year.  
391 Of these sites, 22% had annual average values higher than 0.3 mg l<sup>-1</sup> and over half had average  
392 concentrations above 0.1 mg l<sup>-1</sup>, the lowest concentration used in these experiments with a  
393 statistical effect on egg survival. 26% of sites had OP levels above 0.1 mg l<sup>-1</sup> in every measurement  
394 made throughout the year (Table 4). These results are consistent with the work of Worrell et al.  
395 (2016) who calculated that annual average OP concentrations have declined from 0.19 to 0.1 mg l<sup>-1</sup>  
396 between 1974 and 2012 in England, Wales and Scotland, based on routine monitoring data.  
397 Therefore, whilst concentrations of OP are declining across Europe (Bouraoui and Grizzetti, 2011), in  
398 many streams concentrations remain above those found to exert an effect in the experiments  
399 reported in this study. Fewer sites were sampled for suspended sediment but of the 129 sites with 9  
400 or more measurements in 2015, 9% had average values higher than 25 mg l<sup>-1</sup> and 39% had values  
401 over 10 mg l<sup>-1</sup>, found to effect egg hatching in these experiments (Table 4). OP legal levels are  
402 dependent on site specific characteristics and physio-chemical conditions but it is clear that low- to-  
403 moderately elevated levels can have direct effects on insect egg development, which may be  
404 accelerated at higher concentrations by fungal growth. In addition, elevated OP in rivers alters  
405 primary production leading to important indirect implications for dissolved oxygen concentrations  
406 and water temperature because excessive plant and algae growth can shade the water column,  
407 which may also impact insect egg development (Humpesch 1980; Elliot, 1987; Pritchard et al. 1996;  
408 Bennett 2007; Rotvit and Jacobsen, 2013).

409 The current experimental findings support the growing concern that the annual mean SS guideline  
410 standard of 25 mg l<sup>-1</sup> in the UK is not sufficient (WWF, 2007). This is supported by other studies that  
411 have identified effects of fine sediment on invertebrate survival at levels  $\geq 8$  mg l<sup>-1</sup> in Canadian  
412 freshwaters (Rosenberg and Wiens, 1978; Quinn *et. al.*, 1982). In these experiments, egg masses  
413 were lost because of sediment coverage and the weight of deposited sediment dislodging them,  
414 although it is not clear whether dislodgement occurred during or after egg health had deteriorated,  
415 potentially reducing their adhesive properties. In rivers, this would probably result in the burial  
416 and/or damage of eggs. The coating of eggs with sediment has implications for oxygen transfer  
417 which will be partly controlled by the extent of sediment coverage on the egg surface, as well as the

418 particle size and shape. These parameters will be at least partially dependent on the flow velocity  
419 and sediment properties and are likely to be less well correlated to suspended sediment  
420 concentrations. Therefore, the results support the assertion of Bilotta and Brazier (2008) and Kefford  
421 *et al.* (2010) that standards should move away from turbidity or suspended sediment concentrations  
422 to focus on settlement rates and sediment properties. Similarly, the source of sediment could have  
423 different effects on egg mortality because of its ability to harbour other pollutants, including  
424 phosphorous.

425

#### 426 **4.5. Management implications**

427 Previous research on the larval and adult stage of invertebrates indicates that elevated SS and OP  
428 are pervasive issues in river management (Friberg *et al.* 2010; Jones *et al.* 2012; Bini *et al.* 2014;  
429 Mathers *et al.* 2017). Internationally, OP concentrations remain high and rising in many river  
430 systems, in particular due to agricultural intensification and population increases coupled with the  
431 direct discharge of untreated human waste (Tysman *et al.* 2013; Stokal *et al.* 2016; Yan *et al.* 2016).  
432 Despite reductions in both SS and OP concentrations in river systems across Europe and North  
433 America, many rivers still show clear signs of negative impact (Jarvie *et al.* 2015; Blaas and Kroeze,  
434 2016). This is likely to be partly related to the indirect impacts of OP and SS, their interaction with  
435 other stressors, lags in ecological response, and remobilisation of OP bound to sediments long after  
436 inputs into the river system have been reduced (Jarvie *et al.* 2012). However, the results presented  
437 here suggest that relatively low levels of both SS and OP can negatively affect invertebrate egg  
438 development. Therefore, it is possible that by focusing on the larval and adult stage of invertebrate  
439 development, important information is being missed about the tolerance of species during what is  
440 potentially their most vulnerable developmental stage. More information is needed on the effect of  
441 stressors on egg development as impaired hatching could have significant implications for  
442 invertebrate populations at lower pollutant concentrations than those observed to effect larval and  
443 adult stages of the same species.

444

#### 445 **5. Conclusions:**

446 The effects of environmental pollutants on the eggs of aquatic invertebrates are not well understood  
447 despite the fact that eggs are potentially the most vulnerable life stage of many invertebrates.  
448 Relatively modest levels of SS and OP have highly significant detrimental effects on the mortality of

449 *S. ignita* eggs, with potentially significant implications for populations of mayfly. Fine sediment was  
450 the more pervasive stressor, increasing mortality of eggs exposed to OP enrichment, whereas  
451 elevated OP levels did not significantly increase mortality in comparison to those exposed only to  
452 fine sediment. The direct mechanism for the detrimental effects on eggs is likely to be complex but  
453 suspended sediment settled onto eggs, coating them and under high dosage ( $> 0.2 \text{ mg l}^{-1}$ ) resulting  
454 in dislodgement. High OP levels ( $> 0.2 \text{ mg l}^{-1}$ ) fuelled the growth of hyphomycete, which negatively  
455 affected eggs. The mechanism by which lower levels of OP ( $0.1 - 0.2 \text{ mg l}^{-1}$ ) negatively impacted  
456 eggs, in the absence of hyphomycete growth, is not known. Current legal limits of SS and OP in the  
457 European Union are above those found to have an effect in the experiments reported in the study  
458 and suggests management needs to focus on elevated SS levels. Although levels are dropping across  
459 Europe – substantially in the case of OP – the results of these experiments support growing concern  
460 about current guidelines relating to SS and associated organic contaminants and the need for more  
461 stringent regulation.

462

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466



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712 **Table 1:** Experimental designs for bioassays.

<b>Chemical treatment</b>	<b>Nominal chemical concentration mg/l</b>					
Fine suspended solids (SS)	0	5	10	15	20	25
Orthophosphate (OP)	0	0.05	0.07	0.1	0.2	0.3
0.07 mg l <sup>-1</sup> OP + SS	0	5	10	15	20	25
10 mg l <sup>-1</sup> SS + OP	0	0.05	0.07	0.1	0.2	0.3

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716 **Table 2:** Mean physical-chemical properties for each bioassay.

Parameter	Mean water quality $\pm$ s.d. (n = 10)					
	0	5	10	15	20	25
Nominal SS concentration (mg l <sup>-1</sup> )	0	5	10	15	20	25
Actual SS concentration (mg l <sup>-1</sup> )	<3	5.2 $\pm$ 0.2	10.0 $\pm$ 0.1	14.9 $\pm$ 0.2	20.0 $\pm$ 0.2	24.7 $\pm$ 0.6
Water temperature (°C)	13.4 $\pm$ 4.9	13.4 $\pm$ 4.9	13.5 $\pm$ 4.9	13.4 $\pm$ 5.0	13.3 $\pm$ 5.0	13.3 $\pm$ 4.9
Dissolved oxygen (mg l <sup>-1</sup> )	10.5 $\pm$ 0.4	10.3 $\pm$ 0.4	10.3 $\pm$ 0.4	10.3 $\pm$ 0.4	10.2 $\pm$ 0.2	10.3 $\pm$ 0.4
pH	8.0 $\pm$ 0.1	8.0 $\pm$ 0.1	8.0 $\pm$ 0.1	7.9 $\pm$ 0.3	7.9 $\pm$ 0.1	7.9 $\pm$ 0.1
Nominal OP concentration (mg l <sup>-1</sup> )	0	0.05	0.07	0.1	0.2	0.3
Actual OP concentration (mg l <sup>-1</sup> )	0.04 $\pm$ 0.01	0.05 $\pm$ 0.01	0.07 $\pm$ 0.02	0.11 $\pm$ 0.01	0.20 $\pm$ 0.01	0.30 $\pm$ 0.01
Water temperature (°C)	13.4 $\pm$ 5.1	13.1 $\pm$ 4.9	13.0 $\pm$ 5.0	13.0 $\pm$ 4.9	12.9 $\pm$ 5.0	12.8 $\pm$ 5.0
Dissolved oxygen (mg l <sup>-1</sup> )	10.5 $\pm$ 0.4	10.2 $\pm$ 0.3	10.2 $\pm$ 0.3	10.3 $\pm$ 0.3	10.2 $\pm$ 0.3	10.3 $\pm$ 0.4
pH	7.9 $\pm$ 0.1	7.9 $\pm$ 0.1	7.9 $\pm$ 0.1	7.8 $\pm$ 0.3	7.8 $\pm$ 0.1	7.8 $\pm$ 0.1
Nominal SS concentration (mg l <sup>-1</sup> )	0	5	10	15	20	25
Actual SS concentration (mg l <sup>-1</sup> )	<3	5.2 $\pm$ 0.4	10.0 $\pm$ 0.1	15.0 $\pm$ 0.1	20.1 $\pm$ 0.2	25.0 $\pm$ 0.2
Nominal OP concentration (mg l <sup>-1</sup> )	0.07	0.07	0.07	0.07	0.07	0.07
Actual OP concentration (mg l <sup>-1</sup> )	0.04 $\pm$ 0.01	0.08 $\pm$ 0.01	0.07 $\pm$ 0.01	0.07 $\pm$ 0.004	0.07 $\pm$ 0.003	0.07 $\pm$ 0.01
Water temperature (°C)	13.7 $\pm$ 5.2	13.6 $\pm$ 4.7	13.6 $\pm$ 4.5	13.3 $\pm$ 4.9	13.0 $\pm$ 4.7	12.9 $\pm$ 4.8
Dissolved oxygen (mg l <sup>-1</sup> )	10.4 $\pm$ 0.4	10.0 $\pm$ 0.5	10.2 $\pm$ 0.3	10.2 $\pm$ 0.3	10.2 $\pm$ 0.4	10.0 $\pm$ 0.3
pH	8.0 $\pm$ 0.1	8.1 $\pm$ 0.1	8.0 $\pm$ 0.1	8.0 $\pm$ 0.3	8.0 $\pm$ 0.1	8.2 $\pm$ 0.2
Nominal OP concentration (mg l <sup>-1</sup> )	0	0.05	0.07	0.1	0.2	0.3
Actual OP concentration (mg l <sup>-1</sup> )	0.04 $\pm$ 0.01	0.05 $\pm$ 0.004	0.07 $\pm$ 0.004	0.10 $\pm$ 0.01	0.21 $\pm$ 0.01	0.31 $\pm$ 0.01
Nominal SS concentration (mg l <sup>-1</sup> )	10	10	10	10	10	10
Actual SS concentration (mg l <sup>-1</sup> )	<3	9.9 $\pm$ 0.5	10.0 $\pm$ 0.2	10.2 $\pm$ 0.3	9.9 $\pm$ 0.3	10.0 $\pm$ 0.1
Water temperature (°C)	13.3 $\pm$ 4.6	13.3 $\pm$ 4.7	13.4 $\pm$ 5.2	13.4 $\pm$ 4.7	13.1 $\pm$ 5.0	13.0 $\pm$ 4.9
Dissolved oxygen (mg l <sup>-1</sup> )	10.2 $\pm$ 0.4	10.7 $\pm$ 0.4	11.0 $\pm$ 0.6	10.3 $\pm$ 0.5	10.1 $\pm$ 0.4	10.1 $\pm$ 0.3
pH	8.0 $\pm$ 0.1	8.1 $\pm$ 0.1	8.0 $\pm$ 0.2	7.9 $\pm$ 0.3	8.3 $\pm$ 0.2	8.3 $\pm$ 0.3

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719 **Table 3:** Regression equations and significance values for OP and/or SS concentration against egg  
 720 mortality. Note relationships are all exponential with the exception of OP + SS, where the strongest  
 721 relationship was linear. All regressions are significant ( $p < 0.01$ ).

Treatment	Time (days)	Equation	R <sup>2</sup>
OP	72	$25.495e^{5.265x}$	0.88
	121	$29.162e^{7.225x}$	0.98
	183	$29.324e^{8.189x}$	0.98
SS	72	$22.817e^{0.099x}$	0.99
	121	$25.832e^{0.115x}$	0.97
	183	$27.70e^{0.120x}$	0.98
OP + SS	72	$781.39x + 5.8258$	0.98
	121	$921.45x + 35.319$	0.92
	183	$1155.2x + 51.769$	0.93
SS + OP	72	$22.446e^{0.095x}$	0.96
	121	$27.646e^{0.113x}$	0.98
	183	$36.937e^{0.111x}$	0.98

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724 **Table 4:** Analysis of national routine spot measures of OP and SS made by the Environment Agency  
 725 in 2015 for WFD compliance across England.

		Count	Average (mg l <sup>-1</sup> )	Percentage of sites where the average is			
				> 0.3 mg l <sup>-1</sup>	> 0.1 mg l <sup>-1</sup>	> 25 mg l <sup>-1</sup>	> 10 mg l <sup>-1</sup>
All sites	<b>OP</b>	32549	0.27	21.8	51.6		
All sites	<b>SS</b>	2029	16.6			9.2	31.6

		Count	Average (mg l <sup>-1</sup> )	Percentage of sites where every measurement is			
				> 0.3 mg l <sup>-1</sup>	> 0.1 mg l <sup>-1</sup>	< 0.1 mg l <sup>-1</sup>	< 0.3 mg l <sup>-1</sup>
Sites > 9 samples	<b>OP</b>	1812	0.30	22.2	52.9	21.8	52.7

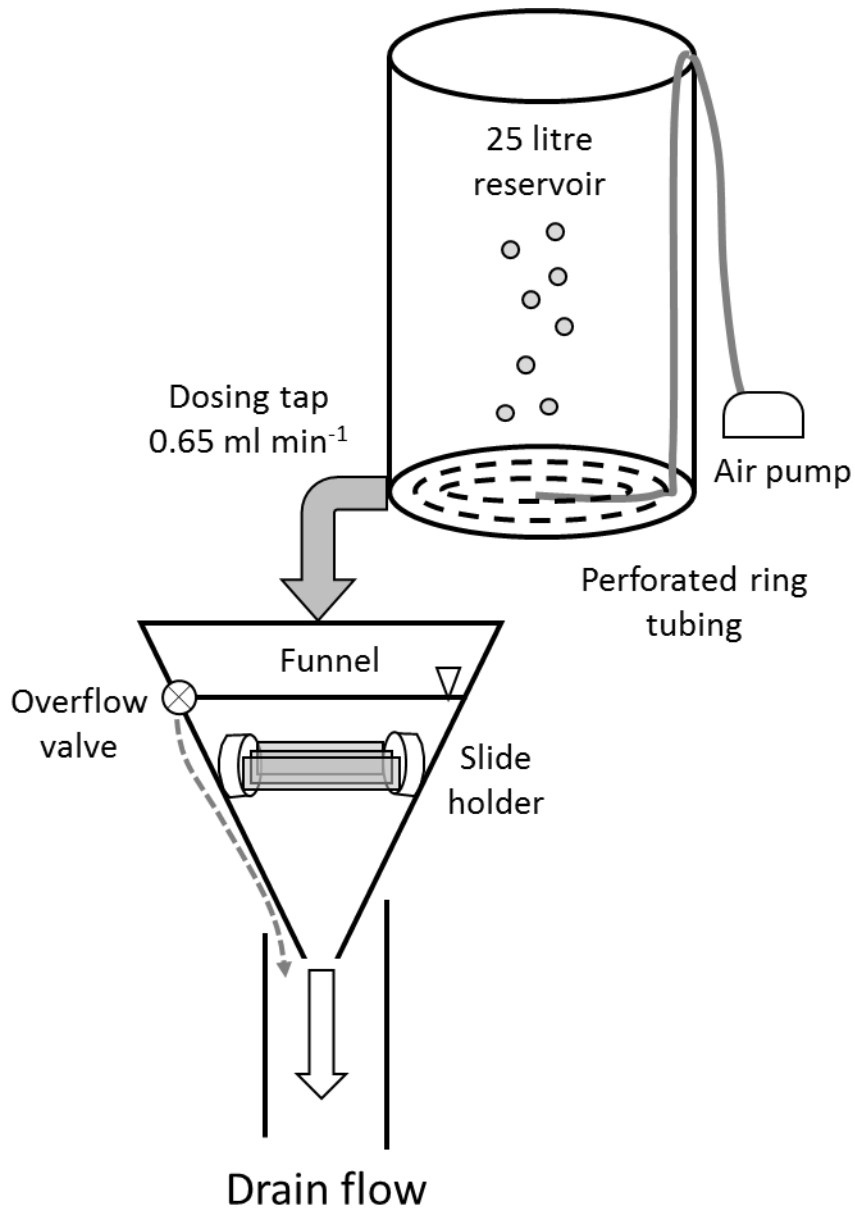
  

		Count	Average (mg l <sup>-1</sup> )	Percentage of sites where every measurement is			
				> 25 mg l <sup>-1</sup>	> 10 mg l <sup>-1</sup>	< 10 mg l <sup>-1</sup>	< 25 mg l <sup>-1</sup>
Sites > 9 samples	<b>SS</b>	129	12.7	9.4	39.0	49.6	10.9

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728 **Figure 1:** Schematic of *S. ignita* egg dosing rigs for controls and treatments. A reservoir with  
729 experiment solution is held above a funnel, within which 3 slides containing *S. ignita* eggs are held in  
730 a slide holder. A perforated ring of tubing on the base of the reservoir ensured complete mixing and  
731 aeration of experimental water.

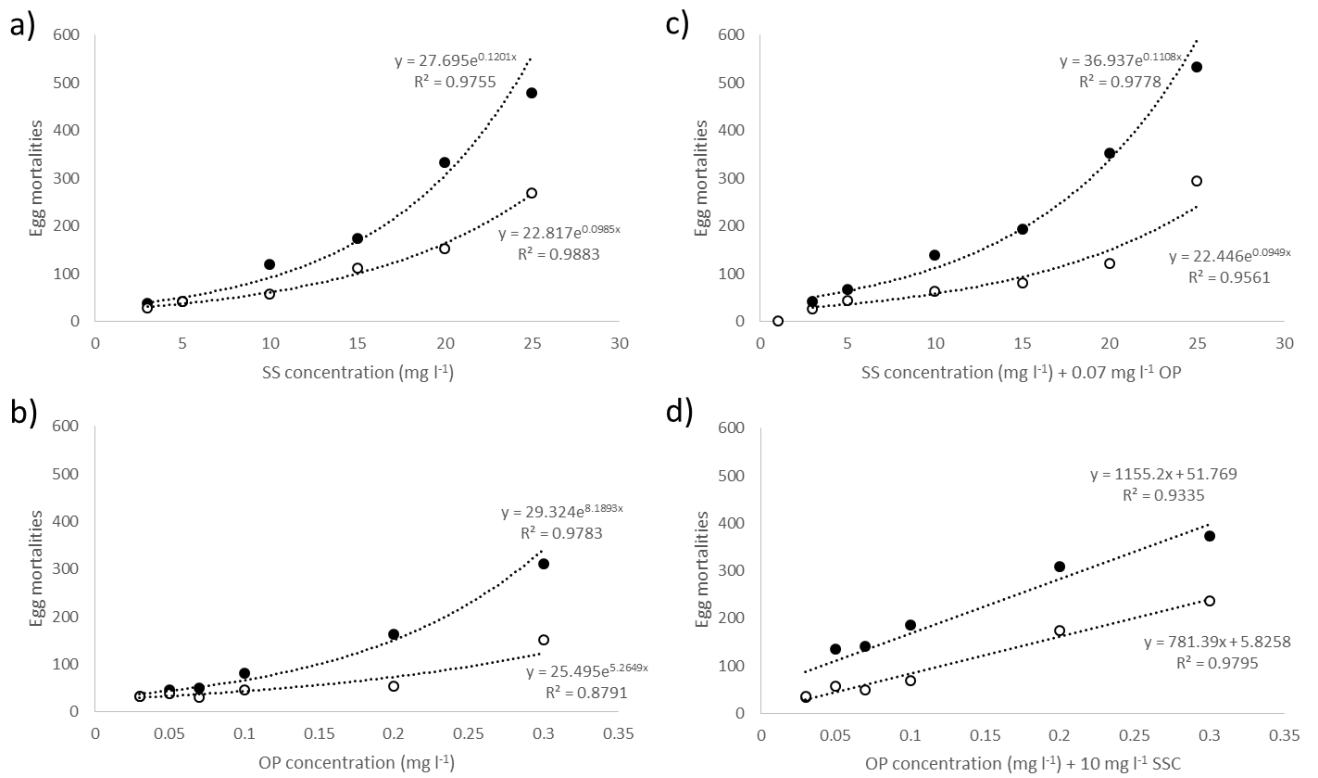


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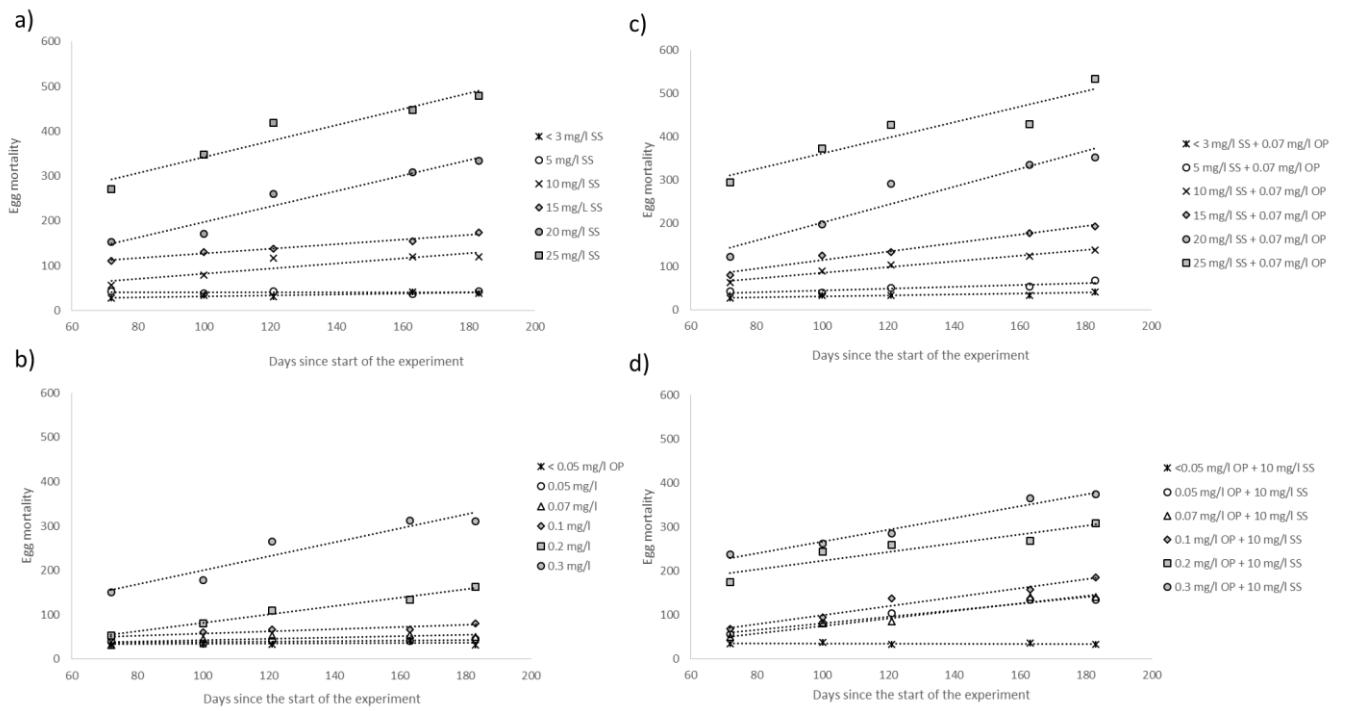
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735 **Figure 2:** Regressions of the mortality of *S.ignita* eggs after 72 days exposure (open circles) and 183  
 736 days exposure (filled circles) against (a) SS, (b) OP), (c) SS in addition to 0.07 mg l<sup>-1</sup> OP, and (d) OP in  
 737 addition to 10 mg l<sup>-1</sup> SS.



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740 **Figure 3:** Mortality of *S. ignita* eggs through time under differing concentrations of (a) SS; (b) OP; (c)  
 741 SS plus 0.07 mg l<sup>-1</sup> OP, and; (d) OP plus 10 mg l<sup>-1</sup> SS.



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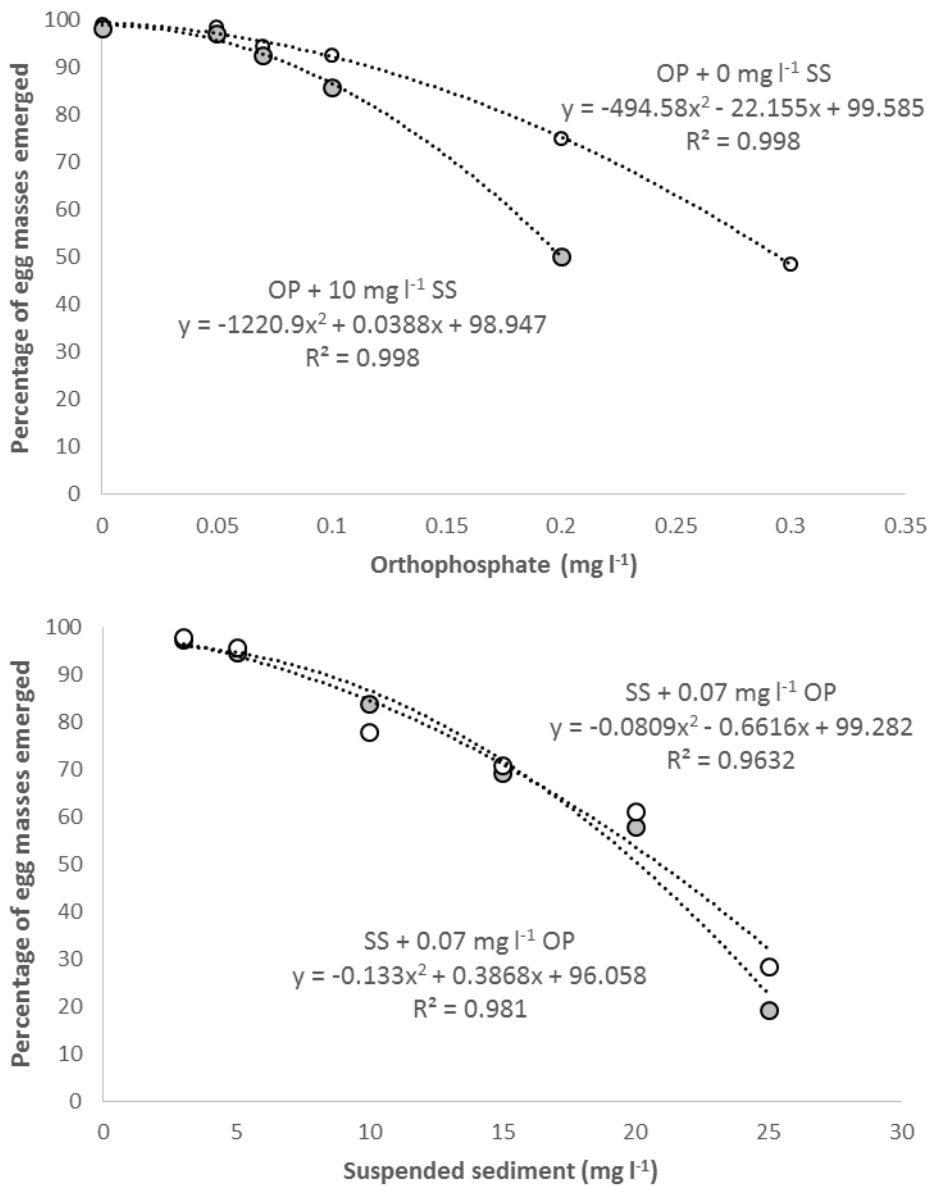
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746 **Figure 4:** Percentage of *S.ignita* egg masses surviving to emergence under different concentrations  
 747 of (a) OP in isolation (open circles) and in combination with 10 mg l<sup>-1</sup> of SS (closed circles) and (b) SS  
 748 in isolation (open circles) and in combination with 0.07 mg l<sup>-1</sup> of OP. Note, at 0.3 mg l<sup>-1</sup> OP plus 10 mg  
 749 l<sup>-1</sup> SS, fungal growth prevented the majority of egg masses from emerging and prevented an accurate  
 750 count of egg mass emergence.

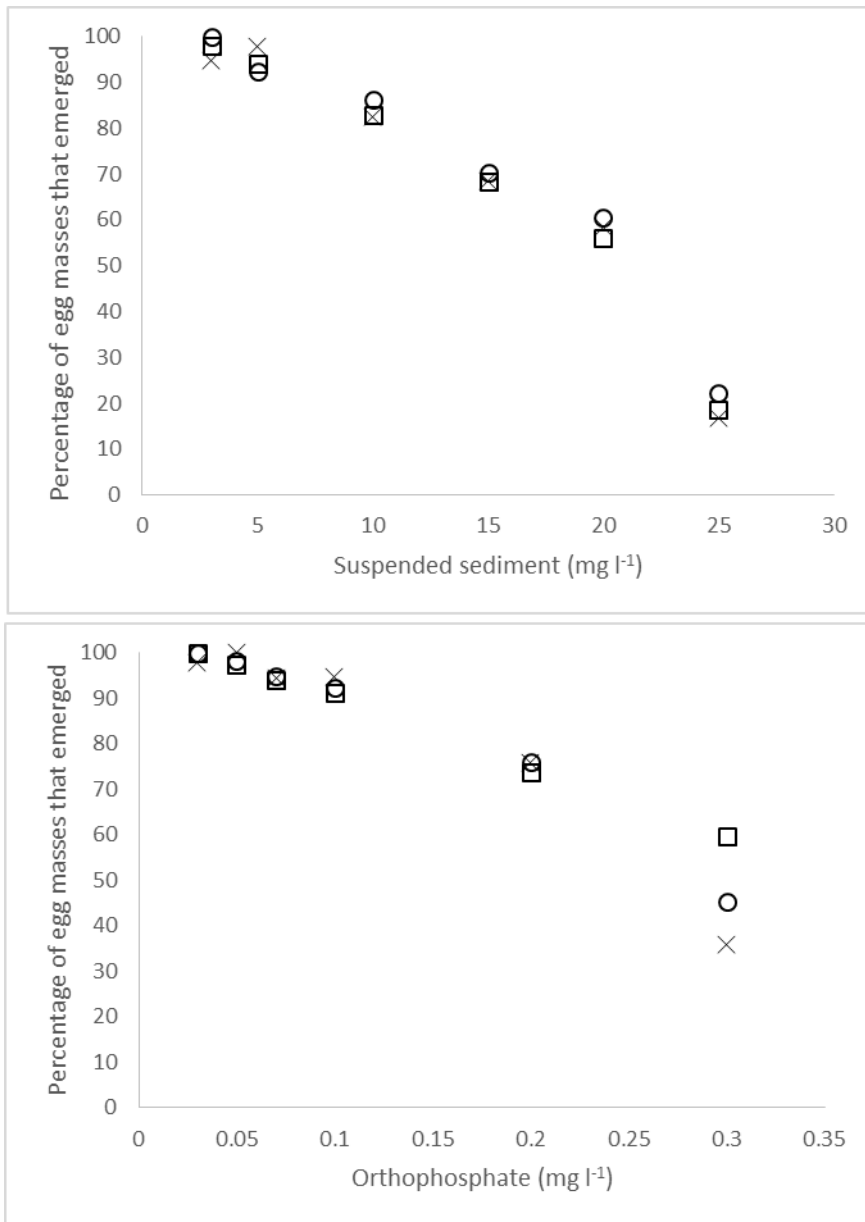


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754 **Figure 5:** Percentage of *S.ignita* egg masses surviving to emergence under different concentrations  
755 of (a) OP (b) SS for each of the 3 slides in each treatment indicated separately.



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758 **SUPPLEMENTARY MATERIAL A:**

759 Image of spent *Serratella ignita* with eggs deposited on glass slides at the bottom of the tray.



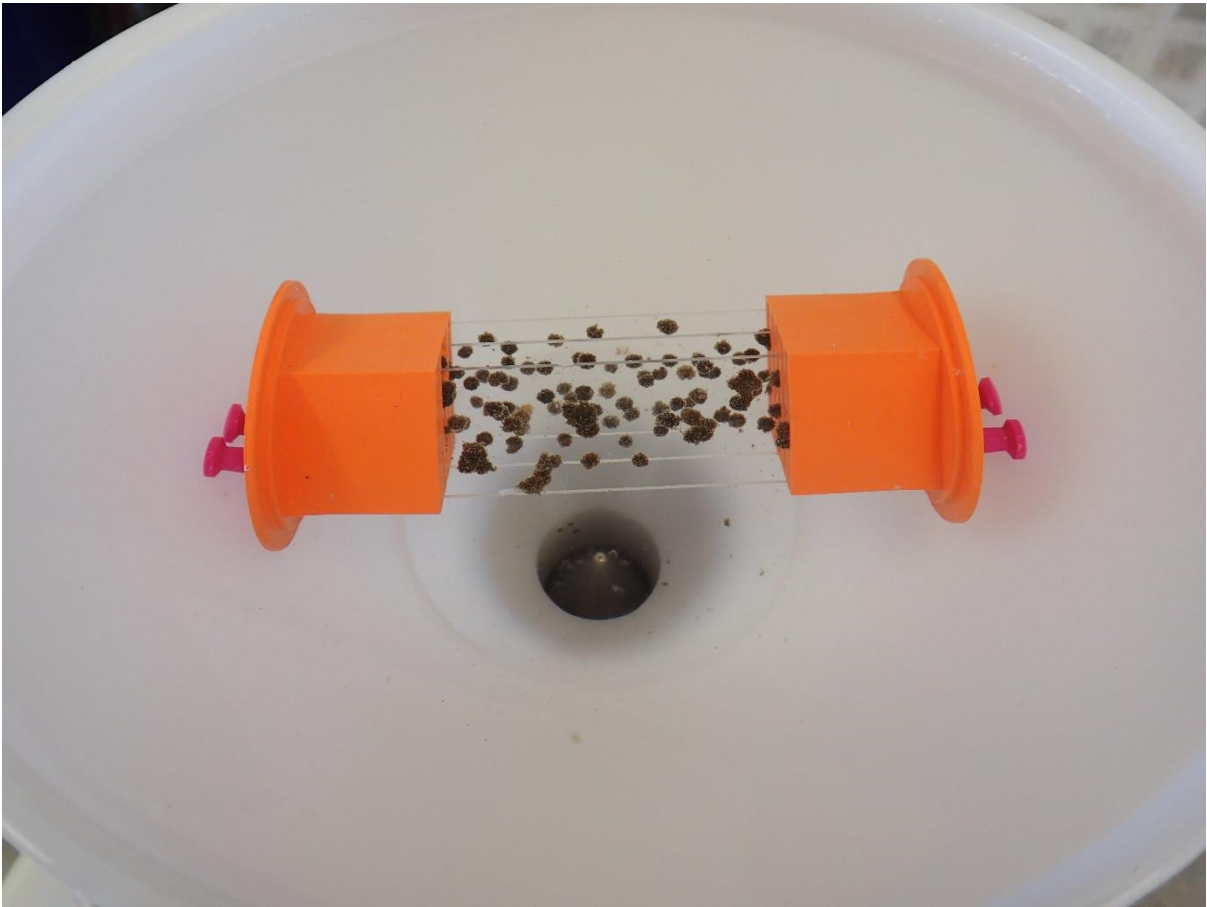
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763 **SUPPLEMENTARY MATERIAL B:**

764 Image of dosing funnel chamber containing glass slides with deposited egg masses.

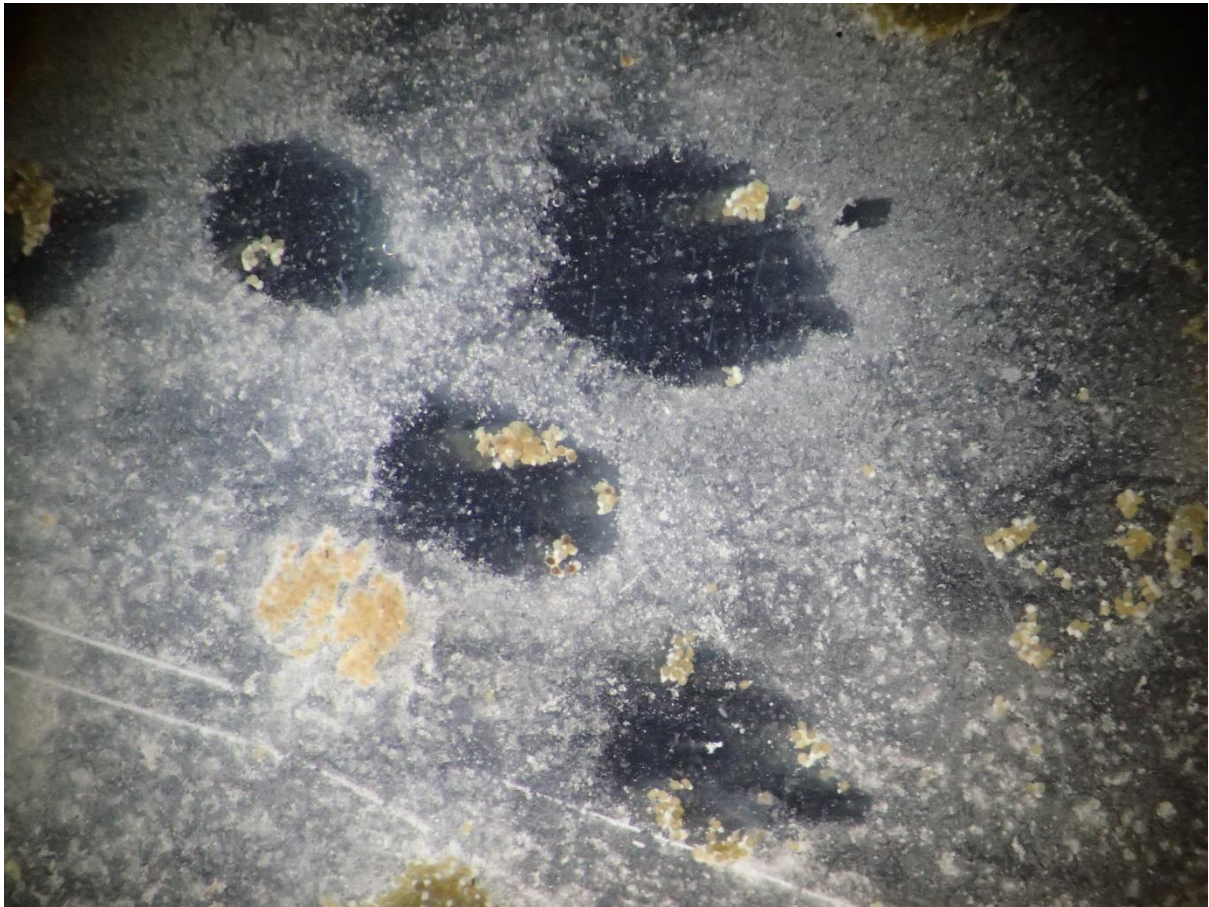


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767 **SUPPLEMENTARY MATERIAL C:**

768 Image of displaced or 'ghost' *S.ignita* egg masses.



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771 **SUPPLEMENTARY MATERIAL D:**

772 Mean physical-chemical properties for control and diluent carbon-limestone filtered tap water.

Parameter	Unit	Sample date (n = 8)							
		29/8/15	26/9/15	31/10/15	28/11/15	19/12/15	30/1/16	19/2/16	26/3/16
Nitrogen: Total as N	mg l <sup>-1</sup>	0.5	0.525	0.435	0.44	0.535	0.501	0.702	0.563
Alkalinity to pH 4.5 as CaCO <sub>3</sub>	mg l <sup>-1</sup>	117	122	129	139	144	154	150	161
Ammoniacal Nitrogen as N	mg l <sup>-1</sup>	<0.030	<0.030	<0.030	<0.0300	<0.0300	<0.0300	<0.0300	<0.0300
Nitrite as N	mg l <sup>-1</sup>	<0.0040	<0.0040	<0.0040	<0.0040	<0.0040	<0.0040	<0.0040	<0.0040
Orthophosphate as P	mg l <sup>-1</sup>	0.044	0.045	0.027	0.033	0.03	0.036	0.04	0.029
pH		7.97	8.03	7.92	7.99	8.07	8.07	8.14	8.27
Suspended Solids at 105° C	mg l <sup>-1</sup>	<3	<3	<3	<3	<3	<3	<3	<3
Boron	µg l <sup>-1</sup>	<100	<100	<100	<100	<100	<100	<100	<100
Calcium	mg l <sup>-1</sup>	49.2	58.1	49	58	59.3	61.9	66.9	65.1
Iron	µg l <sup>-1</sup>	34.9	49.9	45.2	37.6	39.9	32.8	40.9	34.7
Lithium	µg l <sup>-1</sup>	<100	<100	<100	<100	<100	<100	<100	<100
Magnesium	mg l <sup>-1</sup>	2.55	3.46	5.02	3.05	3.91	3.32	3.37	3.86
Manganese	µg l <sup>-1</sup>	<10	<10	<10	<10	<10	<10	<10	<10
Sodium	mg l <sup>-1</sup>	8.19	9.1	8.65	8.17	8.28	8.4	8.34	8.35
Mercury	µg l <sup>-1</sup>	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Hardness : Total as CaCO <sub>3</sub>	mg l <sup>-1</sup>	136	159	143	157	164	168	181	178
Arsenic	µg l <sup>-1</sup>	1	-	-	-	-	-	-	1
Selenium	µg l <sup>-1</sup>	1	-	-	-	-	-	-	1
Cadmium	µg l <sup>-1</sup>	0.1	-	-	-	-	-	-	0.2
Copper	µg l <sup>-1</sup>	0.5	-	-	-	-	-	-	0.115
Lead	µg l <sup>-1</sup>	0.61	-	-	-	-	-	-	0.5
Nickel	µg l <sup>-1</sup>	1.97	-	-	-	-	-	-	0.4

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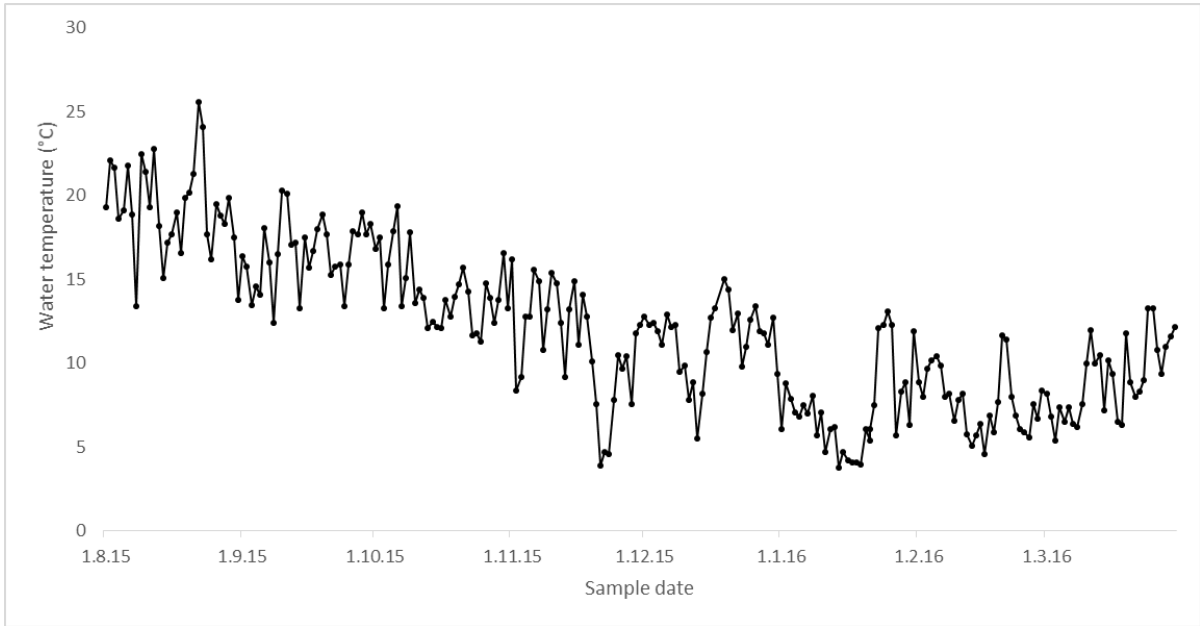
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777 **SUPPLEMENTARY MATERIAL E:**

778 Water temperature in a control *S. ignita* egg chamber.



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