The effects of wastewater effluent on multiple behaviours in the amphipod, *Gammarus pulex*.

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Abstract

The prevalence of pharmaceuticals and personal care products (PPCPs) in lotic habitats is increasing, with the main source of these contaminants being effluent from waste water treatment works (WwTW). There is still much uncertainty about the impacts of these PPCPs at environmentally relevant concentrations and their potential effects on aquatic ecology. Behaviour is a sensitive endpoint which can help evaluate possible population levels effects from changes in physiology. This paper evaluates the effects of WwTW effluent on a range of behaviours in the freshwater invertebrate, *Gammarus pulex*. Effluent taken from the outflow of two WwTW in southern England was used in the study. Behavioural analyses, namely feeding rate, phototaxis, activity, velocity and precopula pairing, were measured in *G. pulex* following a period of one and three weeks after exposure to a 50% or 100% effluent and a control. Mortality remained very low throughout the 3 week experiment (0-10%, n = 20) and no significant changes in moulting frequency were observed ($p > 0.05$). No significant effects on feeding or velocity or phototaxis following 3 weeks of effluent exposures were observed ($p > 0.05$). However, significant reductions were observed in the overall activity over 3 weeks across which appeared to be exacerbated by exposure to effluents. Interestingly, males exposed for 3 weeks to WwTW effluent re-paired with unexposed females significantly faster (4-6x) than control animals. This result was consistent between the effluents taken from the two WwTW. The implications of these behavioural changes are currently unknown but highlight the need for a varied set of tools to study the behavioural changes in wildlife.

Capsule:

The effects of wastewater effluent on the multiple behaviours in the riverine amphipod, *Gammarus pulex*. Study surprisingly finds very little effects on activity-based behaviours but does find effects of reproductive behaviours.

Amphipod, Effluent, *Gammarus pulex*, PPCPs, Pharmaceuticals, River, Ecotoxicology, Behaviour, Pairing, Velocity

Introduction

Globally, aquatic environments are under increasing pressure (Vörösmarty et al.,2010; Loeb, 2016) with pollution being one of the main threats (Dudgeon et al., 2006). One of the main sources of water pollution has been identified as effluent discharge by waste water treatment works (WwTWs) (Wigh et al., 2017), the regulation and monitoring of which is under increasing scrutiny (EEA, 2019). A growing number of pollutants are controlled through their Environmental Quality Standard (EQS) which can be used to reduce the impact of pollutants in the aquatic environment. Assessment for EQS takes time and investment in order to formulate and new chemicals are continually developed. As a result, delays in evaluating EQS values are considerable and the majority of compounds remain undesignated (Crane et al., 2009; Dang et al., 2015). Despite many WwTW effluents containing chemicals that should have an EQS, the effluents are complex and inherently inconsistent, and therefore the impact of individual components of WwTW effluent is difficult to ascertain. Tests such as the Direct Toxicity Assessment (DTA) and the Whole Effluent Toxicity (WET) can characterise the aggregated effects of unknown contaminants in environmental samples (Chapman, 2000: Gruiz et al., 2016). Such tests offer the advantage that they measure the total effects of the discharge (including interactions between components) as well as having direct ecological relevance (Silva et al., 2002; Picado et al., 2008). The disadvantage is that any measured toxicity cannot be expressed in concentration of any components, thus it does not fit into the model-based risk assessment, the foundation of regulatory guidelines. Studies on the impacts of effluents must reflect that there will typically be some dilution of the effluent and organisms might only be exposed to 100% effluent immediately around the discharge point, if at all.

Amphipods are widely accepted as indicator species of environmental impacts (Neuparth et al., 2002; Wang et al., 2004; Costa et al., 2005; Kunz et al., 2010). Effects on their population around WwTW have been reported by several authors (Jones & Johnson, 1992; Jones & Wigham, 1993; Gross et al., 2001; Schirling et al., 2005; Ladewig et al., 2006). These studies do not report evidence of lethal levels of contamination, prompting questions over whether lethality endpoints are the most reliable in detecting long-term and potentially subtle effects (Gavrilescu et al., 2015). The impact of whole WwTW effluent on amphipod osmoregulation (Johnson & Jones, 1990), metabolism (Agnew & Jones, 1986), food consumption (Maltby et al., 2002; Bundschuh & Schulz, 2011b; Bundschuh et al., 2011c), reproduction (Schneider et al., 2015; Wigh et al., 2017), genotoxicity (Wigh et al., 2017) and survival (Woodworth et al., 1999) has been investigated through direct exposure assays and produced mixed results. Many of these endpoints might first impinge on the behaviour of the amphipod which can provide further insights onto the total toxicity (Hellou, 2011).

By definition, behaviour is the external manifestation of an organism's response to physiological and environmental factors (Dell'Omo, 2002). Behaviour offers many benefits as an ecotoxicological endpoint and it is a more sensitive indicator of toxicity than mortality (Nassef et al., 2010; Hellou, 2011). This is an advantage particularly when investigating the potentially subtle effects of contaminants which are typically found in the environment at levels well below lethal concentrations (Norris et al., 1999). Behavioural observations are non-invasive allowing for repeated measurements permitting longer-term studies. Changes in behaviour have very wide implications and effects seen in individuals can infer changes within a population (Boyd et al., 2002) and entire ecosystem (Brodin et al., 2014).

Previous studies on the effect of sewage effluent on behavioural activity is limited. Most commonly, it has been investigated in fish (Schoenfuss et al., 2002; Martinović et al., 2007; Sebire etal., 2011; Brodin et al., 2013; Melvin, 2016; Melvin et al., 2016), and a small number of invertebrate genera; namely polychaetes (Dauer & Conner, 1980) and cladocerans (Van Veen et al., 2002; Mannarino et al., 2010). Amongst amphipod studies, behavioural effects have been documented in animals exposed to contaminated sediments (Oakden et al., 1984; Hellou, 2011; Rastetter & Gerhardt, 2017) and potential components of sewage effluent (De Lange et al., 2006; De Lange et al., 2009; Guler & Ford, 2010; Dietrich et al., 2010; Bossus et al., 2014), but not in the entirety of the effluent itself.

One of the characteristics of amphipods that predisposes them to behavioural studies is that they demonstrate several discrete behaviours. Gammarids display a pre-copulatory amplexus where the male clasps and guards the female immediately before she can mate (post-moult). Since reproduction (and ecdysis) is coordinated by endocrine control and environmental factors, any disruption of these stimuli by environmental contamination might be detected behaviourally (Dietrich et al., 2010a; Hyne, 2011). Thus, pre-copulatory mate guarding has been measured as an endpoint for environmental contaminants (Poulton & Pascoe, 1990; Pascoe et al., 1994; Malbouisson et al., 1995; Watts et al., 2001; Negro et al., 2013; Pedersen et al., 2013; Wisniewska & Szaniawska,2015) and its frequency measured in amphipods after exposure to treated WwTW effluent (Bundschuh & Schulz, 2011). Amphipods are generally negatively phototaxic (Rauque et al., 2011), a behaviour influenced by serotonin and, by extension, the endocrine system. Several studies have found impacts of pharmaceutical exposure at environmentally relevant levels on phototaxic behaviour of amphipods (Guler & Ford, 2010; Bossus et al., 2014). Pharmaceuticals, among many chemicals, are ubiquitous in WwTW effluent (Ashton et al., 2004; Owens, 2015) yet there have been no studies investigating the effect of WwTW effluents on phototaxis in *G. pulex*. Therefore, the purpose of this investigation was to determine the impact of WwTW effluents on the behaviour of the freshwater amphipod *Gammarus pulex*.

Materials and Methods

The effluents of two WwTW were selected for the assay. Chickenhall WwTW discharges into the River Itchen at Eastleigh, Hampshire (UK) and Fullerton WwTW discharges into the Test 3km south of Andover, Hampshire (UK). Both effluents undergo the same treatment consisting of primary, secondary and chemical phosphate stripping (Suppl Table 1). The population equivalents served are 101,692 and 62,194 for Chickenhall and Fullerton respectively. Chickenhall WwTW is one of the sites selected for scrutiny of its effluent by the Chemical Investigations Project, a national undertaking to measure the prevalence of micro pollutants in the UK (ALS, 2014; Suppl Table 2).

Adult *G. pulex* were collected from the source of the River Test; a spring rising near Overton, UK (Latitude: 51.245584, Longitude: -1.239073). The site was given the highest grade for water quality and biology (EA, 2009). Adult males, identified by the presence of genital papillae (Welton, 1979), were selected. Visual checks for acanthocephalan parasites (indicated by the orange acanthella visible in the ventral pereon) were made and any individuals containing the parasite were rejected.

Gammarids were maintained in cages with sediment and water crowsfoot (*Ranunculus aquatilis*) (for refuge) in a flow through system at University Centre Sparsholt, Hampshire (UK), using borehole water at a rate of 2 litres per minute. Temperature and dissolved oxygen (DO) were measured weekly and were consistently 11.3±0.2˚C and over 90% dissolved oxygen saturation (Suppl Table 3). Gammarids were fed *ad libitum* on conditioned alder (*Alnus glutinosa*) as per Bloor and Banks (2006), and 7 days acclimation was allowed prior to any trial.

Effluent was collected in the morning from the discharge channels at Chickenhall and Fullerton WwTW in 25 litre food grade polyethylene carboys (Nalgene, Rochester, NY, USA) and immediately transported back to the laboratory. For the first (1 week) trial it was stored at 4˚C; for the second, it was subdivided into 5 litre polyethylene bottles and frozen at -20˚C. In both trials, samples were allowed to return to 11˚C before use. All experiments used different batches of gammarids apart from the precopular pairing experiments which used males from the manual behaviour experiments to pair with reproductively receptive females.

Mortality and Moulting

Mortality and moulting frequency were recorded through daily observations. Mortality was checked through gently prodding the amphipods in their tanks and observing movements and moutling was recorded as a visible sign of an exoskeleton within the experimental vessels. Since some expected cell counts were less than five, Fisher's exact test (2 x c) was conducted between moulting (and mortality) frequency and concentration of effluent exposure.

Automated Behavioural Trials (Daniovision)

After acclimation, animals were transported to the Institute of Marine Science, Portsmouth in polystyrene transport boxes in borehole water and allowed to recuperate in a climate controlled room (10±0.5˚C) for 24h before being exposed to the effluents. In addition to a control group, 20 gammarids were exposed to 100% and 50% effluent. Effluent was diluted with spring water from the collection site near Overton. Animals were individually maintained in static 50ml food-grade pots (Fixnfast, Berks, UK), fed ad libitum with conditioned alder leaves, and maintained at 10±0.5˚C in a climate controlled room with a photoperiod of 12:12 light dark. Mortality and moulting was recorded daily and water was renewed every 3 days. After 0, 2, 24h and 7 days' exposure, *Gammarus* behavioural analysis was performed using DanionVision™ (Noldus Information Technology, Wageningen, The Netherlands) and its software: EthoVision® XT. Individual animals were placed in to each well of 6-well plates which were filled with their treatment water. Individuals were left for 1 minute inside the DanioVision behavioural Observation Chamber to allow for acclimatisation prior to recording. The velocity (mm/s) and distance (mm) of the amphipods were recorded every 0.016 seconds during 60 second light: dark intervals for 4 minutes. After measurement, animals were returned to their respective 50mL pots. Each pot was individually labelled allowing repeated measures analyses.

Velocity (mm s^{-1}) data from the DanioVision was averaged for every 60 second light and dark exposure for each animal. The initial 60 seconds (dark) were not included in the analysis as the data suggested shrimp were still acclimating to the container and their behaviour was erratic. A three-way mixed (repeated measures) ANOVA was used to test whether there was a main effect of exposure to effluent, and interaction between the subject factors of exposure duration (time) and illumination (light/dark) and between subject factors of effluent concentration (50%, 100% effluent and control). Normality of distribution of the velocity data was confirmed by Shapiro-Wilk's test ($p > 0.05$). Outlying data points (as assessed by inspection of a boxplot) were kept in the analysis because they did not materially affect the results as assessed by a comparison of the results with and without the outliers. Homogeneity of variances was assessed by Levene's test for equality of variances ($p > 0.05$). Sphericity in the three-way interaction was assessed with Mauchly's test; within-subject factors were tested using the Greenhouse-Geisser adjustments when the assumption of sphericity was violated.

Manual Behavioural Observations

Manual Behavioural Observations were made based on the protocol developed by Guler and Ford (2010) and were undertaken at University Centre Sparsholt (UK). Individual gammarids (20 per treatment) were maintained in spring water, 50:50 mix of spring water and final effluent, and 100% effluent as in the previous trial. Animals were fed ad libitum with conditioned alder leaves. All solutions were changed twice per week. Pots (50ml food-grade) containing individual gammarids were maintained on trays and a shallow layer of borehole water at 11.5±0.3˚C. Dissolved oxygen levels were measured daily using a hand-held oxygen meter (model HQ-40D, Hach, USA). Test conditions were maintained under 24h darkness as per Zubrod et al. (2015). Activity was measured by a method adapted from Guler and Ford (2010). At weekly intervals, gammarids were placed in a 15cm glass tube filled with test medium. At 7.5cm along the test chamber a line was marked. After a minimum of 2 minutes acclimation time the activity of the shrimp was measured by counting the number of times the amphipod moved over the halfway line in 60 seconds. With the specimen still in the chamber, half of the chamber was enclosed in an opaque cover, excluding light. The position of the Gammarus was taken every 10 seconds for 2 minutes. If the animal was in the light side it it was considered active and received a score of 1, if it was in the dark it was considered inactive and given a score of 0. Therefore, a score of 12 indicated highly positive phototaxic behaviour, and a score of 0 very negatively phototaxic. The process was repeated for 3 weeks.

Some animals were observed to be very inactive and therefore generated very high or low scores depending on which side they started on. To clarify the situation a ‘preference’ index of the animal was calculated:

$$P = 1-(A\ (6 - L))$$

Where P was the preference (whether they ‘preferred’ to be in the light or dark), A = activity score (from the activity test result), L = photoperiod score (a maximum of 12, therefore equal time spent in both areas would generate a score of 6).

Data from manual observations were analysed by means of a Generalized Linear Model (GzLM) whereby activity, phototaxis and preference indices were the dependant variable and time (0-3 weeks) and concentration (control, 50% and 100%) were the fixed factors. Both activity and phototaxis were modelled using a poison distribution and preference indices using a normal distribution. Additional pairwise comparisons were conducted with Bonferroni corrections.

Feeding Experiment

Calculation of feeding rate was based on the methods of Maltby et al. (2002). Conditioned alder leaves were cut into approx. 2cm square portions (removing the mid-rib), dried at 60°C for 48h, weighed, and placed in the holding pots (50ml, food-grade). The exposure medium was added to rehydrate the leave portions 24h before an adult male *Gammarus* was added to each pot (20 animals per treatment). Five leaf portions were also left in each treatment solution without *Gammarus* to account for decomposition ('correction factor'). After a week's exposure, each *Gammarus* and remaining leaf matter were dried at 60°C for 48h and re-weighed. Feeding rate (FR, mg dry wt food/mg dry wt animal/d) of each *G. pulex* was calculated using equation:

$$FR = (L_1 \text{ x } C_L) – L_2 / W \text{ x } 6$$

where L_1 is the dry weight of food material initially supplied (mg), L_2 is the dry weight of leaf material remaining after 6d (mg), W is the dry weight of *G. pulex* (mg), and C_L is the leaf weight change correction factor given by the mean of the ratio of the final to initial weight of control leaves (e.g. a 95% retention in weight after 6 days would give a factor of 0.95). Feeding rate was assessed with a one- way analysis of variance (ANOVA) after assumptions of normal distribution of residuals and homogeneity of variance were met.

Reproductive Pairing Experiment

The precopulatory guarding experiment was based on Watts et al. (2001). The males undertaking the activity behaviour were tested after 3 weeks of effluent exposure. Similar sized males over 8mm length were selected for the assay since male size has been shown to affect pairing success (Adams & Greenwood, 1983; Bollache & Cézilly, 2004). Females were randomly selected in order to avoid size bias for each treatment. Precopulatory pairs from the stock population that had been maintained in flowing borehole water were selected and separated by placing on a paper towel and, if necessary, gentle separation with tweezers. The female of the pair was placed immediately with the male from the effluent exposure in clean borehole water in 50ml vessels. Animals were placed at opposite sides of the vessel to maintain consistency. The two animals were timed until amplexus was achieved.

The time taken for pairing of exposed males with unexposed females was statistically assessed with a Welch's ANOVA after Levene's test indicated heteroscedasticity. Post hoc differences were assessed with a Games-Howell test.

Results

Mortality and Moulting

There was no difference (Fisher's exact test, $p > 0.05$) in the mortality between the treatments in the first (1 week) trial, nor the second (3 week) trial (Table 1). The frequency of moulting was slightly higher in the controls for both effluent experiments (25% of individuals moulted after 3 weeks which contrasts with 5-10% in the 100% effluent exposures). However, this difference was not significant ($p > 0.05$).

Table 1 Mortality and moulting frequency in *Gammarus pulex* (n = 20) exposed to Chickenhall and Fullerton effluent (50% and 100%) over 3 weeks

Trial	Effluent	Moults			Mortalities		
		Control	50%	100%	Control	50%	100%
1 (1 week)	Chickenhall	0	2	1	0	1	0
	Fullerton	1	0	2	0	0	1
2 (3 week)	Chickenhall	5	4	1	0	1	2
	Fullerton	5	2	2	1	0	1

Automated Behavioural Trials

A three-way mixed ANOVA was carried to determine the effects of exposure time (time), light (light/dark) and the effluent concentration on the average velocity of *Gammarus* (mm s^{-1}) over 60 seconds. Using Chickenhall effluent, there was no statistically significant effect of concentration on average velocity (p > 0.05). The three-way interaction between the exposure duration, light/dark conditions, and effluent concentration on the average velocity of *G. pulex* was not significant, F (4.949, 141.06) = 0.934, p = 0.460, partial η2 = 0.032. There were however significant two way interactions for different treatments (Table 2), particularly the reaction to light/dark over time (weeks). Velocity significantly increased in the light exposure compared to the dark through the duration of the trial in all treatments.

Similarly, there was no significant difference in the velocity (mm/s) between effluent concentrations from Fullerton and the control (p > 0.05). The assumption of sphericity was not met. There was no statistically significant three -way interaction between exposure time, light/dark and effluent concentration on the velocity of *Gammarus*, F (5.525, 157.47) = 2.075, p = 0.065, partial η2 = 0.068. There was a significant increase in velocity when animals were exposed to light over the duration of the trial (Table 3).

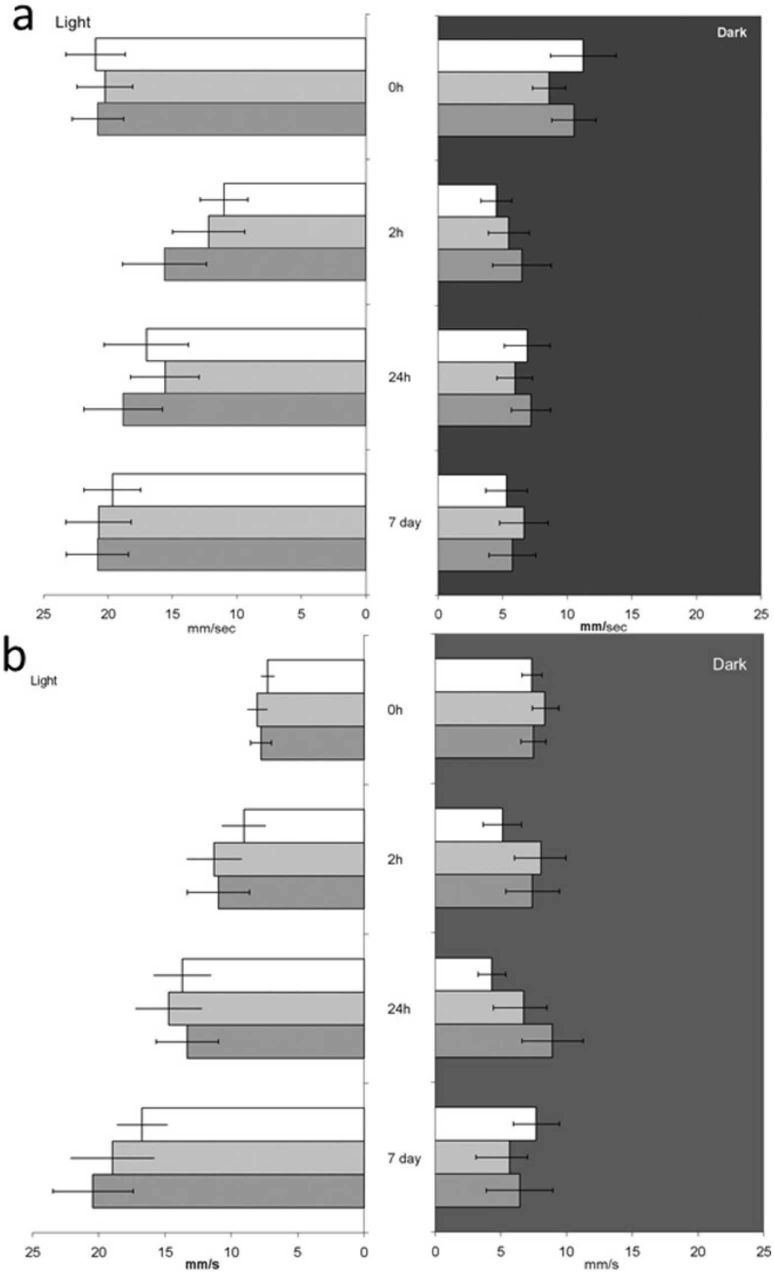

Figure 1 Mean velocity (mm/s 2±SE) of Gammarus pulex over 60 seconds of light (left) and dark (dark). Measurements taken after 0h, 2h, 24h and 7d exposure to Chickenhall (a) Fullerton (b) WwTW effluent. White bars: control, mid grey: 50% dark grey: 100% effluent (n = 60)

Table 2. Analysis of variance of *G. pulex* velocity (n = 60) over 60s light:dark photoperiods and measured at 0h, 2h, 24h and 7d of exposure to 0, 50% and 100% Chickenhall WwTW effluent.

Dependent variable: velocity (mm s^{-1})

	Source	*df*	Mean square	*F*	*P*
Overall	Concentration	2.000	180.987	2.453	0.095
	Light:dark	1.000	3937.29	221.94	<0.001
	Exposure time	2.798	95.588	3.571	0.059
	Error	57	73.722		
Control	Exposure time	2.706	28.596	0.865	0.456
	Light:dark	1.000	898.298	70.995	<0.001
	Time*light:dark	2.263	120.144	3.609	0.031
	Error(time*L:D)	43.005	33.287		
50% effluent	Exposure time	2.442	145.471	7.240	0.001
	Light:dark	1.000	1679.751	100.529	<0.001
	Time*light:dark	2.341	266.837	18.997	<0.001
	Error(time*L:D)	44.476	14.046		
100% effluent	Exposure time	2.205	11.498	0.294	0.767
	Light:dark	1.000	1423.257	59.654	<0.001
	Time*light:dark	2.331	241.103	22.686	<0.001
	Error(time*L:D)	44.294	10.628		

Table 3. Analysis of variance of *G. pulex* velocity (n = 60) over 60s light:dark photoperiods and measured at 0h, 2h, 24h and 7d of exposure to 0, 50% and 100% Fullerton WwTW effluent.

Dependent variable: velocity (mm s^{-1})

	Source	*df*	Mean square	*F*	*P*
Overall	Treatment	2.000	118.33	2.612	0.121
	Light:dark	1.000	9099.23	63.565	<0.001
	Exposure time	1.164	343039.2	768.64	<0.001
	Error	57	242.25		
Control	Exposure time	2.238	40.458	0.842	0.449
	Light:dark	1.000	748.936	16.816	0.001
	Time*light;dark	2.238	141.235	4.455	0.015
	Error(time*L:D)	42.522	31.704		
50% effluent	Exposure time	2.542	83.466	2.902	0.052
	Light:dark	1.000	1285.438	71.676	<0.001
	Time*light;dark	2.031	125.216	4.382	0.019
	Error(time*L:D)	38.595	28.573		
100% effluent	Exposure time	2.505	201.956	5.338	0.005
	Light:dark	1.000	1207.039	60.519	<0.001
	Time*light;dark	2.448	131.952	6.757	0.001
	Error(time*L:D)	46.513	19.527		

Manual behavioural Trials

There was no significant effects of exposure to Chickenhall effluents on activity observed during the manual observations (GzLN $p = 0.777$; Table 1, Figure 1a). During the 3 weeks of exposure the overall activity levels significantly decreased across all exposures ($p < 0.001$), however this reduction in activity was greater in the exposed groups vs the control but did not meet the significant threshold ($p = 0.077$). Conversely, at Fullerton there was a significant impact of effluents by reducing the activity measurements ($p = 0.03$; Figure 2b). Bonferroni corrected pairwise analysis indicated a decrease in activity between the controls and the exposed groups which was significant for the 50% exposure ($p = 0.043$) but not the 100% ($p = 0.133$). Similarly with the Chickenhall experiment, there was an overall reduction in activity over time ($p < 0.001$) however this occurred only in the exposed rather than the control groups resulting in an interaction between concentrations and time ($p < 0.001$). In both experiments the activity at time zero was significantly higher than after both 2 and 3 weeks (Figure 2)

No significant differences in phototaxis were observed between controls and amphipod exposure to effluent from both Chickenhall and Fullerton ($p > 0.05$; Table 1, Figure 3). However, there was a significant effect of time in both experiments with animals becoming less attracted to the light during the three-week exposure ($p < 0.001$). No significant interactions were observed between treatment and time during both experiments ($p > 0.05$).

There was no significant difference in preference scores between effluent concentrations for both Chickenhall and Fullerton effluents ($p > 0.05$; Table 1, Figure 4). With both experiments, as with the other measurements, time had a significant effect with all animals showing lower scores across the 3 weeks of exposure ($p < 0.001$). There was no interaction between time and exposure to the effluents ($p < 0.05$)

Location	**Measurement**	**Fixed Factor**	**Wald Chi-Square**	**df**	**Significance**
Chickenhall	Activity	Concentration	0.505	2	0.777
		Time	59.495	3	**<0.001**
		Concentration*Time	11.396	6	0.077
Fullerton	Activity	Concentration	6.991	2	**0.030**
		Time	24.624	3	**<0.001**
		Concentration*Time	27.145	6	**<0.001**
Chickenhall	Phototaxis	Concentration	3.849	2	0.146
		Time	123.142	3	**<0.001**
		Concentration*Time	11.645	6	0.070
Fullerton	Phototaxis	Concentration	5.687	2	0.058
		Time	54.729	3	**<0.001**
		Concentration*Time	1.707	6	0.945
Chickenhall	Preference	Concentration	0.257	2	0.879
		Time	46.113	3	**<0.001**
		Concentration*Time	12.359	6	0.054
Fullerton	Preference	Concentration	0.545	2	0.761
		Time	39.354	3	**0.000**
		Concentration*Time	4.680	6	0.586

Table 1: Results of Generalized Liner Model with Actvity, Phototaxis and Preference measurements as dependant variables and Time (0-3 weeks), Concentrations (Control, 50 and 100% effluent) as

fixed factors. Activity and Phototaxis were moth modelled using a poison distribution and Preference Tests using a normal distribution.

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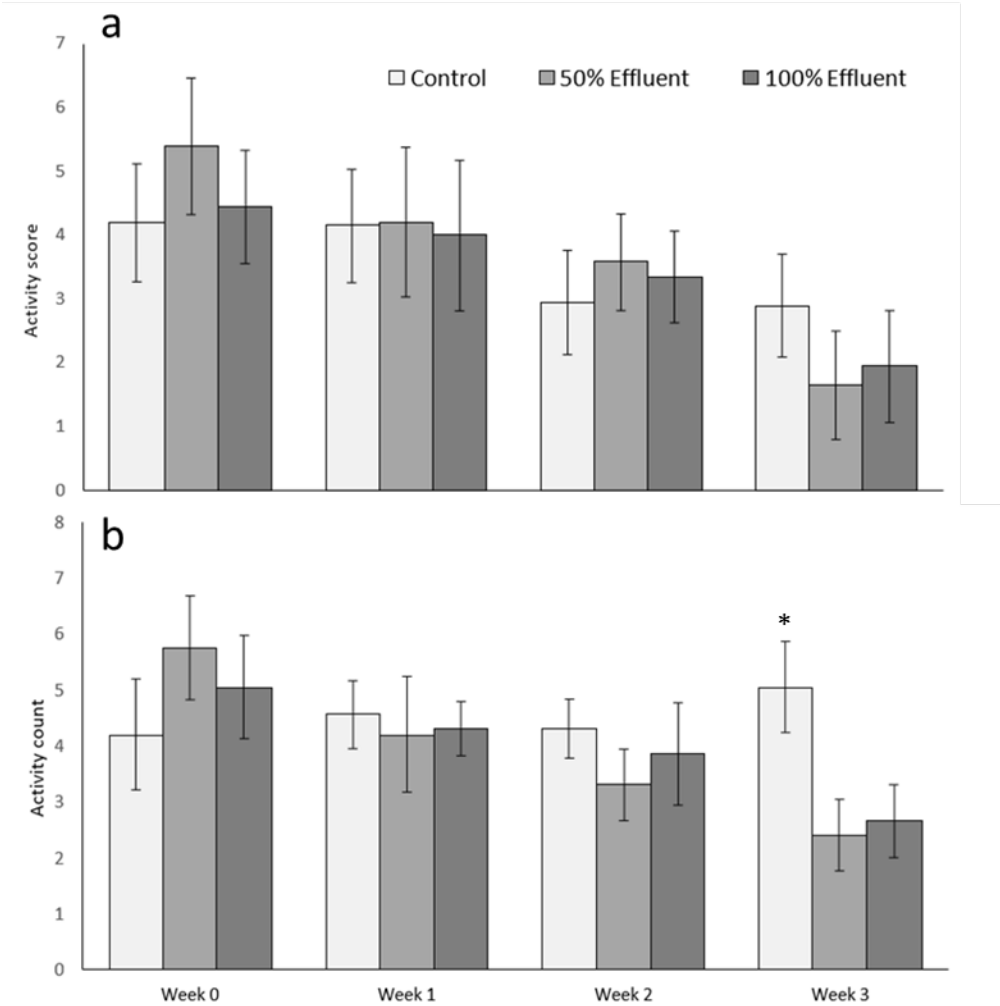

Figure 2. Mean activity count of Gammarus (± 2SE) after exposure to Chickenhall (a) and Fullerton (b) WwTW effluent (n = 20 per treatment, * $p < 0.05$ Bonferroni corrected pairwise analysis)

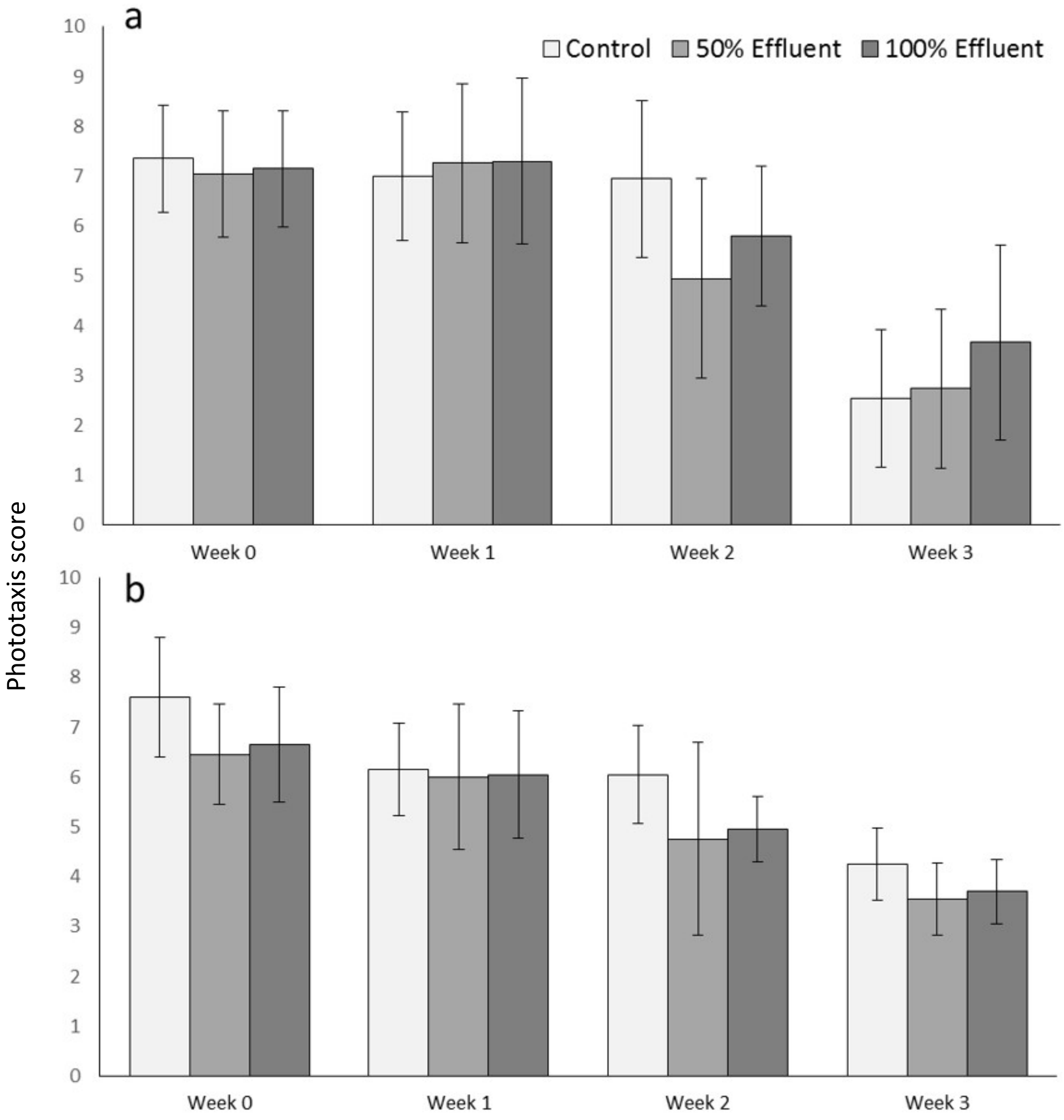

Figure 3. Mean phototaxis scores (± 2SE) of *Gammarus pulex* after exposure 0-3 weeks to Chickenhall (a) and Fullerton (b) WwTW effluent (n = 20 per treatment).

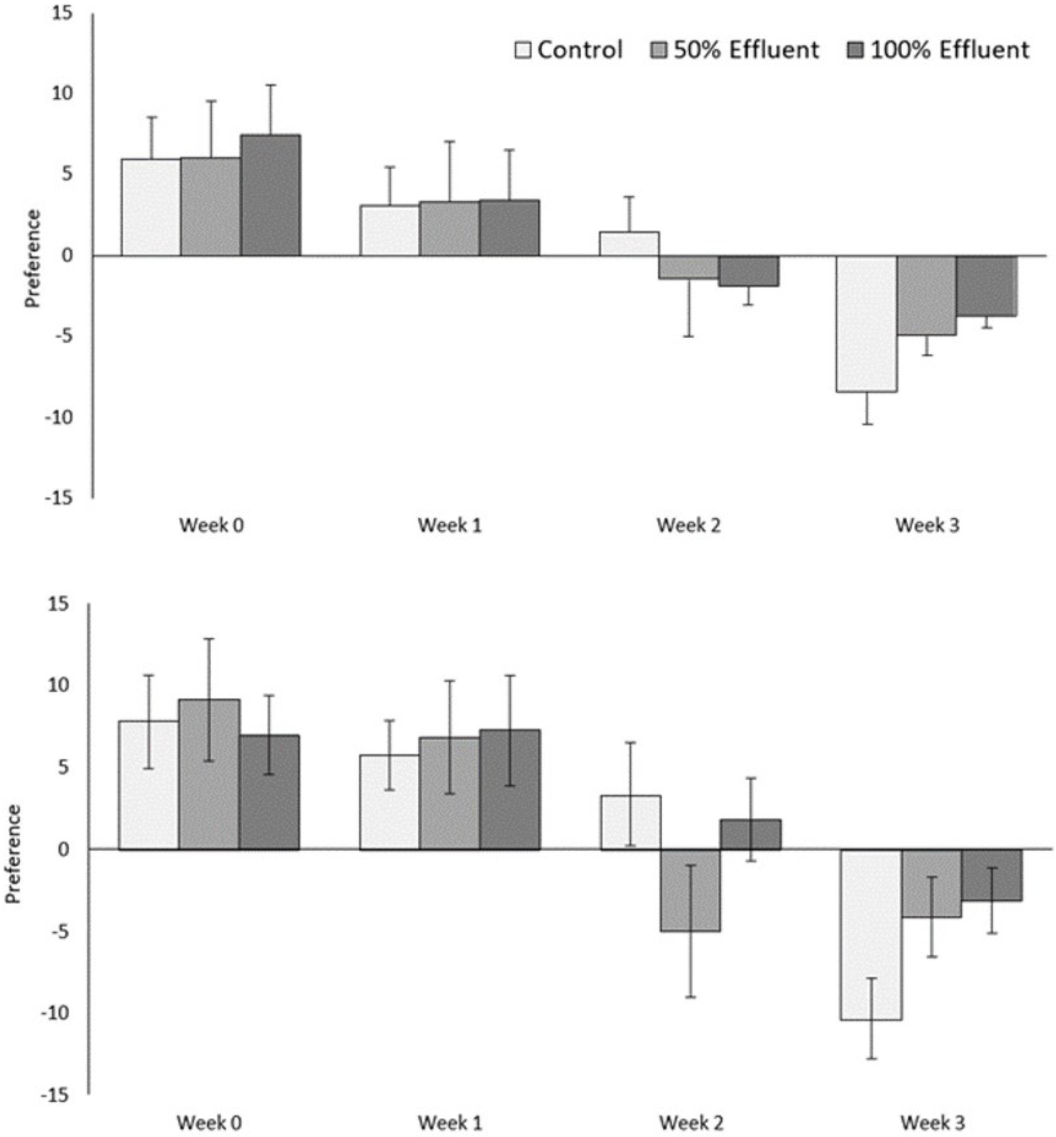

Figure 4 Mean preference index (± 2SE) of Gammarus pulex after exposure to Chickenhall (a) and Fullerton (b) WwTW effluent over 3 weeks (n = 20 per treatment).

Pairing Trials

The time taken for males exposed to effluent for 21 days to pair with unexposed females was statistically significantly different between controls and exposure to Fullerton (Welch's F (2, 33.638) = 9.216, $p = 0.001$) and Chickenhall (F (2, 33.709) = 4.345, $p = 0.021$) effluent. In both cases, the effluent-exposed males were significantly ($p < 0.05$) quicker at pairing with females than the control males; there was no significant difference between 50% and 100% effluent (Figure 5a,b).

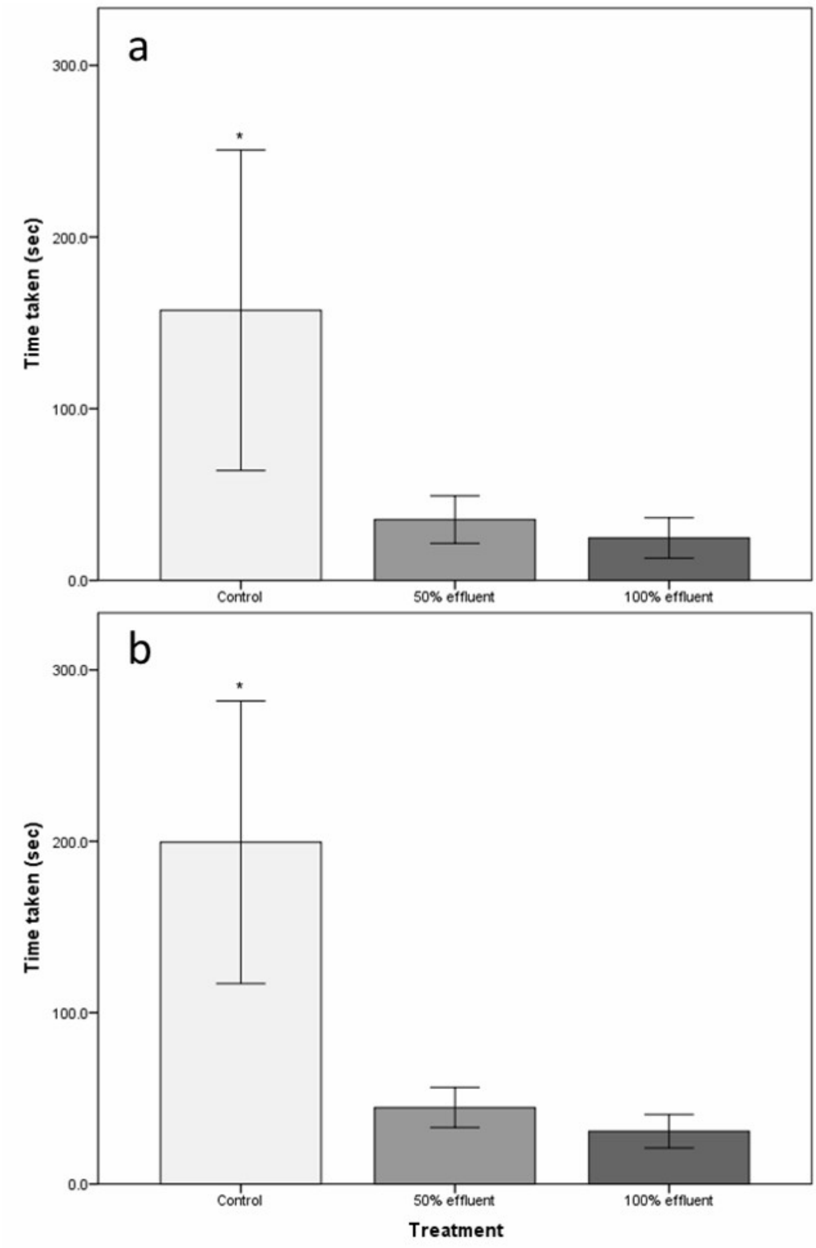

Figure 5: Mean time (± 2SE) taken for males exposed to Chickhall (a) and Fullerton (b) WwTW effluent (for 21 days) to pair with (unexposed) females (* indicates significant differences following Welch's ANOVA and Games-Howell post hoc test, p <0.05) (n = 60).

Feeding

There was no statistically significant difference in feeding rate between the effluent exposed and control groups for Fullerton ($F_{(2, 57)} = 0.212$ $p = 0.810$) or Chickenhall ($F_{(2, 57)} = 0.019$ $p = 0.981$) effluent. Similarly, final body weight was not significantly different between treatments ($p > 0.05$) (data not shown).

Discussion

The behavioural responses of *G. pulex* to exposure of whole effluents from WwTWs have not been previously documented. This research evaluates, for the first time, the effects of whole WwTW effluent on amphipod behaviour. Mortality was low throughout these experiments (<10%) therefore one might assume the amphipods were in good health. Interestingly, moulting frequency was less in both the effluent exposures compared with the controls although this result was not significant different. Whether this might indicate a suppression of moulting would be an interesting avenue to explore with a greater sample size.

Typically, with the automated behavioural analyses the gammarid amphipods responded well to the lights coming on within the Daniovision system although no differences were observed between the effluent exposures and the control. A motile response of amphipods in response to effluent exposure has been reported in other studies, though usually in terms of escaping from, or avoiding, the contamination (Lenihan et al., 1995; De Lange et al., 2006b; Tidona et al., 2009). A compulsion to escape might manifest itself as a general increase in activity, at least in the short term: both Gerhardt (1995) and Nørum et al. (2011) describe an otherwise atypical initial increase in amphipod activity after exposure to toxins. Peeters et al. (2009) found this atypical behaviour to continue for up to 7h post exposure and concluded that at least 2h was required for acclimation. On the other hand, acclimatised animals may lack the sensitivity of their naïve counterparts (McGee et al., 1998; Timofeyev et al., 2006). When viewed in the context of the subsequent responses, the initial burst of velocity is not maintained or repeated, even only two hours later, suggesting that if this was avoidance behaviour it dissipates rapidly and there is no long-term physiological change.

During the automated experiments conducted after 7 days, there was an increase in activity during the 'lights-on' periods recorded within the Daniovision. Interestingly, our manual observations of behaviour recorded an overall decrease in activity over the duration of the 3-week experiments. Within their habitat, many variables have been found to affect amphipod behaviour such as sediment, water depth and flow, and refuge abundance (Dahl & Greenberg, 1996; Ford & Paterson, 2001; Vadher et al., 2015; Vander Vorste et al., 2016; Maazouzi et al., 2017). These experiments were conducted with different animals and at different times of the year, therefore, a change in all of these factors may well have had behavioural implications which may have interacted with the effluent effects.

In this study a degree of variation was seen in the initial velocity of Gammarids in different effluents. Activity of animals in the Chickenhall trials at the 0h measurement was approximately twice that of their Fullerton counterparts. The reaction of the control animals precludes a contaminant effect, thus other variables must be considered. One of the primary merits of evaluating behavioural change in ecotoxicology is its sensitivity to environmental variation (Hellou, 2011). However, that sensitivity may be manipulated by other factors whose influence needs to be controlled to reduce error. Therefore, in this study, other variables that are known to illicit a behavioural response were kept constant in all treatments: namely maturity (Maltby, 1995), sex (McCahon & Pascoe, 1988; Maltby & Naylor, 1990; Peeters et al., 2009; Sornom et al., 2010; Barros et al., 2017) and parasitism (Tain et al., 2006; Franceschi et al., 2010). *Gammarus* behaviour was shown to be impacted by low dissolved oxygen Costa (1967), whilst other studies have found temperature significantly affects the response of amphipods to contaminants (Neuparth et al., 2002; Vellinger et al., 2012a). In the current investigation, oxygen levels did not fall below 90% saturation in any treatment and pH and temperature of the maintenance conditions were consistent and optimal (Wijnhoven et al., 2003).

For the longer-term (3 week) manually observed assays, activity was seen to decrease with time. Other studies have similarly reported a decline in *G. pulex* activity with time (Peeters et al., 2009a; De Castro-Català et al., 2017; Vanucci-Silva et al 2019), though the cause or mechanism remains unclear but suggested have included a habituation to experimental conditions. Interestingly, this reduction in activity was greater in the exposed groups vs the controls however this was only significant in the Fullerton effluents. A decline in activity is a common response to toxicity, due to direct sensory or neurological impediment, or metabolic disruption with a concomitant drain on the energy budget (Scott & Sloman, 2004). Amphipod behaviour has been observed to decline in response to contaminated sediments (Morris & Keough,2001; De Lange et al., 2006b), acid mine effluent (de Bisthoven et al., 2006), lead and copper (Gerhardt, 1995), cadmium (Wallace & Estephan, 2004), xenoestrogens (Gerhardt, 2007) and pesticides (Nørum et al., 2010; Nørum et al., 2011; Berghahn et al., 2012). If this is a response to contaminants in the effluent the delay could be due to what has been characterised as 'loading stress' (Wilson et al., 1994; Gerhardt, 1995), indicating an exhaustion of the compensatory responses engaged to maintain homeostasis. Therefore, very low levels of contaminants typically take time to have an effect (Roex et al., 2000; Liess & Ohe, 2005). Given this result was not observed during the automated measurements and with other effluent it should be considered with caution.

In the only study specifically addressing the intraspecific variation in amphipod behaviour, Peeters et al. (2009) found within males a distinction could be made between very active and less active specimens, and though 70% of tested individuals behaved similarly, 20% showed no consistency and 10% routinely showed behaviour opposite to their cohort. Leading the authors to conclude that inter-individual variation in behaviour must be taken into account when using behaviour as an endpoint in ecotoxicological bioassays. Certainly, other trials have observed the issue (Gerhardt, 1995; de

Bisthoven et al., 2006; Guler & Ford, 2010). Possibly, such variation might be attenuated by trimming, winsorising or otherwise transforming the data, but this would be done to remove a characteristic of the data, and therefore render them less representative (Field, 2013). Increasing *N* could reduce statistical variance if it were still possible to test all specimens at the same time points.

An invariable observation in all pertinent studies is that light is an overwhelmingly strong influence on amphipod behaviour (Holmes, 1901; Peeters et al., 2009a; Guler & Ford, 2010; Bossus et al., 2014; Kohler et al 2018a,b) which is supported by this investigation. *Gammarus* were up to 23.9% more active under light conditions than dark in equivalent treatments, which might be interpreted as predator avoidance (Boyd et al., 2002; Guler & Ford, 2010) and, as such, a vital response, any impact on which could produce wider population effects. No single effect of effluent could be seen in the current studies over the short or long term. The preference measurement shows that time has a significant effect in increasing the negative phototaxis of all specimens, which could have been an artefact of the 24h dark conditions the amphipods were maintained in. However, similar patterns can be seen in the observations of de Bisthoven et al. (2006) who found that under control conditions, *G. pulex* became more active at night after 7 days of 12:12 light:dark conditions, though the cause or mechanism behind this pattern was not established. Guler and Ford (2010) also found a decline in the phototaxis score in their controls and treatments after 3 weeks exposure in 12:12 L:D photoperiod. Indeed, Michels et al. (2000) found the same pattern in *D. pulex* after only 6 hours exposure, though they attributed this to hunger.

The precopulatory disruption assay was established by Poulton and Pascoe (1990) who proposed that the time taken for precopula amphipods to separate is indirectly correlated with the concentration of pollutant to which they are exposed. As a measure of toxicity variations on this test have been repeatedly used in measuring the effect of heavy metals (Poulton & Pascoe, 1990; McCahon & Poulton, 1991), herbicides (Pascoe et al., 1994), and pesticides (Malbouisson et al., 1995; Cold & Forbes, 2004; Negro et al., 2013; Pedersen et al., 2013) and they all concur that re-pairing is less likely after exposure to toxins. The results of the current study show interestingly the opposite, male *Gammarus* exposed to either effluents took significantly less time to pair than controls. No changes in feeding or weight gain were observed during these studies, which might imply more nourishment for testicular development. All specimens including controls were kept isolated during the 3-week exposure suggesting that sperm within the seminal vesicles were not being used. The stimulation for pairing is unknown and the cause of impeded re-pairing is not clear, but is generally assumed to be due to some degree of toxic stress (Poulton & Pascoe, 1990; Malbouisson et al., 1995), endocrine disruption (Wisniewska & Szaniawska,2015) or a secondary effect from a decline in feeding and energy intake (Cold & Forbes, 2004; Bundschuh & Schulz, 2011) broadly due to toxicity. However, where amphipods have been exposed to less noxious chemicals, the precopulatory test is less categorical. For instance, in *Gammarus tigrinus* significant separation effects only at near lethal ethinylestradiol concentrations (Wisniewska and Szaniawska, 2015). Watts et al. (2001) observed no effect of environmentally relevant concentrations of the xenoestrogens ethinylestradiol (EE) and bisphenol A on pair re-forming of *G. pulex,* so the reported effect of other environmental contaminants is particularly interesting. It is also possible that the varying inter-moult stages of the males in this experiment could have been a contributing factor to the outcome.

G. pulex placed downstream of discharge points (for 24h) separated more quickly, though the results were not significant (Pascoe et al., 1994). Other studies have shown a deleterious impact on sperm integrity in caged amphipods downstream of WwTW (Lacaze et al., 2011) or when directly exposed to effluents (Wigh et al., 2017). Bundschuh and Schulz (2011a) found a reduction in the proportion of precopula pairs in effluent exposed *Gammarus fossarum*, but only after 8 weeks continual exposure to effluents. Trials on fish have also shown a lack of effect of WwTW effluent on reproductive behaviour (Schoenfuss et al., 2002; Sebire et al., 2011). Why the effluents appear to have a stimulatory effect is less clear although one might speculate an effect occurring directly or indirectly on the endocrine system.

Typically, contaminants are reported to have an inhibitory effect on reproduction (Lagadic et al., 1994; Baird et al., 2007). Field studies report a stimulatory (Schneider et al., 2015) as well as inhibitory (Ladewig et al., 2006) effect on the proportion of ovigarous females after exposure to WwTW effluent, though this is typically attributed to alterations of the female reproductive physiology. In a similar vein, environmentally relevant concentrations of PPCPs such as fluoxetine, fluvoxamine, and paroxetine have been found by numerous studies to stimulate reproduction in bivalves (Fong, 1998; Fong et al., 1998; Fong et al., 2003; Fong & Molnar, 2008; Lazzara et al., 2012), including reproductive behaviour (Bringolf et al., 2010), and increase fecundity in *Daphnia* (Flaherty & Dodson, 2005). There are some studies showing an increase in reproductive behaviour after exposure to sewage effluents (Schröder & Peters, 1988b, 1988a), though their experimental design and conclusions have been criticised (Jones & Reynolds, 1997). Potentially, then, is that there is another, unknown stimulus is having an effect on the *G. pulex* in this trial. An increased male body size is known to encourage pairing (Bollache & Cézilly,2004; Wellborn & Bartholf, 2005), but there was no significant difference found in the body weight of the treatments in this trial. Alternatively, some studies have reported that invertebrates in contaminated sites show earlier maturation and increased reproductive effort

(Donker et al., 1993; Spurgeon & Hopkin, 1999; Li et al., 2005). The size of maturity was not measured, but could form future investigations.

In summary, the lack of acute toxicity or behavioural changes in *G. pulex* exposed to 100% final effluent was surprising and an indication of the improvements made to WwTW effluent, although not without precedent. Other studies have found no significant negative impact of WwTW effluent on algae, invertebrates or fish (Dauer & Conner, 1980; Hoeger et al., 2004; Santos et al., 2008; Lundström, et al., 2010; Bundschuh & Schulz, 2011). Nevertheless, impacts of exposure to WwTW effluents were observed in this study and it would be interesting to observe longer term exposures given the effects appeared to manifest themselves mainly after 3 weeks exposure. Namely, a very prominent effect on their reproductive behaviour replicated across effluents recovered from two WwTW and reductions manual recorded activity. The causal mechanisms and implications of these behavioural changes will make an interesting new avenue for research.

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