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

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Abstract:

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

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

1. Introduction

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

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

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

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

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

larvae, *Seratella ignita* (Ephemerellidae: Ephmeroptera) under experimental conditions. This was achieved by investigating whether:

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

2. Methodology:

2.1. Target Organism

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

2.2. Experimental Set-Up and Overview

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

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

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

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

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

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

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

2.4. Bioassay design and egg monitoring

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

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

2.5. Chemical dosing and testing

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

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

(Supplementary material D). Dissolved oxygen and pH were also recorded once a month across all the treatments. Water samples were taken from all of the bioassay test chambers once a month across the 8 month experimental period using an overflow valve fitted into the treatment rigs (Figure 1). All samples for chemical analysis were sent to the UKAS Accredited National Laboratory Service for monthly analyses of Total Nitrogen, Ammoniacal Nitrogen, Nitrite, Alkalinity (to pH 4.5 as CaCO3), Orthophosphate, pH, Suspended Solids (at 105 ^{o}C), Boron, Calcium, Iron, Lithium, Magnesium, Manganese, Sodium, Water hardness (Total as CaCO3), Arsenic, Selenium, Cadmium, Copper, Lead, Mercury, Nickel and Zinc (Supplementary material E). The test treatments were also tested monthly for respective actual dosed SS and OP levels.

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

2.6. Statistical analysis

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

3. Results:

3.1. Egg mortality within bioassays

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

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

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

3.2. Egg mass emergence in bioassays

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

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

4. Discussion:

4.1. Effects of multiple stressors

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

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

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

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

4.2. The effect of suspended sediment on eggs

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

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

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

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

4.3. Phosphorous effects on egg development

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

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

4.4. Concentrations of OP and SS in rivers

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

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

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

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

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

4.5. Management implications

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

5. Conclusions:

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

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

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465

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710

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

Chemical treatment	Nominal chemical concentration mg/l					
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^{-1} OP + SS	0	5	10	15	20	25
10 mg l^{-1} SS + OP	0	0.05	0.07	0.1	0.2	0.3

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

Parameter	Mean water quality ± s.d. (*n* = 10)					
Nominal SS concentration (mg l^{-1})	0	5	10	15	20	25
Actual SS concentration (mg l^{-1})	<3	5.2 ± 0.2	10.0 ± 0.1	14.9 ± 0.2	20.0 ± 0.2	24.7 ± 0.6
Water temperature (°C)	13.4 ± 4.9	13.4 ± 4.9	13.5 ±4.9	13.4 ± 5.0	13.3 ± 5.0	13.3 ± 4.9
Dissolved oxygen (mg l^{-1})	10.5 ± 0.4	10.3 ± 0.4	10.3 ±0.4	10.3 ± 0.4	10.2 ±0 .2	10.3 ± 0.4
pH	8.0 ± 0.1	8.0 ± 0.1	8.0 ± 0.1	7.9 ± 0.3	7.9 ± 0.1	7.9 ± 0.1
Nominal OP concentration (mg l^{-1})	0	0.05	0.07	0.1	0.2	0.3
Actual OP concentration (mg l^{-1})	0.04 ± 0.01	0.05 ± 0.01	0.07 ± 0.02	0.11 ± 0.01	0.20 ± 0.01	0.30 ± 0.01
Water temperature (°C)	13.4 ± 5.1	13.1 ± 4.9	13.0 ± 5.0	13.0 ± 4.9	12.9 ± 5.0	12.8 ± 5.0
Dissolved oxygen (mg l^{-1})	10.5 ± 0.4	10.2 ± 0.3	10.2 ± 0.3	10.3 ± 0.3	10.2 ± 0.3	10.3 ± 0.4
pH	7.9 ± 0.1	7.9 ± 0.1	7.9 ± 0.1	7.8 ± 0.3	7.8 ± 0.1	7.8 ± 0.1
Nominal SS concentration (mg l^{-1})	0	5	10	15	20	25
Actual SS concentration (mg l^{-1})	<3	5.2 ± 0.4	10.0 ± 0.1	15.0 ± 0.1	20.1 ± 0.2	25.0 ± 0.2
Nominal OP concentration (mg l^{-1})	0.07	0.07	0.07	0.07	0.07	0.07
Actual OP concentration (mg l^{-1})	0.04 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.004	0.07 ±0 .003	0.07 ± 0.01
Water temperature (°C)	13.7 ±5.2	13.6 ± 4.7	13.6 ± 4.5	13.3 ± 4.9	13.0 ± 4.7	12.9 ± 4.8
Dissolved oxygen (mg l^{-1})	10.4 ±0.4	10.0 ± 0.5	10.2 ± 0.3	10.2 ± 0.3	10.2 ± 0.4	10.0 ± 0.3
pH	8.0 ± 0.1	8.1 ± 0.1	8.0 ± 0.1	8.0 ± 0.3	8.0 ± 0.1	8.2 ± 0.2
Nominal OP concentration (mg l^{-1})	0	0.05	0.07	0.1	0.2	0.3
Actual OP concentration (mg l^{-1})	0.04 ± 0.01	0.05 ± 0.004	0.07 ± 0.004	0.10 ± 0.01	0.21 ± 0.01	0.31 ± 0.01
Nominal SS concentration (mg l^{-1})	10	10	10	10	10	10
Actual SS concentration (mg l^{-1})	<3	9.9 ± 0.5	10.0 ± 0.2	10.2 ± 0.3	9.9 ± 0.3	10.0 ± 0.1
Water temperature (°C)	13.3 ± 4.6	13.3 ± 4.7	13.4 ± 5.2	13.4 ± 4.7	13.1 ± 5.0	13.0 ± 4.9
Dissolved oxygen (mg l^{-1})	10.2 ± 0.4	10.7 ±0.4	11.0 ± 0.6	10.3 ±0.5	10.1 ± 0.4	10.1 ± 0.3
pH	8.0 ± 0.1	8.1 ± 0.1	8.0 ± 0.2	7.9 ± 0.3	8.3 ± 0.2	8.3 ± 0.3

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

Treatment	Time (days)	Equation	R^2
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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723 **Table 4:** Analysis of national routine spot measures of OP and SS made by the Environment Agency
724 in 2015 for WFD compliance across England.

		Count	Average (mg l^{-1})	Percentage of sites where the average is			
				> 0.3 mg l^{-1}	> 0.1 mg l^{-1}	> 25 mg l^{-1}	> 10 mg l^{-1}
All sites	**OP**	32549	0.27	21.8	51.6		
All sites	**SS**	2029	16.6			9.2	31.6
		Count	Average (mg l^{-1})	Percentage of sites where every measurement is			
				> 0.3 mg l^{-1}	> 0.1 mg l^{-1}	< 0.1 mg l^{-1}	< 0.3 mg l^{-1}
Sites > 9 samples	**OP**	1812	0.30	22.2	52.9	21.8	52.7
		Count	Average (mg l^{-1})	Percentage of sites where every measurement is			
				> 25 mg l^{-1}	> 10 mg l^{-1}	< 10 mg l^{-1}	< 25 mg l^{-1}
Sites > 9 samples	**SS**	129	12.7	9.4	39.0	49.6	10.9

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

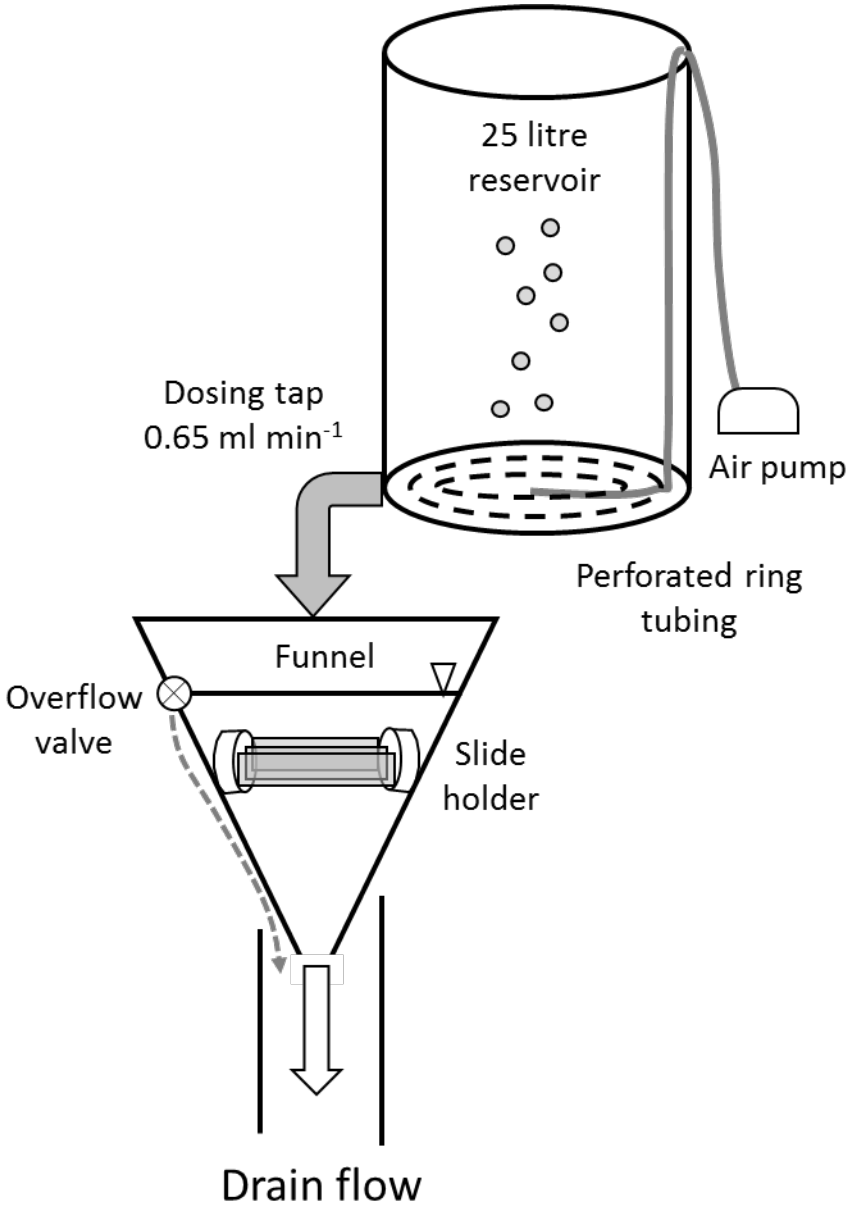
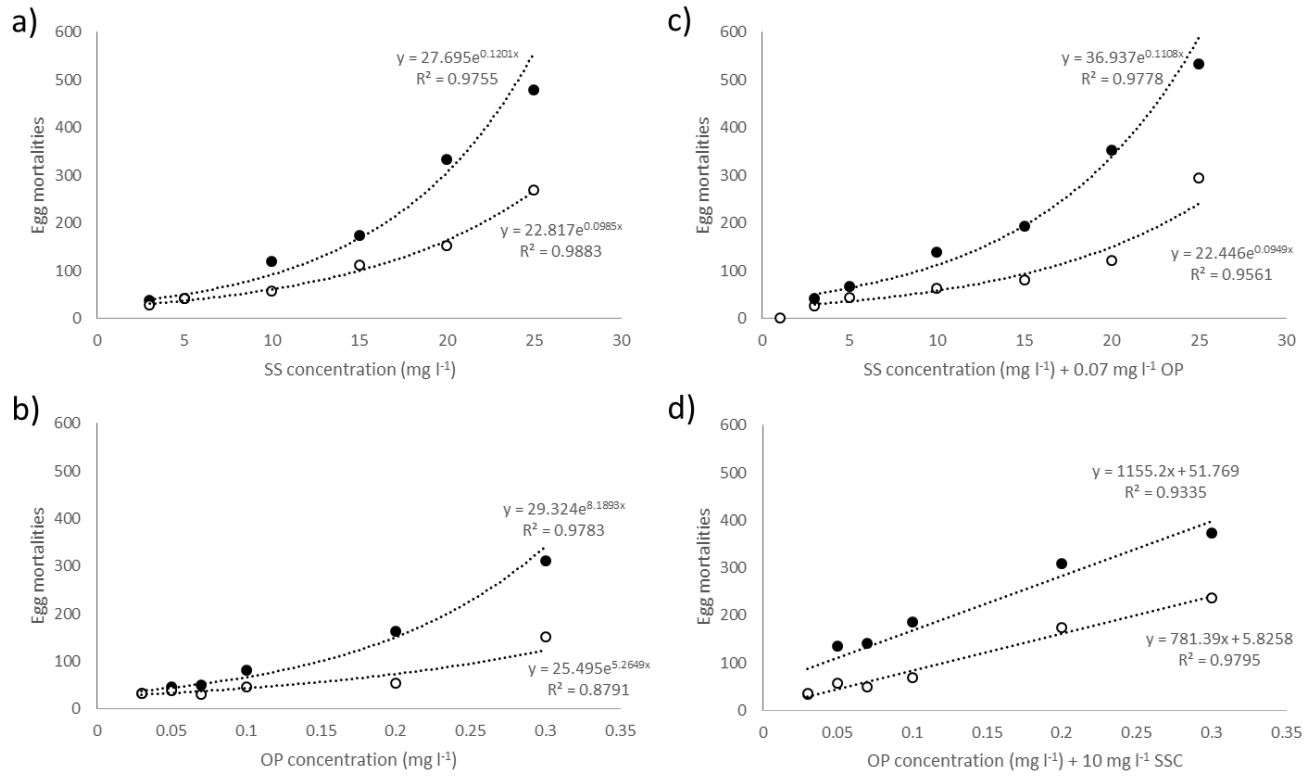


Figure 2: Regressions of the mortality of *S.ignita* eggs after 72 days exposure (open circles) and 183 days exposure (filled circles) against (a) SS, (b) OP), (c) SS in addition to 0.07 mg l^{-1} OP, and (d) OP in addition to 10 mg l^{-1} SS.



739 **Figure 3:** Mortality of *S. ignita* eggs through time under differing concentrations of (a) SS; (b) OP; (c)
740 SS plus 0.07 mg l^{-1} OP, and; (d) OP plus 10 mg l^{-1} SS.

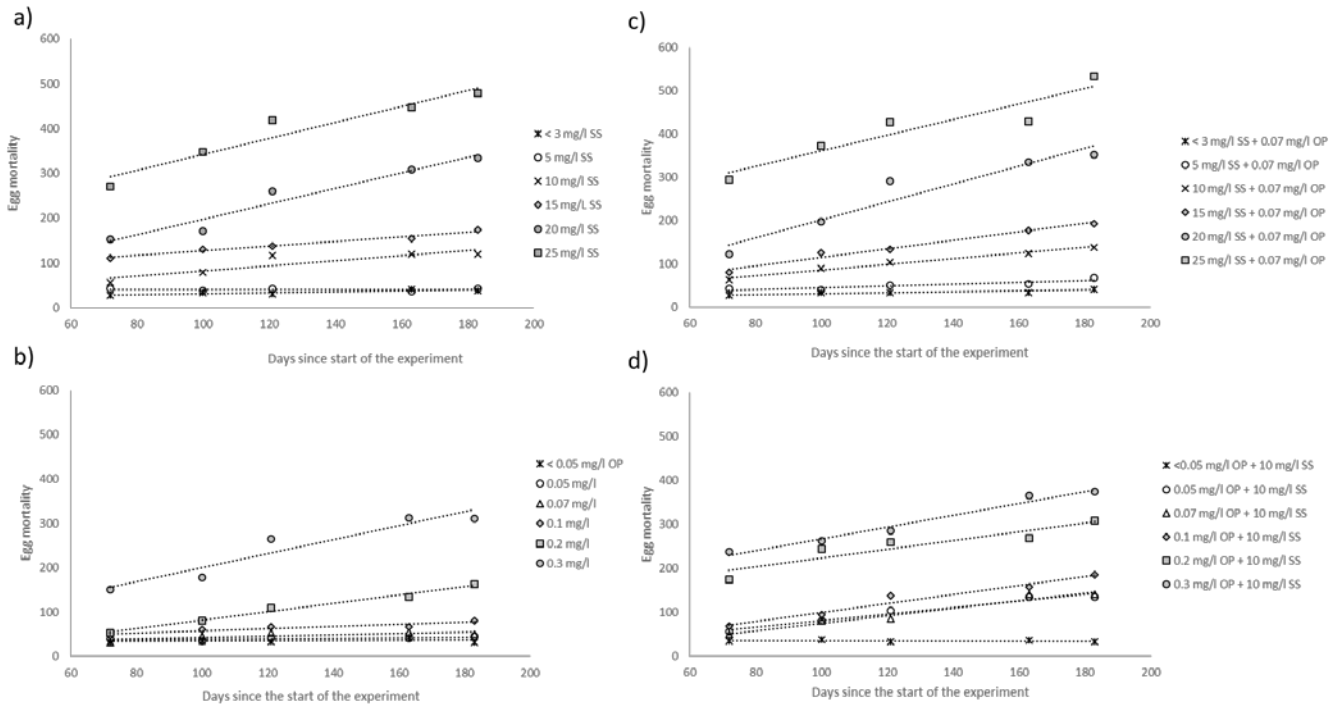


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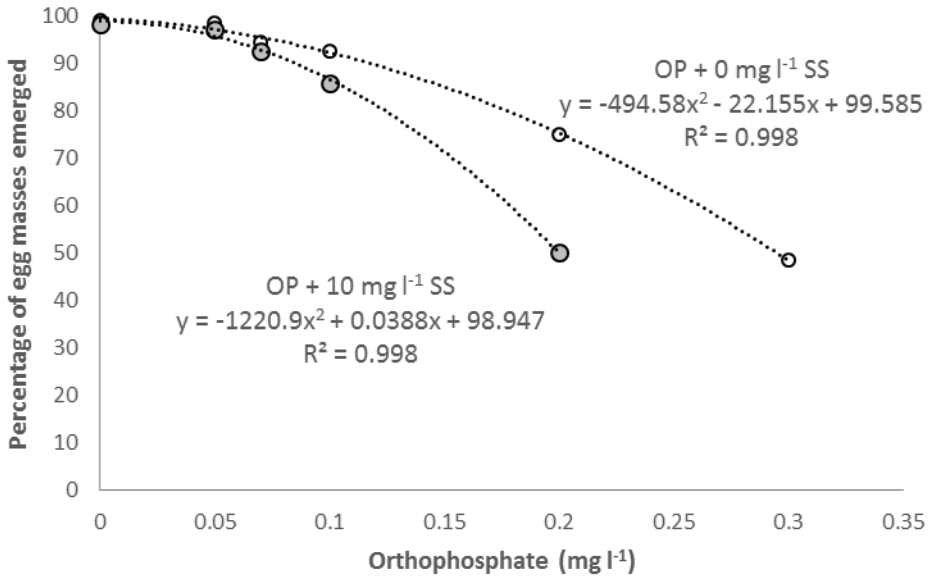
Figure 4*:* Percentage of *S.ignita* egg masses surviving to emergence under different concentrations of (a) OP in isolation (open circles) and in combination with 10 mg l^{-1} of SS (closed circles) and (b) SS in isolation (open circles) and in combination with 0.07 mg l^{-1} of OP. Note, at 0.3 mg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



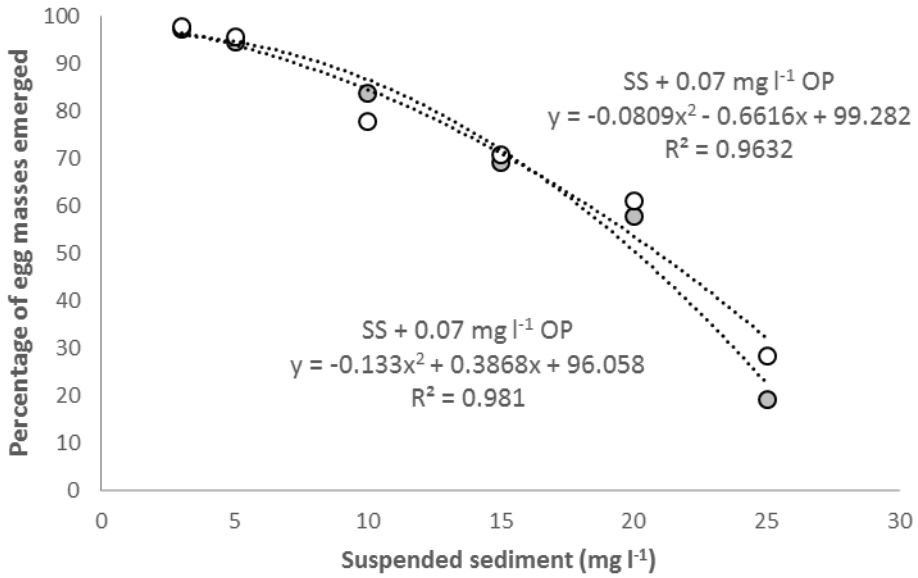
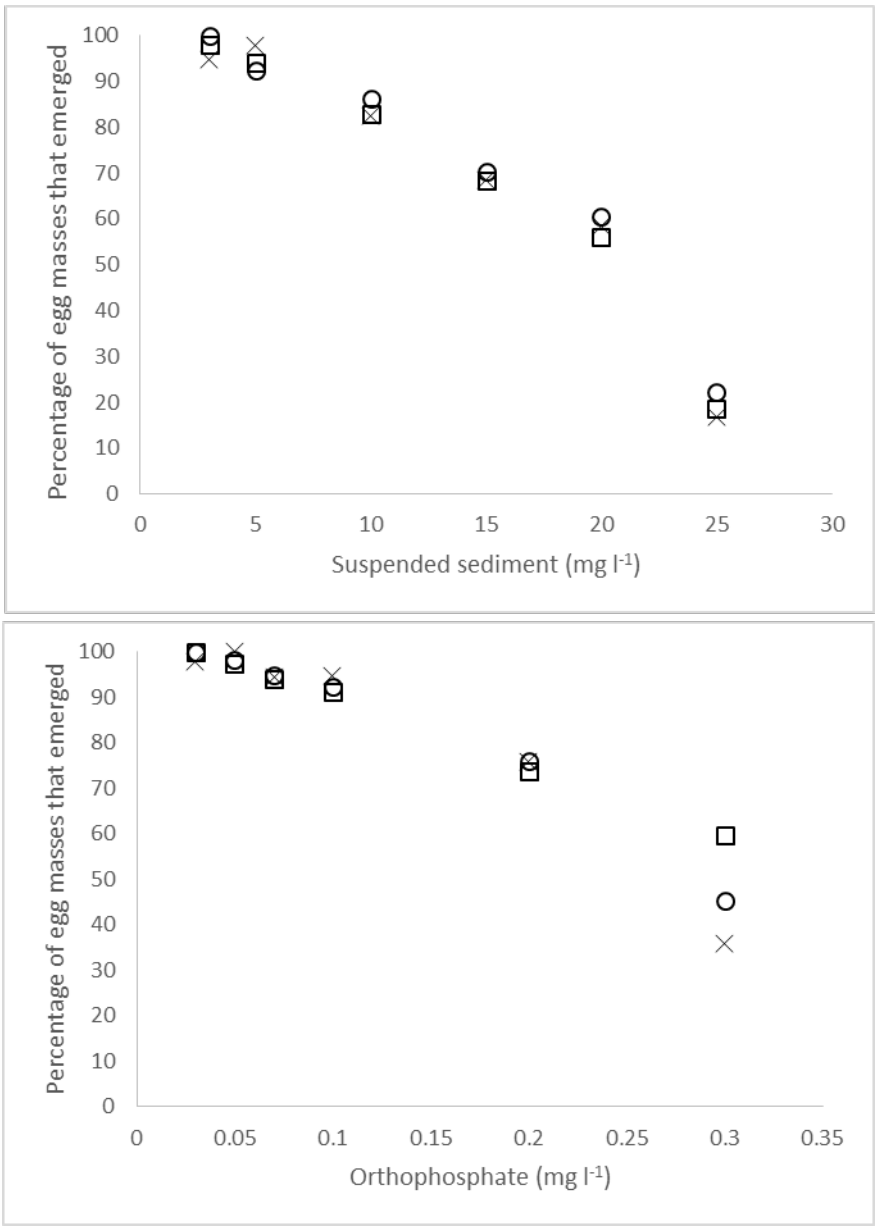
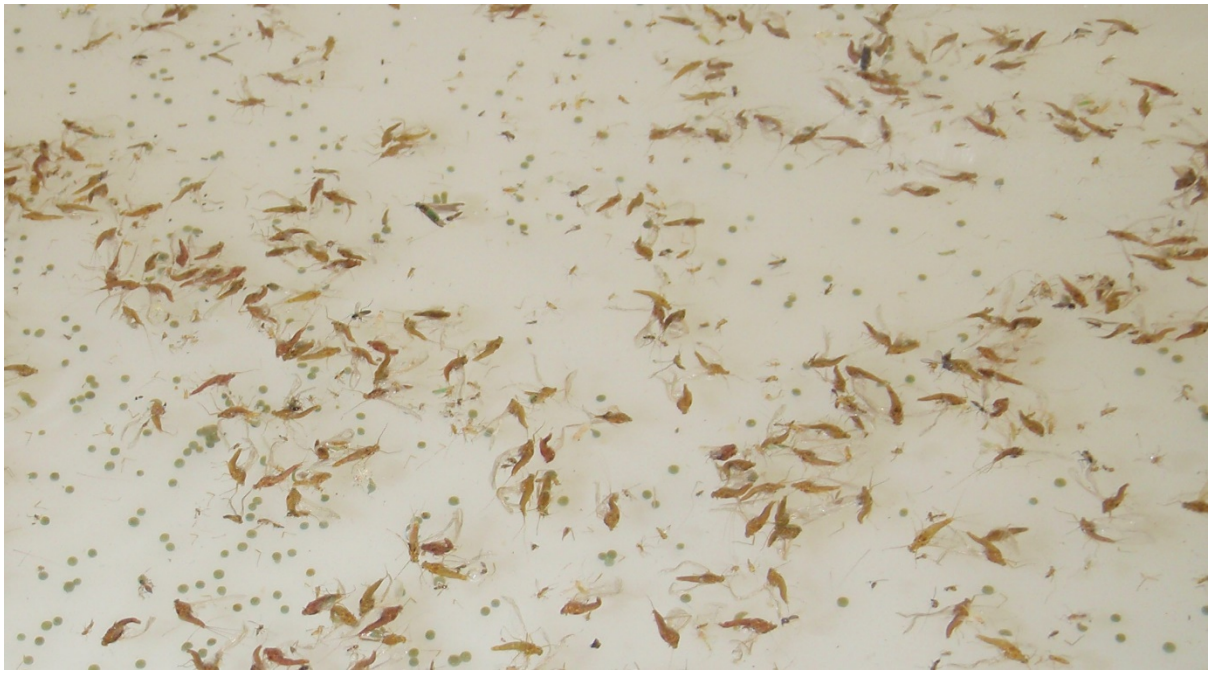


Figure 5: Percentage of *S.ignita* egg masses surviving to emergence under different concentrations of (a) OP (b) SS for each of the 3 slides in each treatment indicated separately.



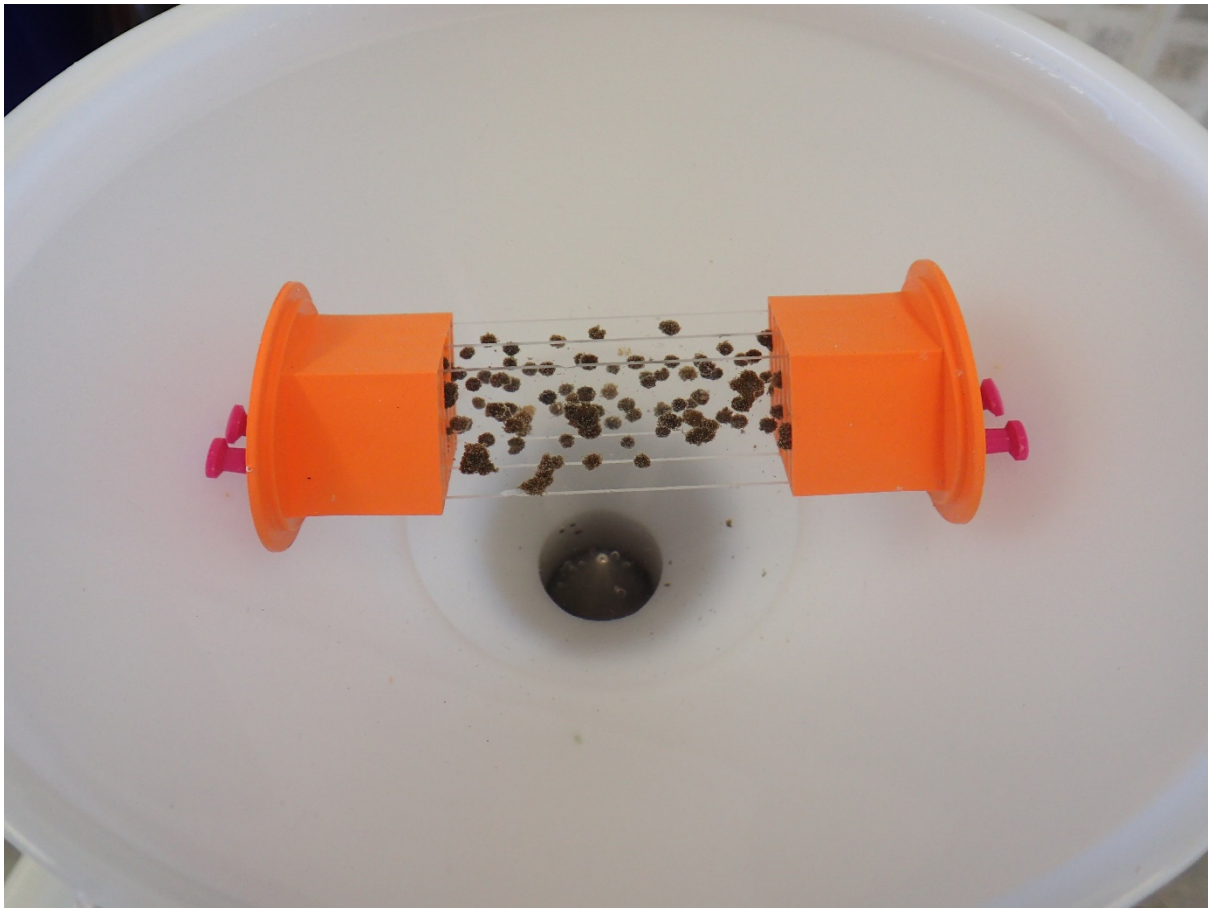
SUPPLEMENTARY MATERIAL A:

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



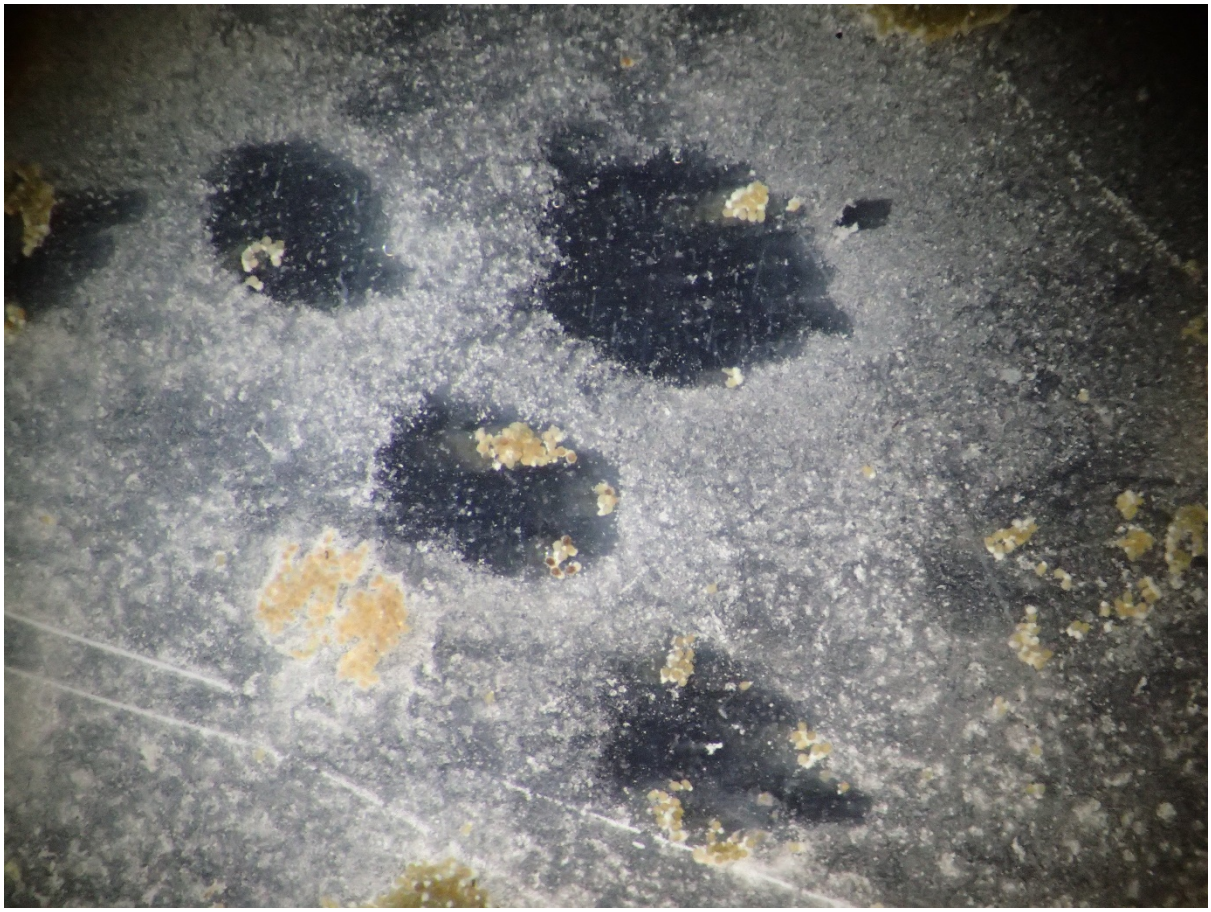
SUPPLEMENTARY MATERIAL B:

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



SUPPLEMENTARY MATERIAL C:

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



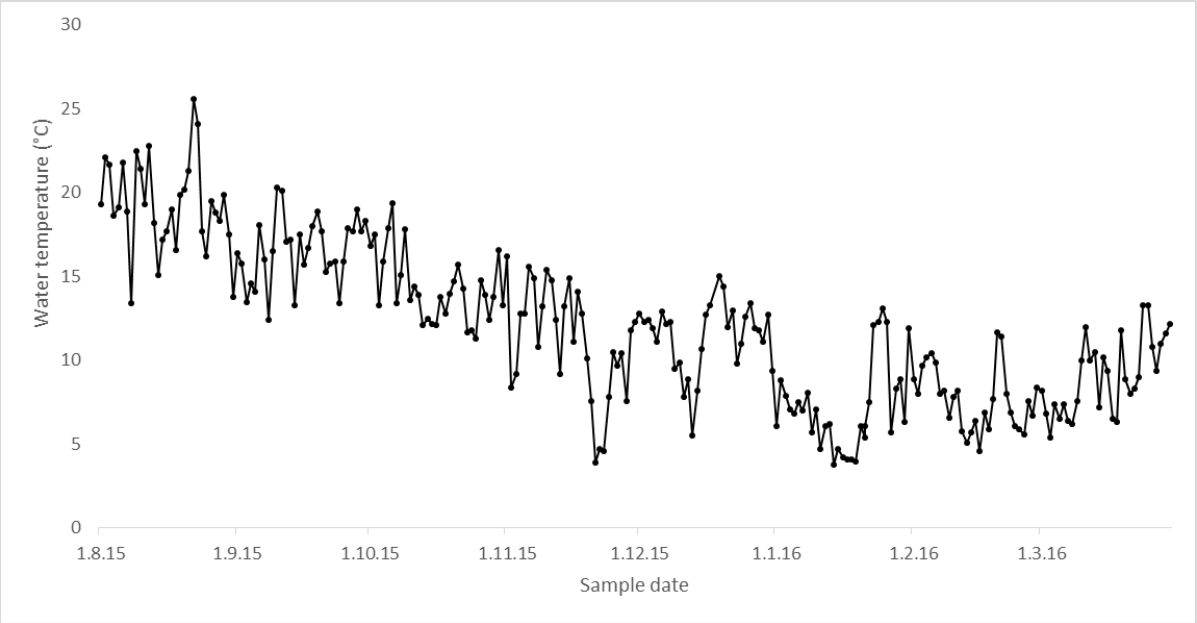
SUPPLEMENTARY MATERIAL D:

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^{-1}	0.5	0.525	0.435	0.44	0.535	0.501	0.702	0.563
Alkalinity to pH 4.5 as CaCO3	mg l^{-1}	117	122	129	139	144	154	150	161
Ammoniacal Nitrogen as N	mg l^{-1}	<0.030	<0.030	<0.030	<0.0300	<0.0300	<0.0300	<0.0300	<0.0300
Nitrite as N	mg l^{-1}	<0.0040	<0.0040	<0.0040	<0.0040	<0.0040	<0.0040	<0.0040	<0.0040
Orthophosphate as P	mg l^{-1}	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^{-1}	<3	<3	<3	<3	<3	<3	<3	<3
Boron	µg l^{-1}	<100	<100	<100	<100	<100	<100	<100	<100
Calcium	mg l^{-1}	49.2	58.1	49	58	59.3	61.9	66.9	65.1
Iron	µg l^{-1}	34.9	49.9	45.2	37.6	39.9	32.8	40.9	34.7
Lithium	µg l^{-1}	<100	<100	<100	<100	<100	<100	<100	<100
Magnesium	mg l^{-1}	2.55	3.46	5.02	3.05	3.91	3.32	3.37	3.86
Manganese	µg l^{-1}	<10	<10	<10	<10	<10	<10	<10	<10
Sodium	mg l^{-1}	8.19	9.1	8.65	8.17	8.28	8.4	8.34	8.35
Mercury	µg l^{-1}	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Hardness : Total as CaCO3	mg l^{-1}	136	159	143	157	164	168	181	178
Arsenic	µg l^{-1}	1	-	-	-	-	-	-	1
Selenium	µg l^{-1}	1	-	-	-	-	-	-	1
Cadmium	µg l^{-1}	0.1	-	-	-	-	-	-	0.2
Copper	µg l^{-1}	0.5	-	-	-	-	-	-	0.115
Lead	µg l^{-1}	0.61	-	-	-	-	-	-	0.5
Nickel	µg l^{-1}	1.97	-	-	-	-	-	-	0.4

776 ***SUPPLEMENTARY MATERIAL E:***

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



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