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4 **The impact of natural and anthropogenic Dissolved Organic Carbon (DOC), and pH on**

5 **the toxicity of triclosan to the crustacean *Gammarus pulex* (L.).**

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16 **Abstract**

17 Regulatory ecotoxicology testing rarely accounts for the influence of natural water chemistry

18 on the bioavailability and toxicity of a chemical. Therefore, this study identifies whether key

19 omissions in relation to Dissolved Organic Carbon (DOC) and pH have an impact on

20 measured effect concentrations (EC). Laboratory ecotoxicology tests were undertaken for

21 the widely used antimicrobial compound triclosan, using adult *Gammarus pulex* (L.), a wild-

22 type amphipod using synthetic fresh water, humic acid solutions and wastewater treatment

23 works effluent. The toxicity of triclosan was tested at two different pHs of 7.3 and 8.4, with

24 and without the addition of DOC and 24 and 48 hour EC values with calculated 95%

25 confidence intervals calculated. Toxicity tests undertaken at a pH above triclosan's pKa and

26 in the presents of humic acid and effluent, containing 11 and 16 mg L^{-1} mean DOC

27 concentrations respectively, resulted in significantly decreased triclosan toxicity. This was

28 most likely a result of varying triclosan speciation and complexation due to triclosan's pKa

29 and high hydrophobicity controlling its bioavailability. The mean 48 hour EC50 values varied

30 between 0.75 ±0.45 and 1.93 ±0.12 mg L^{-1} depending on conditions. These results suggest

31 that standard ecotoxicology tests can cause inaccurate estimations of triclosan's

32 bioavailability and subsequent toxicity in natural aquatic environments. These results

33 highlight the need for further consideration regarding the role that water chemistry has on the

34 toxicity of organic contaminants and how ambient environmental conditions are incorporated

35 into the standard setting and consenting processes in the future.

36 **Keywords**: Triclosan; effluent; pH; toxicity; bioavailability; dissolved organic carbon

37

1.   INTRODUCTION

Prior to the 21st century, the impact of chemical contamination largely focused on conventional priority chemicals (Daughton and Ternes, 1999). A group of chemicals of increasing concern, but which have received comparatively little attention are 'emerging contaminants', namely; Pharmaceuticals and Personal Care Products (PPCP) (Peck, 2006). These chemicals are released into the environment, primarily through wastewater either via incomplete removal from Wastewater Treatment Works (WwTW), or through storm water discharges. Despite a lack of detailed knowledge regarding their safety and fate, PPCP have increasingly been documented as ubiquitous contaminants and a possible threat to aquatic environments (Liu and Wong, 2013). This study focuses on the antimicrobial agent triclosan, which is predominantly found in personal care products. Triclosan is an environmentally relevant chemical which has been identified as an emerging contaminant in recent years (Gardner *et al.*, 2012). It is active against both Gram-positive and Gram-negative bacteria, (Suller and Russell, 2000) as well as some fungi and protozoa (Yazdankhah *et al.*, 2006). Triclosan has been used in consumer products since 1968 with increasing popularity. It has been reported that up to 450 tonnes per year are used within the EU (Scientific Committee on Consumer Safety, 2010), with approximately 85% used in more than 140 personal care products in concentrations up to 0.3% by wet weight (Sutton *et al.*, 2008; European Commission, 2009). Almost all (96%) of triclosan containing products are disposed of through the domestic drainage system (Reiss *et al.*, 2002), therefore, entering the aquatic environment via WwTW final effluents or emergency overflows.

Laboratory toxicity tests confirm that triclosan is toxic to a range of aquatic organisms and that it may cause adverse environmental effects (Jones *et al.*, 2002).   As such, it now comes under Annex VIII of the Water Framework Directive (WFD) as a Specific Pollutant within the UK (Aldous *et al*, 2012) with an annual average Environmental Quality Standard (EQS) of 0.10 µg L^{-1} (Aldous *et al*, 2012). It has been reported that in the UK, greater than 50% of sampled WwTW effluents (162 in total) exceed this EQS (Gardner *et al.*, 2012). Triclosan has been detected in river waters across the globe at concentrations ranging from <0.1 to 1023 ng L^{-1} (Bendz *et al.*, 2005; Peng *et al.*, 2008).

These EQS are based on available triclosan toxicity data, derived using standardised methodologies which rarely account for natural water chemistry and its effect on triclosan. Therefore, results may not represent the true natural bioavailable triclosan exposure at a given concentration. Consequently, inappropriate EQS may be produced that over or under protect a waterbody. This issue is widely recognised for metals in the aquatic environment and resulted in the introduction of the Biotic Ligand Model (BLM) (Environment Agency,

73   2009). It could be suggested that a similar approach should be implemented for organics to
74   provide the most relevant standards which reflect sound science and still protect aquatic
75   ecology.

76   Increased organic matter content in waterbodies has been stated to reduce triclosan's
77   bioavailability due to complexes caused by its high hydrophobicity being too large or too
78   polar to cross cell membranes (Chalew and Halden, 2009). Therefore, natural and
79   anthropogenic DOC in river bodies may have a mitigating effect on its toxicity.

80   There is limited experimental evidence concerning the toxicity of triclosan in the presence of
81   DOC. Behera *et al.* (2010) however, established that an increase in humic acid (HA; a major
82   constituent of natural DOC) concentration in the aqueous phase resulted in increased HA-
83   complexed triclosan, thus causing a decrease in free triclosan. Humic acids have also been
84   reported to decrease toxicity when undertaking tests with other organic xenobiotic
85   compounds to *Brachydanio rerio*, *Daphnia magna* and *Ampelisca abdita* (Lorenz *et al.*,
86   1996). Other studies have reported decreased organism bioaccumulation and
87   bioconcentration of organic chemicals in the presence of DOC (Haitzer *et al.*, 1999a).

88   The pH of water has also been shown to affect the toxicity of triclosan due to its pKa.
89   Triclosan has a measured pKa of 8.0, which sits within the range of pH observed in natural
90   waters (Figure 1). With increasing acidity triclosan becomes increasingly protonated and
91   loses the negative charge associated with the molecule, which in turn increases its
92   bioavailability and hence toxicity (Orvos *et al.*, 2002). This variable toxicity is likely to be
93   because lipid membranes are generally permeable to un-ionised species, whereas, relatively
94   impermeable to ionised species (Lipnick, 1995). Regulatory ecotoxicity test methods (e.g.
95   OECD and ISO test guidelines) are intended to be reliable and repeatable for the
96   international acceptance of data, which is achieved through a high degree of control over the
97   abiotic and biotic factors; often by simplifying experimental conditions (Boudou and Ribeyre,
98   1997). For example, most toxicity tests are undertaken with synthetic water, avoiding many
99   components of natural water bodies and their effects on bioavailability (Boudou and Ribeyre,
100 1997). These tests may therefore not reflect key exposure variables in the natural
101 environment. Despite triclosan's wide use, there have been limited published studies
102 focusing on its toxicity and fate in non-standard laboratory conditions, and many studies do
103 not report pH when presenting toxicity results (Orvos *et al.*, 2002).

104 Consequently, ecotoxicology tests using *Gammarus pulex* (Crustacea: Amphipoda) in the
105 presence of natural (humic acid) and anthropogenic (sewage effluent) DOC and varied pH
106 were used to determine their effect on triclosan toxicity. *G. pulex* was selected as the test
107 organism because it is abundant in rivers and is easy to collect, handle and maintain

108 (Vellinger *et al.*, 2012). It has been used for sublethal testing, such as growth (Maltby *et al.*,
109 2002), reproduction (Welton and Clarke, 1980) activity (Gerhardt *et al.*, 1994), and acute
110 endpoints (Güven *et al.*, 1999). Furthermore, *G. pulex* is known to be sensitive to a range of
111 stresses (Felten *et al.*, 2008). Triclosan was specifically chosen as, like many other personal
112 care products which are of growing concern to regulators, it is a polar compound with ionic
113 characteristics, with a pKa in the range where environmentally relevant pH values significant
114 affect its behaviour and bioavailability.
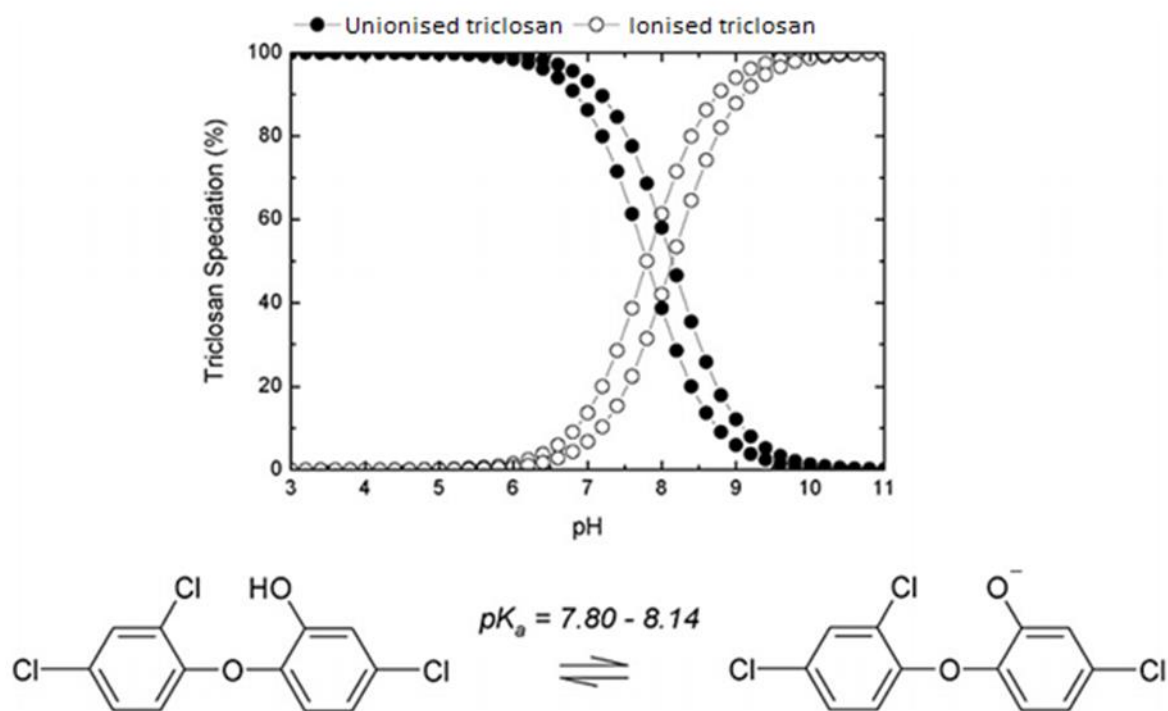
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117 **Figure 1. Speciation of triclosan as a function of the solution pH. Calculation based on**
118 **pKa values of 7.80 and 8.14 (Nghiem and Coleman, 2008 (Edited)).**

119

120 Results from this study provide a valuable insight towards understanding of the role that
121 water chemistry can have on the toxicity of organic chemicals. The data generated through
122 this study are especially helpful for determining if approaches to setting environmental
123 standards should account for potential varying bioavailability. This increased knowledge
124 benefits those regulating and complying with aquatic environment standards, by providing
125 the most relevant standards that neither cause wasteful mitigation measures nor
126 expenditure, while ensuring the environment is suitably protected.

2.   MATERIALS AND METHODS

2.1   DOC sources

Sewage effluent was collected on 27th June 2015 from Callington WwTW in Cornwall UK, grid reference SX 34044 68905, which utilises primary and final settlement tanks and biological (activated sludge) treatment and serves a population of approximately 6000 (Hammond, 2007). The effluent was taken to the laboratory within 24 hours and stored at 10°C for a maximum of five days. Three additional 50 ml samples were collected and refrigerated until DOC analysis. The 50 ml sampling bottles were glass and the effluent was filtered on return to the laboratory using a leached plastic filtration kit, a hand vacuum pump (Nalgene, Mityvac) and 47 mm 0.7µm microfiber GF/F filters (Whatman). The effluent DOC was measured as 16 mg-C L^{-1} using a high temperature catalytic oxidation method (Shimadzu Ltd). Tests using humic acid utilised Technical grade humic acid (Sigma-Aldrich) at 20 mg L^{-1}. This was estimated to be equivalent to 11 mg L^{-1} C based on literature, particularly a comprehensive review by Tan (2014), which estimated concentration of carbon within HA at approximately 55%. **2.2 *G. pulex* collection and laboratory acclimatisation**

G. pulex were collected from a small stream, part of the Lower River Tavy, situated on the east of Dartmoor National Park, Devon UK (grid reference SX 51455 74039). The site was selected due to its lack of WwTW discharges upstream and viable *G. pulex* population. *G. pulex* were collected prior to each test using kick sampling with a standard 1 mm mesh Freshwater Biological Association net downstream to catch disturbed organisms. Once in the laboratory, *G. pulex* were placed into one of two prepared 15L plastic holding tanks containing synthetic freshwater (SFW) (hardness: 77 mg L^{-1} ±25, DO: 80% ±5.5, pH: 7.8 ±0.3), made to ASTM (1980) specification (Table A1 in ESI). The prewashed (10% HCl with high purity water rinse) aerated tanks were housed in a 15°C temperature controlled room at a 12h photoperiod cycle, with decaying leaves, predominantly *Fagus sylvatica*, collected from the same habitat used as a food source and provided when required. Supplementary organic carrot was provided to ensure adequate food availability. All *G. pulex* were acclimatised for a minimum of 4 days prior to testing. The physicochemical parameters of the water were monitored daily using an Oakton Acorn series pH monitor and a YSI Pro2030 Meter (dissolved oxygen, conductivity and temperature). Water samples were collected every seven days for determining hardness using ICP-OES and nutrient levels (ammonia, nitrite and nitrate) using an API freshwater testing kit. Every three days, 20% water changes (by volume) were made for the duration of the study.

2.3 Laboratory Experiments

Prior to testing, all equipment was cleaned in a 10% HCl bath for 24 hours before being washed with Milli-Q water. All glassware (field and laboratory work) was soaked in 2% Decon for 24 hours and then soaked in a 10% HCl bath for 24 hours, before being washed with Milli-Q water. All glassware and filters for DOC determination were combusted at 450°C for six hours to remove remaining organic residues. All tests were undertaken in 1L griffin glass beakers (Fisherbrand). All chemicals used in the laboratory tests are presented in Table A2 of the ESI.

2.3.1 *G. pulex* toxicity testing conditions

The organisms were not fed during the 48 hour toxicity tests and test solutions were not changed. Ten adult *G. pulex* with a body length between 8-12 mm were consistently used at each concentration, excluding juveniles based on classifications used by Naylor *et al.*, (1990). This length was taken from the base of the first antennae to the base of the telson. All organisms appeared healthy, behaved normally and had extremely low mortality in holding tanks before use. A formal test protocol has not been developed for acute *G. pulex* testing and this study had a unique set of variables, therefore, adaptations of existing toxicological methods were followed (OECD, 2004; OECD, 2012).

2.3.2 Toxicity range finding test

A 48 hour static toxicity test was undertaken to identify a suitable concentration range for the main study. A 1000 mg L^{-1} triclosan stock standard was made in methanol and used to make concentrations based on OECD (2012) test guidelines, made up with pre-aerated SFW. These nominal concentrations were: solvent control (3.2 ml L^{-1} methanol), 0.01, 0.032, 0.056, 0.1, 0.32, 0.56, 0.8, 1.0 and 3.2 mg L^{-1}. These were then mixed using a pre-cleaned glass stirring rod. The stock standard was stored in a refrigerator and used within two days.

Ten adult *G. pulex* were randomly taken from the holding tank and placed in each beaker, which were covered with clear PETE plastic to reduce contamination and evaporation loss. Observations for immobilisation (failure to respond to mechanical stimulation) were made after 24 and 48 hours. Dead organisms were removed immediately. Initial water samples were taken to confirm nominal concentrations of triclosan. Dissolved oxygen, pH, salinity, and conductivity were measured at 0, 24 and 48 hours using methods discussed. Test validation was based on a control mortally rate ≤10%, with tests rejected if this was exceeded.

2.3.3 Acute static 48 hour toxicity test

The acute static 48 hour toxicity tests followed the range finding study methodology, but for the determined suitable concentration range of: solvent control, 0.032, 0.1, 0.32, 0.56, 0.8, 1, 1.8, 2.6 and 3.2 mg L^{-1}. This test was repeated four times at the 'natural' pH of the SFW (8.4 ±0.09), and four times at a neutral pH (7.3 ±0.18), referred to as Test series #1 and #2 respectively. Neutral pH was maintained using 5mM solutions of 3-(N-morpholino)propanesulfonic acid (MOPS), and the pH was adjusted using HCl. MOPS was selected to use as unlike other pH buffers tested, it displayed no effects on *G. pulex* at the required concentration and has been previously tested for aquatic invertebrate studies (De Schamphelaere *et al.*, 2004). Water samples were collected at the start and end of the test then stored in a freezer until analysis to confirm nominal concentrations of triclosan.

2.3.4 Acute static 48 hour toxicity test with the addition of humic acid

These tests followed the acute 48 hour toxicity test methodology (section 2.3.3), however, 20 mg L^{-1} HA (11 mg L^{-1} as C) was added to each of the concentrations. A 1000 mg L^{-1} HA stock standard was prepared using HA and NaOH (350 mg L^{-1}), with Milli-Q water. Once the beakers containing HA and SFW had been spiked with triclosan, they were mixed and left for two hours to allow time for triclosan to be complexed before *G. pulex* were added. This test was repeated four times at both 'natural' and neutral pH, referred to as Test series #3 and #4 respectively.

2.3.5 Acute static 48 hour toxicity test in the presence of WwTW effluent

These tests followed the acute 48 hour toxicity test methodology (section 2.3.3), however, WwTW effluent replaced the SFW. Once the beakers containing the effluent had been spiked with triclosan, they were mixed and left for two hours to allow for triclosan complexation before *G. pulex* were added. This test was repeated four times at a mean pH of 8.3, referred to as Test series #5.

2.3.6 Zinc reference toxicant (positive control)

To ensure *G. pulex* were responding to toxicants as expected, an acute zinc positive control was undertaken following Environment Agency (2007) guidance. This is a test applying identical conditions but for a toxicant with a known response in order to demonstrate acceptable laboratory performance. This test followed the acute 48 hour toxicity test methodology, at pH 7.8 and concentrations: control, 1.0, 3.2, 10, 32 and 52 mg Zn L^{-1} diluted from a stock solution of zinc sulfate heptahydrate ($ZnSO_4.7H_2O$). Zinc measurements were

225 made on dissolved samples (filtered through 0.01% HCl acid washed 0.45 μm cellulose

226 acetate filters) at 0 and 48 hours, preserved with 2 ml L^{-1} of concentrated nitric acid.

2.4 Laboratory analysis

2.4.1 Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES)

229 The concentrations of zinc in the positive control test and calcium within the stock tanks

230 were determined using an ICP-OES instrument (Thermo Scientific, iCAP 7400) calibrated

231 between 0 and 50 mg L^{-1} and utilising procedural blanks and three replicate samples.

2.4.2 High-Performance Liquid Chromatography (HPLC)

233 Triclosan was determined using a Shimadzu LC20AD liquid chromatograph, Shimadzu

234 SIL20A HT Autosampler, Shimadzu SPD20A UV-vis spectrophotometer (230nm). A

235 Phenomenex C18 4.6X150 mm reversed-phase column with guard column was used for all

236 determinations. Isocratic elution using 70:30 acetonitrile (HPLC grade, 99.99%) and Milli-Q

237 water mobile phase was used at a 1ml min^{-1} flow rate. Calibration was achieved from the

238 peak areas of triplicate determinations standards made up in methanol (0, 0.5, 1, 3, 5, 10 mg

239 L^{-1}). Standards prepared in varying HA and effluent solutions confirmed a lack of matrix

240 interference.

2.5 Statistical analysis

242 Observed responses for *G. pulex* immobilisation at measured concentrations were used to

243 model predicted dose response curves and 95% confidence intervals using a Probit Analysis

244 (sigmoidal function) within SigmaPlot® 12.5. The process was undertaken for each test

245 repeat for both 24 and 48 hour results and the modelled data output was used to derive

246 mean test EC10, EC20 and EC50 values. Minitab® statistical software was used to

247 undertake a One-Way Analysis of Variance (ANOVA) with grouping Information using the

248 Tukey post hoc test to identify statistical significance between results. This statistical method

249 was used after confirming normal data distribution using the Anderson-Darling Normality test

250 for all EC values being tested for all testing conditions. Supporting statistical values (DF, SS,

251 MS, F and P) derived during the ANOVA analysis are presented in Table A3 within the ESI.

3 RESULTS

253 Daily measurements of environmental parameters, and weekly measurements of nutrient

254 levels and hardness (based on Ca content) of the holding tanks collected during the study

255 have been summarised in Table A4 of the ESI. The conditions in both holding tanks were
256 very similar and the values were within a suitable range for *G. pulex*.

3.1 Analytical data

258 All measured zinc mean concentrations were within 12% of nominal concentrations and
259 were used to calculate EC50 values (OECD, 2012). Control samples were lower than the
260 Limit of Detection (LOD), calculated by multiplying the SD of the lowest calibration standard,
261 1 mg L^{-1}, by three. Procedural blanks were also lower than the LOD, displaying extremely
262 low contamination. Check standards indicated no instrumental drift during analysis.

263 The LOD for triclosan analysis using the HPLC instrument was 0.006 mg L^{-1} calculated as
264 per the zinc LOD, with procedural blanks calculated as less than the LOD. Measured mean
265 triclosan concentrations compared well with nominal values and were used for all toxicity
266 calculations and showed no degradation over the course of the 48 hour tests.

3.2 Toxicity data

3.2.1 Zinc positive control results

269 The results for the 48 hour zinc positive control test were generated using SigmaPlot® to
270 produce modelled predicted dose response curves and 95% confidence intervals by means
271 of a sigmoidal function. Tables A5 and A6 in the ESI display percentage immobilisation and
272 effect concentrations respectively. An EC50(48h) of 3.23 mg Zn L^{-1} was calculated. Lower
273 concentrations were calculated for EC10 (1.08 mg Zn L^{-1}) and EC20 (1.94 mg Zn L^{-1}) values
274 at 48 hours. No *G. pulex* were immobilised in the control beaker.

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3.2.2 Triclosan toxicity test results

The observed mean toxicity data from four repeat tests, are shown in Table 1. Percentage mortality data for 24 and 48 hours are provided in Tables A7 and A8 and plotted for Test series #2 as an example in Figure A1 of the ESI respectively. These results showed mean *G. pulex* immobilisation of ≤1% in control exposures. More *G. pulex* were immobilised at 48 hours compared with 24 hours, as would be expected. EC values were calculated for each individual experiment repeat and the mean of these individual values is displayed.

Table 1. Mean EC values for triclosan to *G. pulex* (Dartmoor wild-type).

| Test series #1: SFE, pH 8.39				
Time Exposed	**Response**	**EC10 (mg L^{-1}) (±95% CI)**	**EC20 (mg L^{-1}) (±95% CI)**	**EC50 (mg L^{-1}) (±95% CI)**
24hours	Immobilisation	1.02 (0.9-1.15)	1.17 (1.07–1.27)	1.42 (1.33-1.50)
48hours	Immobilisation	0.74 (0.60-0.86)	0.91 (0.81–1.02)	1.22 (1.12–1.36)
Mean measured concentrations: 0.000, 0.102, 0.295, 0.598, 0.787, 0.990, 1.738, 2.57, 3.35mg TCS L^{-1} **Mean environmental parameters (SD)**: pH 8.39 (±0.08) (N= 120); Conductivity (mS) 0.0006 (±0.0007) (N= 120); Temperature (^{o}C) 14.9 (±0.28) (N= 120); DO (%) 81.9 (±3.95) (N= 120); Salinity (PPT) 0 (±0) (N= 120).				
Test series #2: SFW, pH 7.25				
24hours	Immobilisation	0.66 (0.61–0.70)	0.72 (0.68–0.74)	0.82 (0.80-0.85)
48hours	Immobilisation	0.53 (0.46–0.61)	0.62 (0.55–0.66)	0.75 (0.70–0.79)
Mean measured concentrations: 0.0, 0.033, 0.107, 0.35, 0.62, 0.81, 1.07, 2.16, 2.71, 3.38mg TCS L^{-1} **Mean environmental parameters (SD)**: pH 7.25 (±0.18) (N= 120); Conductivity (mS) 0.0008 (± 0.0001) (N= 120); Temperature (^{o}C) 14.8 (±0.2) (N= 120); DO (%) 77.1 (±4.6) (N= 120); Salinity (PPT) 0 (±0) (N= 120).				
Test series #3: SFW + 11 mg-C L^{-1} as Humic acid, pH 8.35				
24hours	Immobilisation	1.84 (1.81–1.89)	1.91 (1.88–1.92)	2.02 (2.01-2.03)
48hours	Immobilisation	1.16 (0.93–1.42)	1.36 (1.16–1.55)	1.71 (1.56–1.83)
Mean measured concentrations: 0.0, 0.033, 0.107, 0.34, 0.52, 0.75, 0.85, 1.7, 2.35, 3.17 mg TCS L^{-1} **Mean environmental parameters (SD)**: pH 8.35 (±0.09) (N= 120); Conductivity (0.0003mS) (±0.0005) (N= 120); Temperature (^{o}C) 14.6 (±0.4) (N= 120); DO (%) 80.0 (±4.4) (N= 120); Salinity (PPT) 0 (±0) (N= 120).				
Test series #4: SFW + 11 mg-C L^{-1} as Humic acid, pH 7.27				
24hours	Immobilisation	0.78 (0.67–0.90)	0.91 (0.83–0.99)	1.13 (1.07-1.24)
48hours	Immobilisation	0.63 (0.55–0.77)	0.75 (0.63–0.87)	0.97 (0.88–1.11)
Mean measured concentrations: 0.0, 0.037, 0.11, 0.39, 0.65, 0.95, 1.20, 2.16, 3.10, 3.71mg TCS L^{-1} **Mean environmental parameters (SD)**: pH 7.27 (±0.20) (N= 120); Conductivity (mS) 0.0009 (±0.0008) (N= 120); Temperature (^{o}C) 14.7 (± 0.3) (N= 120); DO (%) 79.0 (±4.8) (N= 120); Salinity (PPT) 0 (±0) (N= 120).				
Test series #5: 100% WwTW effluent, 16 mg-C L^{-1}, pH 8.26				
24hours	Immobilisation	1.52 (1.17–1.84)	1.83 (1.54–2.06)	2.36 (2.11-2.53)
48hours	Immobilisation	1.01 (0.78–1.24)	1.35 (1.18–1.52)	1.93 (1.81–2.08)
Mean measured concentrations: 0, 0.027, 0.077, 0.36, 0.62, 0.79, 0.99, 2.02, 2.42, 3.43mg TCS L^{-1} **Mean environmental parameters (SD)**: pH 8.26 (±0.16) (N= 120); Conductivity 0.0008 (±0.0009) (N= 120); Temperature (^{o}C) 14.7 (±0.02) (N= 120); DO (%) 74.4 (±6.8) (N= 120); Salinity (PPT) 0 (±0) (N= 120).				

288 Figure 2 provides the fitted effect curves for each of the 48 hour test series based on four
289 repeats, with accompanying 95% confidence intervals, 24 hour immobilisation plots are
290 provided in Figure A2 of the ESI.

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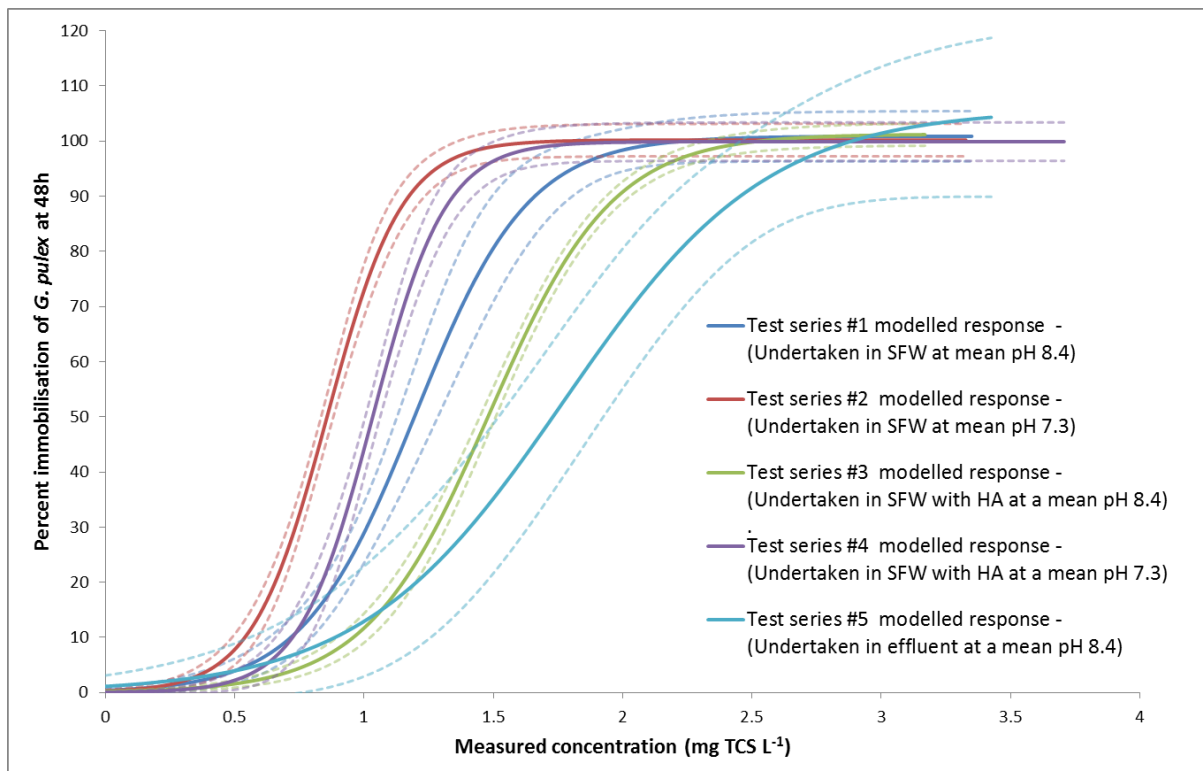
Figure 2. Mean modelled toxicological results and their 95% confidence Intervals (CI)
(dashed line) from SigmaPlot® based on data from four repeats of five tests
for triclosan to *G. pulex*.

298 All graphs show a typical sigmoid curve associated with concentration-response curves and
299 allow accurate determination of a variety of effect concentrations including EC10, EC20 and
300 EC50.

3.2.3 Effect of pH

302 Test series #1, undertaken in SFW at a mean pH of 8.4, was calculated to have an
303 EC50(48h) of 1.22 mg L^{-1}. This was almost 50% higher than the mean EC50(48h) during
304 Test series #2, also undertaken in SFW but at a lower mean pH (7.3), which was calculated
305 as 0.82 mg L^{-1}. The difference between these mean EC50 values was found to be
306 statistically significantly different when comparing results using an ANOVA with Tukey

analysis. Similar results were discovered when tests were undertaken with equal HA concentrations (11 mg-C L^{-1}), but varying mean pH of 8.4 and 7.3 (Test series #3 and #4). At a mean pH of 7.3, the EC50(48h) value was calculated as 0.97 mg L^{-1}, whereas at a mean pH of 8.4 the EC50(48h) value was increased by 76% to 1.71 mg L^{-1}. These mean EC50 values were also shown to be statistically significantly different. Therefore, these results show that an increased pH from 7.3 to 8.4 causes a increased EC50 value.

For EC20 and EC10 results the same pattern was observed, with mean EC values being lower at a mean pH of 7.3 compared with a mean pH of 8.4. The ANOVA and Tukey analysis however, revealed that EC10 and EC20(48h) values for the eight tests undertaken in SFW at varying pH (Test series #1 and #2) were not statistically significantly different. All mean EC20 and EC10 values were found to be statistically significantly different for Test series #3 compared with Test series #4.

3.2.4 Effect of Dissolved Organic Carbon

The effect of DOC was investigated by replicating Test series #1 and #2 conditions with the addition of 20 mg L^{-1} of HA (Test series #3 and #4). All tests with HA displayed a marked increase in mean EC values when compared with tests with alike conditions (Table 1). For example, the mean EC50(48h) of Test series #1 was increased by >40% to 1.71 mg L^{-1} in the presence of HA in Test series #3. This effect was also observed for EC20 and EC10 results, displaying a mean increase in EC values of >50%. These differences between Test series #3 compared with Test series #1, undertaken at a mean pH of 8.4, displayed a statistically significant reduction in EC values based on results from ANOVA with Tukey analysis. Conversely, EC values calculated for Test series #4, the addition of HA at a mean pH of 7.3, were not found to be statistically significantly different to the comparable test without HA (Test series #2) for any mean EC values calculated (Table 1). However, the mean EC values for Test series #4 displayed the same pattern of increased EC values as discussed. For example, the mean EC50(48h) for Test series #4 was 0.97 mg L^{-1}, higher than the mean EC50(48h) for Test series #2 of 0.75 mg L^{-1} carried out at the same pH.

Test series #5 was undertaken at a mean pH of 8.3 using 100% WwTW effluent rather than SFW. The results show the same pattern as that produced using HA, with the effluent resulting in higher mean EC values than any tests undertaken without additional DOC. These results were also statistically significantly higher for all EC values, except EC10(48h), when compared with Test series #1. This effluent test also produced similar EC values (statistically insignificant) to the corresponding test (wrt pH) with the addition of HA (Test series #3). The variance observed suggests that the type and/or concentrations of DOC can

341 have an effect on the toxicity of triclosan. For instance, mean EC50(48h) values were
342 calculated as 1.71 mg L^{-1} and 1.93 mg L^{-1} for Test series #3 and #5 respectively.

343 Overall, the displayed results demonstrate a clear pattern with statistical significance that the
344 toxicity tests undertaken at a higher pH and in the presence of additional DOC have
345 increased EC values (Figure 2).

346

347
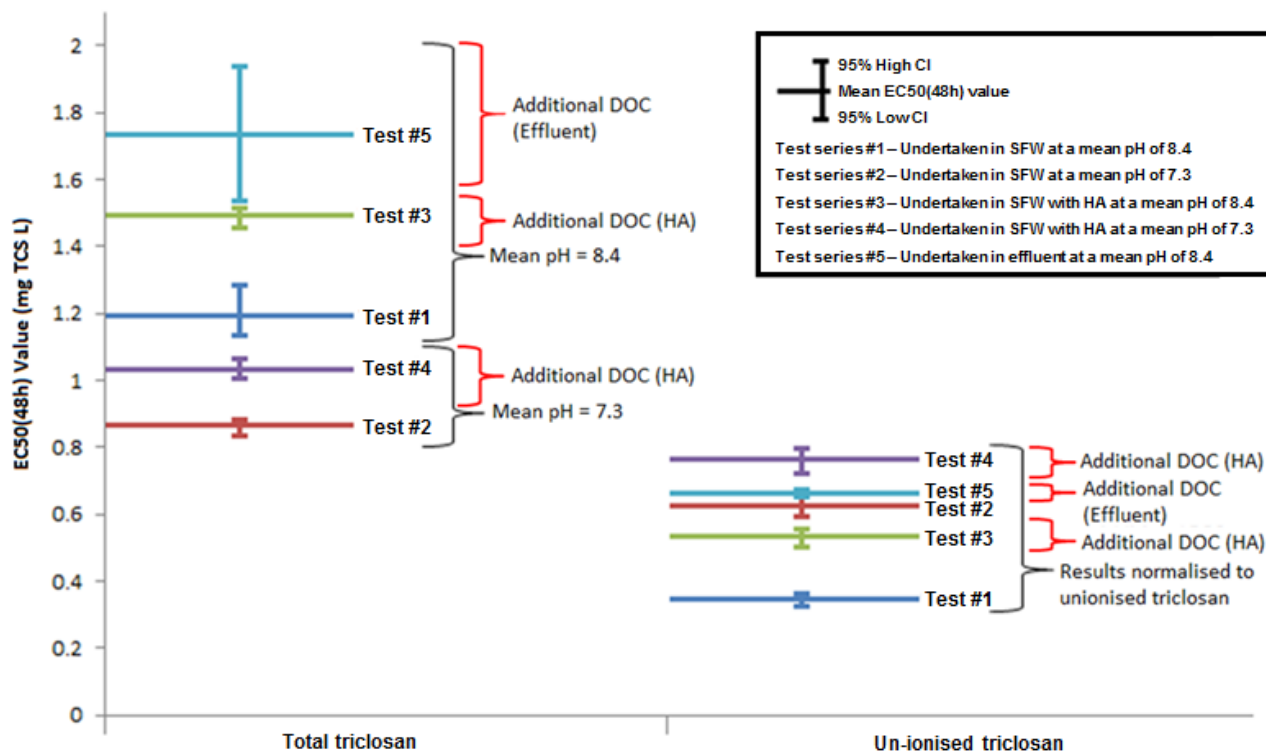
Figure 3. Comparison of variation between EC50(48h) values calculated using both total and un-ionised triclosan concentrations for each of the different test conditions (error bars = 95% confidence intervals)

351

3.2.5 Effect of triclosan speciation

353 The percentage of ionised and un-ionised triclosan in a solution can be calculated by using
354 the pKa (taken as 8.00) and the measured pH. This is significant as, for example, with one
355 pH unit change between pH values 7-9 a 40% change in triclosan species is observed
356 (Figure 1). Consequently, the concentration of un-ionised triclosan (most toxic species) was
357 calculated for all experimental data and the EC50s were recalculated (Figure 3, Figure A3
358 and Table A9 of ESI). These EC values and associated 95% CI were not calculated for each
359 repeat, but for the mean of the four tests results. Because a single number of immobilised *G.*

pulex at each triclosan concentration was used, EC values displayed in Table A9 vary slightly from those displayed in Table 1.

The decrease in un-ionised triclosan EC50 values is comparable to the calculated decrease in triclosan concentration based only on this species at each pH. At more neutral pH (Test series #2 and #4), the EC50 value changes by approximately 25-27%, whereas at a pH>8 (Tests series #1, #3 and #5), the EC50 value changes by approximately 61-71%. These results, displayed in Figures 4 and A3 of the ESI, become visibly more compressed, showing less widely varying values. However, they displayed only slightly smaller percentage difference compared with total triclosan EC50 results; with relative standard deviations being 26.7% and 27.8% respectively.

4   DISCUSSION

4.1   Control tests

Zinc was used as a positive control reference toxicant based on Environment Agency (2007) guidance and its comprehensive ecotoxicology database. The current study calculated a mean EC50(24h) value of 8.18 mg Zn L^{-1} (95% conf. int. = 6.24-10.12 mg Zn L^{-1}) for *G. pulex* and showed excellent agreement with previous studies reporting EC50(24h) of between 7.57 and 8.77 mg Zn L^{-1} depending on the source of *G. pulex* and based on results pooled for a range of life stages (Naylor *et al.*, 1990); thus providing confidence in applying the test to triclosan.

The sparingly soluble nature of triclosan required the use of methanolfor spiking purposes. Solvent control tests displayed a mean *G. pulex* immobilisation of ≤1%, complying with the <10% immobilisation acceptance criteria recommended by OECD (2004).

4.2   Effect of pH

The results from this current study have displayed that pH can have a significant effect on the toxicity of triclosan to *G. pulex*. Test series #1, undertaken in SFW at a mean pH of 8.4, resulted in a statistically significantly higher EC50 value when compared to Test series #2, undertaken at a mean pH of 7.3. This suggests that triclosan is more toxic to *G. pulex* at pH 7.3, when all other environmental parameters were maintained. Similar results were obtained when undertaking tests with equal HA concentrations (11 mg-C L^{-1}), displaying a mean pH of 7.3 (Test series #4) to be statistically significantly more toxic than pH 8.4 (Test series #3). The effect of pH on EC values is summarised in Table 2. The larger increase in

392 EC values between tests containing HA could be a result of pH also influencing the surface
393 charge of HA, with lower pH causing increased triclosan sorption (Behera *et al.*, 2010).

394 If the effect of pH was linear, a theoretical EC50 value under SFW conditions of just 0.52 mg
395 L^{-1} at pH 6.5 and 0.29 mg L^{-1} at pH 6 would be observed. These acidic pH ranges have been
396 most frequently reported in North West, South West and Welsh UK regions. This information
397 could be used by regulators to prioritise efforts at these locations where effluent discharge
398 containing triclosan would cause a particularly high risk.

399 **Table 2. The effect of pH on the calculated EC values for comparable tests**

	Test series EC value		EC value percentage increase	Mean EC value percentage increase (SD)
	Test series #2 (Mean pH 7.3)	Test series #1 (Mean pH 8.4)		
EC50	0.75	1.22	62.7	
EC20	0.62	0.91	46.8	49.7 (11.8)
EC10	0.53	0.74	39.6	
(20 mg L^{-1} HA)	Test series #4 (Mean pH 7.3)	Test series #3 (Mean pH 8.4)		
EC50	0.97	1.71	76.3	
EC20	0.75	1.36	81.3	80.6 (3.95)
EC10	0.63	1.16	84.1	

400

401 The effect of pH on triclosan is a result of its pKa (approximately 8), which is an equilibrium
402 constant describing the degree of ionisation at a particular pH. When the mean pH is 7.3
403 (Test series #2 and #4) approximately 83% of triclosan is un-ionised and at its most toxic,
404 compared with only 28% un-ionised at a pH of 8.4 (Test series #1 and #3). This is significant
405 as lipid membranes are generally impermeable to ionised species, therefore, triclosan
406 toxicity is mainly associated with the un-ionised form (Lipnick, 1995; Lyndall *et al.*, 2010). If
407 triclosan cannot cross the lipid membrane its bioavailability is reduced, supporting the
408 current study's results. Orvos *et al.* (2002) reports similar findings of increased EC50(48h)
409 values of approximately 133% from pH 7.4 - 7.6 to 8.2 - 8.5. This was larger than the 63%
410 increase between Test series #2 and #1 and 76% increase between Test series #4 and #3.
411 However, different species (*Ceriodapnia dubia* neonates) and test conditions were used
412 which may have caused this variation.

413 Although normalisation of EC50 for un-ionised triclosan reduces the variance between EC
414 values for similar test conditions (e.g. SFW with and without added HA) it does not eliminate
415 it (Figure 3). This suggests that the varying toxicity between tests is not purely a result of pH.
416 This would be expected for tests containing HA as these chemicals would also behave
417 differently at varying pH, for example causing sorbent protonation. Therefore, this suggests

418 that DOC is still having an effect even when normalising toxicity to un-ionised triclosan. This
419 cannot explain the difference between Test series #1 and #2 un-ionised triclosan EC values.
420 Other studies have also not reported equal un-ionised triclosan EC50 values, however, they
421 have been closer (Orvos *et al.*, 2002). Possible reasons for this include analytical error in
422 measuring pH and the fact that the pH tended to increase between 0 and 48 hours as a
423 result of aeration purging carbon dioxide from the solution, causing increased exposure to
424 un-ionised triclosan at the beginning of tests. These issues could be significant, as a change
425 in pH value of 0.2 could result in a 10% difference in calculated un-ionised triclosan
426 concentration, therefore, having the potential to bring the un-ionised EC50 values closer
427 together. As normalising the results assumes only un-ionised triclosan uptake, reported
428 uptake of ionised substances would also result in unequal EC values (Saarikoski *et al.*,
429 1986). Furthermore, triclosan uptake through the digestive system, with a pH reported
430 between 4.5-7.5 for *G. pulex* (Monk, 1977), would cause the more toxic un-ionised form to
431 prevail.

432 Although the same effect of pH is observed at EC20 and EC10 values, it was not always
433 found to be statistically significant owing to the larger variation in immobilisation at low
434 concentrations. Furthermore, when comparing 24 and 48 hour EC values, the ratio is larger
435 for tests undertaken at a lower pH suggesting that the effect of immobilisation occurs faster.

4.3   Effect of Dissolved Organic Carbon

437 Tests with HA and WwTW effluent displayed an increase in mean EC values when
438 compared with tests without their addition (Table 3). This could be a result of complexes
439 caused by triclosan's high hydrophobicity, therefore, its sorption and removal from the
440 dissolved phase (Nakada *et al.*, 2010). This would cause the contaminant, in this case
441 triclosan, and DOC to result in complexes that are too large or polar to cross biological
442 membranes, which therefore reduces triclosan's availability to biota and mitigates its toxicity
443 (Chalew and Halden, 2009).

444 An increased HA concentration in the aqueous phase causes increased amounts of HA-
445 complexed triclosan, subsequently, causing a decrease in free triclosan (Behera *et al.*,
446 2010). This would explain the reduction in triclosan toxicity when HA was added to Test
447 series #3 and #4, which has been reported previously when working with other organic
448 compounds (Lorenz *et al.*, 1996). WwTW effluent will have a more varied DOC composition
449 from both anthropogenic and natural sources which will also cause triclosan complexation.
450 Studies have stated that triclosan concentrations added to effluent, which should affect

451   daphnids, is removed or detoxified, supporting effluents mitigating capacity observed in this
452   current study (Orvos *et al.*, 2002).

453

455 **Table 3. The effect of DOC on the calculated 48hour EC values for comparable test**
456 series

pH 8.4	Test series EC value		EC value percentage increase	Mean EC value percentage increase (SD)
	Test #1	Test #3 (11mg-C L^{-1} HA)		
EC50	1.22	1.71	40.2	
EC20	0.91	1.36	49.5	48.8 (8.3)
EC10	0.74	1.16	56.8	
pH 7.3	Test #2	Test #4 (11mg-C L^{-1} HA)		
EC50	0.75	0.97	29.3	
EC20	0.62	0.75	21.0	23.1(5.5)
EC10	0.53	0.63	18.9	
pH 8.4	Test #1	Test #5 (100% effluent)		
EC50	1.22	1.93	58.2	
EC20	0.91	1.35	48.4	47.7 (10.9)
EC10	0.74	1.01	36.5	

457

458 It is reported that sorption of triclosan is pH dependent, due to the deprotonation of the
459 hydroxyl group (Wilson *et al.*, 2009). Ionised triclosan will generally have greater water
460 solubility as it will be dissociated in the aqueous phase and therefore less likely to partition to
461 DOC (Aldous *et al*, 2012). Therefore, more triclosan is expected to be bioavailable during
462 tests undertaken at pH 8.4 than at pH 7.3 in the presence of DOC. However, as previously
463 discussed, pH causes varying toxicity. These conflicting effects could possibly cancel each
464 other out (Lyndall *et al.*, 2010). Figure 4 summarises these key effects.
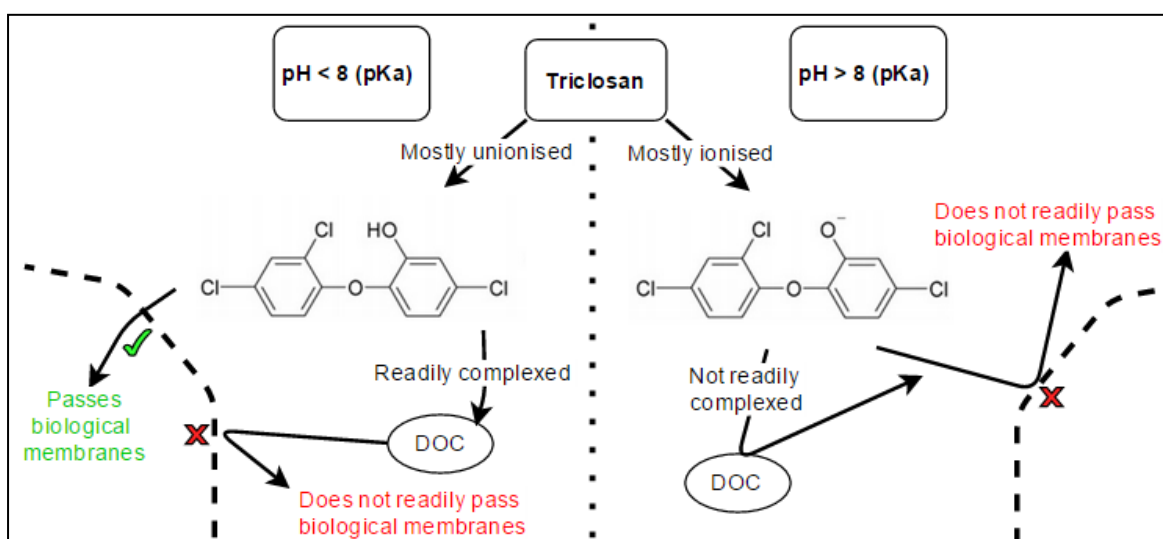
465

466 **Figure 4. A conceptual diagram displaying the effect of pH and DOC on the**
467 **bioavailability of triclosan in solution**

468 Although this difference in percentage reduction is statistically significant, the relationship
469 between pH, DOC and triclosan is complex and supplementary data would be required to
470 conclude the definitive cause of these results.

471 Test series #5, undertaken at a mean pH of 8.3 using WwTW effluent, displayed similar
472 results to Test series #3 with the higher mean EC50 value. This is not statistically significant,
473 although reflects the higher DOC in effluent samples (16 compared with 11 mg-C L^{-1}). The
474 suspended solids present in the effluent (the only test to contain suspended solids) were not
475 sufficiently high at 17 mg L^{-1} to impact on available triclosan based on its observed
476 partitioning characteristics (estimated as a maximum of 13.7% adsorption based on a log
477 Koc of 9200 l Kg^{-1})

478 The similarity between Test series #3 and #5 EC results could possibly be because HA is a
479 major DOC component of treated wastewater (Katsoyiannis and Samara, 2007). This HA
480 readily complexes organic compounds, resulting in the mitigation observed in Test series #3
481 (McDonald *et al.*, 2004). Conversely, different DOC components have a varying ability to
482 form complexes (Chalew and Halden, 2009). Consequently, despite the DOC concentration
483 and suspended solids content being higher in effluent, it may not be as effective as HA
484 alone. This varying effectiveness of different DOC sources, even when at similar
485 concentrations, has been previously observed for other organic chemicals such as
486 benzo[a]pyrene (Haitzer *et al.*, 1999b).

487 Effluent also contains a complex mixture of inorganic and organic compounds which have
488 been shown to exhibit toxicity (Orvos *et al.*, 2002). These could cause additive or synergistic
489 toxic effects with triclosan (Canivet and Gibert, 2002; Kolpin *et al.*, 2002; Chalew and
490 Halden, 2009), which has been shown to have an increased effect than triclosan alone
491 (Yang *et al.*, 2008). This further supports the lack of difference between Test series #3 and
492 #5, regardless of the higher DOC and suspended solid concentration.

493 Overall, although WwTW effluent may be the major source of triclosan, the organic carbon
494 present acts to mitigate its toxicity.

495 **4.4   Importance of results and environmental relevance**

496 Based on the results of this study, toxicity tests undertaken at higher pH have the potential to
497 underestimate triclosan toxicity. This is particularly relevant for triclosan as its pKa is within
498 the pH range of natural surface waters, which will therefore have a huge influence on its
499 speciation, fate and behaviour (Singer *et al.*, 2002). Other similar chemicals, such as
500 chlorophenols, exhibit comparable behaviour with respect to the effect of pH (Sinclair *et al.*,

501 1999). Therefore, the results from this study have implications for the way that other organic
502 chemicals, including pharmaceuticals now listed under the Water Framework Directive as
503 Priority or Priority Hazardous Substances, should also be tested and regulated.

504 The data presented here suggest that results from standard laboratory toxicity tests, which
505 neglect the effect of DOC, will potentially overestimate triclosan's toxicity which could lead to
506 overly stringent EQS and tighter consent conditions for effluent discharges by ignoring
507 effects of speciation on bioavailability. Studies have recognised the need for more realistic
508 exposure scenarios, such as mesocosms (Crane *et al.*, 1999). The methodology undertaken
509 in this current study provides a similar bridge between 'clean' standardised laboratory
510 experiments and those undertaken in the field, with less complexity and cost. It may also
511 reduce uncertainties associated with extrapolating data from laboratory to field exposures
512 (Bloor and Banks, 2006). Furthermore, HA provides reasonably good environmental
513 relevance as it often comprises >10% of DOC in most natural waters (Thurman, 1985).

514 The effect of pH and DOC has been identified when setting EQS for metals, resulting in the
515 introduction of the BLM (Environment Agency, 2009). Based on the results of this study, it
516 could be suggested that a similar approach should be implemented for organics to provide
517 the most relevant standards. Based on the extreme cases compared here, (Test series #1 vs
518 Test series #5), the results display a 157% increase in mean EC50 value. From a
519 toxicological point of view, based purely on the current study results, it would seem that
520 typical triclosan concentrations in the natural freshwater environment would pose minimal
521 acute toxicity risk to *G. pulex*; even if an assessment factor of 1000 was to be applied to
522 laboratory ecotoxicology results.

5   CONCLUSIONS

524 This study set out to identify whether key omissions in routine ecotoxicology testing, in
525 relation to DOC and pH, have an impact on calculated EC values; specifically for triclosan to
526 adult *G. pulex* (Dartmoor wild-type). This was to identify whether approaches to assessing
527 environmental compliance of organic contaminants should account for their potential varying
528 bioavailability.

529 The results from toxicity tests undertaken in this current study displayed a good degree of
530 accuracy, precision and reliability, and demonstrated acceptable inter- and intra-laboratory
531 performance. Broadly speaking and based purely on the current results, it would seem that
532 typical triclosan concentrations in the natural environment would pose minimal acute toxicity
533 risk to *G. pulex*; which were found to display relatively low triclosan sensitivity.

534 A mean EC percentage increase between tests undertaken at pH 7.3 compared to 8.4 was
535 calculated as 70%. This showed that toxicity tests undertaken at a pH above triclosan's pKa
536 have the potential to underestimate its toxicity to *G. pulex*. This has been suggested to be
537 caused by speciation between ionised and un-ionised triclosan, causing varying
538 bioavailability depending on its ability to transfer across lipid membranes. Many studies have
539 been shown not to report the pH when undertaking triclosan toxicity tests. Therefore, varying
540 potential bioavailability renders the results incomparable to one another and may not
541 express the true sensitivity of an organism. By normalising these results to un-ionised
542 triclosan, it could be further suggested the toxicity of triclosan was attributed to this
543 bioavailable species and that pH was not the only factor causing an effect.

544 The addition of DOC to tests was displayed to mitigate toxicity, which was likely to be a
545 result of complexes caused by triclosan's high hydrophobicity. This results in its removal
546 from the dissolved phase and the inability to cross lipid membranes, rendering it unavailable
547 to biota. Results therefore suggested that standard laboratory toxicity tests, which often
548 neglect the effect of DOC, could overestimate triclosan toxicity. As waterbodies contain DOC
549 concentrations not untypical of the levels tested here, these tests may potentially cause
550 overly stringent EQS by ignoring natural effects on bioavailability. This study's results have
551 shown that EQS for triclosan derived from standard tests could be as much as 58% more
552 stringent than those based on tests with DOC.

553 Consequently, both pH and DOC should be more carefully considered, particularly when
554 undertaking toxicity tests with organic chemicals with pKa's within the aquatic pH window
555 (typically pH 5 to 9). This would ensure the most environmentally applicable EQS can be
556 produced and applied to discharge consents.

557

558 **REFERENCES**

559 Aldous, E., Rockett, L. and Johnson, I., 2012. *Proposed EQS for Water Framework Directive*
560 *Annex VIII substances: triclosan (For consultation).*Water Framework Directive – United
561 Kingdom Technical Advisory Group, Edinburgh.

562 ASTM, 1980. *Standard practice for conducting acute toxicity tests with fishes,*
563 *macroinvertebrates and amphibians. E-279-80.* American Standard for Testing and
564 Materials, Philadelphia.

565 Behera, S. K. and Oh, S. and Park, H., 2010. Sorption of triclosan onto activated carbon,
566 kaolinite and montmorillonite: Effects of pH, ionic strength, and humic acid. *Journal of*
567 *Hazardous Materials*, **179**, 684-691.

568 Bendz, D., Paxéus, N. A., Ginn, T. R. and Loge, F. J., 2005. Occurrence and fate of
569 pharmaceutically active compounds in the environment, a case study: Höje River in Sweden.
570 *Journal of Hazardous Materials*, **122**, 195–204.

571 Bloor, M. C. and Banks, C. J., 2006. An evaluation of mixed species in-situ and ex-situ
572 feeding assays: The altered response of *Asellus aquaticus* and *Gammarus pulex*.
573 *Environment International*, **32**, 22-27.

574 Boudou, A. and Ribeyre, F., 1997. Aquatic Ecotoxicology: From the Ecosystem to the
575 Cellular and Molecular Levels. *Environmental Health Perspectives*, **105**, 21-35.

576 Canivet, V. and Gibert, J., 2002. Sensitivity of epigean and hypogean freshwater
577 macroinvertebrates to complex mixtures. Part I: Laboratory experiments. *Chemosphere*, **46**,
578 999-1009.

579 Chalew, T. E. A. and Halden, R. U., 2009. Environmental Exposure of Aquatic and
580 Terrestrial Biota to Triclosan and Triclocarban. *Journal of The American Water Resource*
581 *Association*, **45**, 4-13.

582 Crane, M., Attwood, C., Sheahan, D. and Morris, S., 1999. Toxicity and Bioavailability of the
583 organophosphorus insecticide pirimiphos methyl to the freshwater amphipod *Gammarus*
584 *pulex* L. in laboratory and mesocosm systems. *Environmental Toxicology and Chemistry*, **18**,
585 1456-1461.

586 Daughton, C. G. and Ternes, T. A., 1999. Pharmaceuticals and personal care products in
587 the environmental: agents of subtle change?. *Environmental Health Perspectives*, **107**, 907-
588 938.

589 De Schamphelaere, K. A. C., Heijerick, D. G. and Janssen, C. R., 2004. Comparison of the
590 Effect of Different pH Buffering Techniques on the Toxicity of Copper and Zinc to *Daphnia*
591 *magna* and *Pseudokirchneriella subcapitata*. *Ecotoxicology*, **13**, 697-705.

592 Environment Agency, 2007. *The direct toxicity assessment of aqueous environmental*
593 *samples using the juvenile Daphnia magna immobilisation test, Methods for the Examination*
594 *of Waters and Associated Materials*. Environment Agency, Bristol.

595 Environment Agency, 2009. *Using biotic ligand models to implement environmental quality*
596 *standards for metals under the Water Framework Directive, Science Report –*
597 *SC080021/SR7b*. Environmental Agency, Bristol.

598 European Commission, 2009. *Opinion on Triclosan – SCCP/1192/08*. European
599 Commission, Brussels.

600 Felten, V., Charmantier, G., Charmantier-Daures, M., Aujoulat, F., Garric, J. and Geffard, O.,
601 2008. Physiological and behavioural responses of *Gammarus pulex* exposed to acid stress.
602 *Comparative Biochemistry and Physiology*, **147C**, 189-197.

603 Gardner, M., Comber, S., Scrimshaw. M. D., Cartmell, E., Lester, J. and Ellor, B., 2012. The
604 significance of hazardous chemicals in wastewater treatment works effluents. *Science of the*
605 *Total Environmental*, **437**, 363-372.

606 Gerhardt, A., Svensson, E., Clostermann, M. and Fridlund, B., 1994. Monitoring of
607 behavioral patterns of aquatic organisms with an impedance conversion technique.
608 *Environment International*, **20**, 209–219.

609 Güven, K., Özbay, C., Ünlü, E. and Satar, A., 1999. Acute Lethal Toxicity and Accumulation
610 of Copper in *Gammarus pulex* (L.) (Amphipoda). *Turkish Journal of Biology*, **23**, 513-521.

611 Haitzer, M., Höss, S., Traunspurger, W. and Steinberg, C., 1999a. Relationship between
612 concentration of dissolved organic matter (DOM) and the effect of DOM on the
613 bioconcentration of benzo[a]pyrene. *Aquatic Toxicology*, **45**, 147-158.

614 Haitzer, M., Burnison, B. K., Höss, S., Traunspurger, W., and Steinberg, C., 1999b. Effects
615 of quantity, quality, and contact time of dissolved organic matter on bioconcentration of
616 benzo[a]pyrene in the nematode *Caenorhabditis elegans*. *Environmental Toxicology and*
617 *Chemistry*, **18**, 459-465.

618 Hammond, C., 2007. Callington Sewage Treatment Works – £3.2m upgrade for catchment
619 growth. *UK Water Projects*, **2007**, 127-128.

620 Jones, O. A. H., Voulvoulis, N. and Lester, J. N., 2002. Aquatic Environmental Assessment
621 of the Top 25 English Prescription Pharmaceuticals. *Water Research*, **36**, 5013-5022.

622 Katsoyiannis, A. and Samara, C., 2007. The fate of dissolved organic carbon (DOC) in the
623 wastewater treatment process and its importance in the removal of wastewater
624 contaminants. *Environmental Science and Pollution Research*, **14**, 284-292.

625 Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B. and
626 Buxton, H. T., 2002. Pharmaceuticals, Hormones, and Other Organic Wastewater
627 Contaminants in U.S. Streams, 1999-2000: A National Reconnaissance. *Environmental*
628 *Science and Technology*, **36**, 1202-1211.

629 Lipnick, R. L., 1995. Structure-Activity Relationships. In: Rand, G. M., *Fundamentals of*
630 *Aquatic Toxicology - Effects, Environmental Fate, and Risk Assessment*. Taylor & Francis,
631 Washington, pp. 609-655.

632 Liu, J. and Wong, M., 2013. Pharmaceuticals and personal care products (PPCPs): A review
633 on environmental contamination in China. *Environment International*, **59**, 208-224.

634 Lorenz, R., Brüggemann, R., Steinberg, C. E. W. and Spieser, O. H., 1996. Humic material
635 changes effects of terbutylazine on behaviour of Zebrafish (*Brachydanio Rerio*).
636 *Chemosphere*, **33**, 2154-2158.

637 Lyndall, J., Fuchsman, P., Bock, M., Barber, T., Lauren, D., Leigh, K., Perruchon, E. and
638 Capdevielle, M., 2010. Probabilistic Risk Evaluation for Triclosan in Surface Water,
639 Sediment, and Aquatic Biota Tissues. *Integrated Environmental Assessment and*
640 *Management*, **6**, 419-440.

641 Maltby, L., Clayton, S. L., Wood, R. M. and McLoughlin, N., 2002. Evaluation of the
642 *Gammarus pulex* in situ feeding assay as a biomonitor of water quality; robustness,
643 responsiveness and relevance. *Environmental Toxicology and Chemistry*, **21**, 361-368.

McDonald, S., Bishop, A. G., Prenxler, P. D. and Robards, K., 2004. Analytical chemistry of freshwater humic substances. *Analytica Chimica Acta*, **527**, 105-124.

Monk, D. C., 1977. The digestion of cellulose and other dietary components, and pH of the gut in the amphipod *Gammarus pulex* (L.). *Freshwater Biology*, **7**, 431-440.

Nakada, N., Yasojima, M., Okayasu, Y., Komori, K. and Suzuki, Y., 2010. Mass balance analysis of triclosan, diethyltoluamide, crotamiton and carbamazepine in sewage treatment plants. *Water Science and Technology*, **61**, 1739-1747.

Naylor, C., Pindar, L. and Calow, P., 1990. Inter- and intraspecific variation in sensitivity to toxins; the effects of acidity and zinc on the freshwater crustaceans *Asellus aquaticus* (L.) and *Gammarus pulex* (L.). *Water Research*, **24**, 757-762.

Nghiem, L. D. and Coleman, P. J., 2008. NF/RO filtration of the hydrophobic ionogenic compound triclosan: Transport mechanisms and the influence of membrane fouling. *Separation and Purification Technology*, **62**, 709-716.

Peng, X., Yu, Y., Tang, C., Tan, J., Huang, Q. and Wang, Z., 2008. Occurrence of steroid estrogens, endocrine-disrupting phenols, and acid pharmaceutical residues in urban riverine water of the Pearl River Delta, South China. *Science of the Total Environment*, **397**, 158-166.

OECD, 2004. OECD Guideline for Testing of Chemicals, *Daphnia* sp Acute Immobilisation Test, 202. Available at: http://www.oecd-ilibrary.org/. [Accessed: 25th July 2015].

OECD, 2012. *Daphnia magna* Reproduction Test. OECD Test Guideline 211, adopted 2nd October 2012. Available at: http://www.oecd-ilibrary.org/. [Accessed 3rd August 2015].

Orvos, D. R., Versteeg, D. J., Inauen, J., Capdevielle, M., Rothenstein, A., 2002. Aquatic Toxicity of Triclosan. *Environmental Toxicology and Chemistry*, **21**, 1338-1349.

Peck, A. M., 2006. Analytical methods for the determination of persistent ingredients of personal care products in environmental matrices. *Analytical and Bioanalytical Chemistry*, **386**, 907-939.

Reiss, R., Mackay, N., Habig, C. and Griffin, J., 2002. An ecological risk assessment for triclosan in lotic systems following discharge from wastewater treatment plants in the United States. *Environmental Toxicology and Chemistry*, **21**, 2483-2492.

Saarikoski, J., Lindstrom, M., Tyynila, M., and Viluksela, M., 1986. Factors affecting the absorption of phenolics and carboxylic acids in guppy (*Poecilia reticulate*). *Ecotoxicology and Environmental Safety*, **11**, 158-173.

Scientific Committee on Consumer Safety, 2010. *Opinion on triclosan – Antimicrobial Resistance*. European Union, Brussels.

Sinclair, G. M., Paton, G. I., Meharg, A. A. and Killham, K., 1999. Lux-biosensor assessment of pH effects on microbial sorption and toxicity of chlorophenols. *FEMS Microbiology Letters*, **174**, 273–278.

681   Singer, H., Müller, S., Tixier, C. and Pillonel, L., 2002. Triclosan: Occurrence and Fate of a
682   Widely Used Biocide in the Aquatic Environment: Field Measurements in Wastewater
683   Treatment Plants, Surface Waters and Lake Sediments. *Environmental Science &*
684   *Technology*, **36**, 4998-5004.

685   Suller, M. T. E. and Russell, A. D., 2000. Triclosan and antibiotic resistance in
686   Staphylococcus aureus. *Journal of Antimicrobial Chemotherapy*, **46**, 11–18.

687   Sutton, R., Naidenko, O., Chwialkowski, N. and Houlihan, J., 2008. Pesticide in Soap,
688   Toothpaste and Breast Milk – Is It Kid-Safe?. Environmental Working Group, Washington.
689   Available at: http://www.thienna.com/img/PesticideinProducts.pdf. [Accessed: 2nd June
690   2015].

691   Tan, K, H., 2014. *Humic Matter in Soil and the Environment – Principles and Controversies.*
692   CRC Press, Roca Raton.

693   Thurman, E. M., 1985. *Organic Geochemistry of Natural Waters*. Springer, Berlin.

694   Vellinger, C., Parant, M., Rousselle, P., Immel, F., Wagner, P. and Usseglio-Polatera, P.,
695   2012. Comparison of arsenate and cadmium toxicity in a freshwater amphipod (*Gammarus*
696   *pulex*). *Environmental Pollution*, **160**, 66-73.

697   Welton, J. S. and Clarke, R. T., 1980. Laboratory studies on the reproduction and growth of
698   the amphipod *Gammarus pulex* (L.). *Journal of Animal Ecology*, **49**, 581-592.

699   Wilson, B., Chen, R. F., Cantwell, M., Contz, A., Zhu, J. and Olsen, C. R., 2009. The
700   partitioning of Triclosan between aqueous and particulate bound phases in the Hudson River
701   Estuary. *Marine Pollution Bulletin*, **59**, 207-212.

702   Yang, L., Ying, G., Su., H., Stauber, J. L., Adams, M. S. and Binet, M. T., 2008. Growth-
703   inhibiting effects of 12 antibacterial agents and their mixtures on the freshwater microalga
704   *pseudokirchneriella subcaritata*. *Environmental Toxicology and Chemistry*, **27**, 1201-1208.

705   Yazdankhah, S. P., Scheie, A. A., Hoiby, E. A., Lunestad, B. T., Heir, E., Fotland, T. O.,
706   Naterstad, K. and Kruse, H., 2006. Triclosan and antimicrobial resistance in bacteria: an
707   overview. *Microbial Drug Resistance*, **12**, 83–90.

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The impact of natural and anthropogenic Dissolved Organic Carbon (DOC), and pH on the toxicity of triclosan to *Gammarus pulex* (L.).

Christopher J. Rowett[a], Thomas H. Hutchinson[b] and Sean D.W. Comber[a],†

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Table A1. Content of the SFW used throughout this study, made using deionised water.

SFW content	Source	Quantity (gl^{-1})
$MgSO_4$	Fisher Scientific (Loughborough, UK) Laboratory reagent grade	0.245
$NaHCO_3$	Fisher Scientific (Loughborough, UK) Laboratory reagent grade	0.195
KCl	Fisher Scientific (Loughborough, UK) Laboratory reagent grade	0.008
$CaSO_4$	ACROS Organics (New Jersey) >98%	0.09

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Table A2. Chemicals used during this study, their grades and source.

Chemical	Grade	Source
Triclosan	Certified Reference material	Sigma–Aldrich (Gillingham, UK)
Methanol	HPLC grade (99.99%)	Fisher Scientific (Loughborough, UK)
Acetonitrile	HPLC grade (99.99%)	Fisher Scientific (Loughborough, UK)
Hydrochloric acid	ACS reagent standard	Sigma–Aldrich (Gillingham, UK)
Humic acid	Technical grade	Sigma–Aldrich (Gillingham, UK)
QC26 Elements Standard	Certificate of Analysis +/- 0.5%	CPI International (Santa Rosa, USA)
3-(N-morpholino) propanesulfonic acid	≥99.5%	Sigma–Aldrich (Gillingham, UK)
Sodium hydroxide	ACS reagent grade pellets (≥97.0%)	Sigma–Aldrich (Gillingham, UK)
Zinc sulfate heptahydrate	Analytical reagent >99.5	BDH Chemicals (Poole, UK)
Nitric acid	ACS reagent standard	Sigma–Aldrich (Gillingham, UK)

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732 **Table A3 Supporting statistics for ANOVA comparisons .**

EC value test	Source	Degrees of freedom (DF)	Sum of Squares (SS)	Mean Square (MS)	F-statistic	p-value
EC10(24h)	Test	4	4.0562	1.0140	27.68	0.000
	Error	15	0.5495	0.0366	-	-
	Total	19	4.6057	-	-	-
EC10(48h)	Test	4	1.1093	0.2773	6.24	0.004
	Error	15	0.6668	0.0445	-	-
	Total	19	1.7761	-	-	-
EC20(24h)	Test	4	4.3878	1.0970	39.06	0.000
	Error	15	0.4213	0.0281	-	-
	Total	19	4.8091	-	-	-
EC20(48h)	Test	4	1.8841	0.4710	16.91	0.000
	Error	15	0.4178	0.0279	-	-
	Total	19	2.3019	-	-	-
EC50(24h)	Test	4	5.2561	1.3140	44.35	0.000
	Error	15	0.4445	0.0296	-	-
	Total	19	5.7006	-	-	-
EC(48h)	Test	4	4.0028	1.0007	39.89	0.000
	Error	15	0.4182	0.0279	-	-
	Total	19	-	-	-	-

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735 **Table A4. Summary of the environmental parameters within holding tanks 1 and 2.**

	Tank 1			Tank 2		
	Mean	SD	n	Mean	SD	n
pH	7.8	0.3	38	7.8	0.3	38

Dissolved Oxygen (%)	80.2	5.6	38	80.0	5.4	38
Conductivity (µS)	262.2	110.1	38	255.1	97.4	38
Temperature (^{o}C)	14.7	0.3	38	14.7	0.3	38
Salinity (PPT)	0.07	0.05	38	0.07	0.07	38
Hardness (mgl^{-1} as CaCO₃)	80.0	26.2	6	73.3	23.8	6
Ammonia (mgl^{-1})	>0.25	0	6	>0.25	0	6
Nitrite (mgl^{-1})	>0.25	0	6	>0.25	0	6
Nitrate (mgl^{-1})	>0.25	0	6	>0.25	0	6

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738 **Table A5. Percentage immobilisation of *G. pulex* at 24 and 48 hours for the zinc**
739 **positive control test**

Zinc concentration (mg Zn l^{-1})	Percentage mortality	
	24hours	**48hours**
Control	0	0
1	0	10
3.2	20	50
10	70	90
32	100	100
52	100	100

Mean environmental parameters for 0, 24 and 48hours (SD): pH 7.8 (± 0.4); Temperature (^{o}C) 15.3 (± 0.4); Dissolved oxygen (% oxygen saturation) 79.8 (± 3.3); Conductivity (mS) 0.0001 (± 0). Date of study: 16th June 2015 – 18th June 2015.

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744 **Table A6. EC values calculated for the zinc positive control test using**
745 **observed immobilisation result for *G. pulex* and measured zinc**
746 **concentrations.**

Time Exposed	Response	EC$_{10}$ (mgl^{-1}) (±95% CI)	EC$_{20}$ (mgl^{-1}) (±95% CI)	EC$_{50}$ (mgl^{-1}) (±95% CI)
24hours	Immobilisation	2.23 (<0–5.28)	4.52 (1.94–6.89)	8.18 (6.24-10.12)

48hours	Immobilisation	1.08 (<0–2.37)	1.94 (0.86–2.80)	3.23 (2.58–4.31)

Mean measured concentrations: 0, 0.98, 3.34, 10.5, 32.6, 55.12mg Zn l^{-1}
Mean environmental parameters: pH 7.8 (± 0.4); Temperature (^{o}C) 15.3 (± 0.4);
Dissolved oxygen (% oxygen saturation) 79.8 (± 3.3); Conductivity (mS) 0.0001
(± 0).

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Table A7. Mean percentage *G. pulex* immobilisation during 24 hour exposure to triclosan calculated from four repeat tests (n = 4 for all test results) (NB. Measured test concentrations are displayed in Table 5.4).

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Nominal Conc. (mgl^{-1})	Test series #1 percentage mortality		Test series #2 percentage mortality		Test series #3 percentage mortality		Test series #4 percentage mortality		Test series #5 percentage mortality	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0.0	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.032	0.0	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.100	0.0	0.00	0.0	0.0	0.0	0.0	2.5	5.0	0.0	0.0
0.320	0.0	0.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.560	5.0	5.8	10.0	8.2	0.0	0.0	0.0	0.0	0.0	0.0
0.800	5.0	5.8	20.0	28.3	0.0	0.0	17.5	5.0	0.0	0.0
1.00	7.5	5.0	60.0	21.6	2.5	5.0	35.0	19.2	10.0	14.1
1.80	85.0	5.8	95.0	5.8	37.5	22.2	100.0	0.0	40.0	25.8
2.60	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	77.5	12.6
3.20	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	95.0	5.6

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Table A8. Mean percentage *G. pulex* immobilisation during 48 hour exposure to triclosan calculated from four repeat tests (n = 4 for all test results) (NB. Measured test concentrations are displayed in Table A6).

Nominal Conc. (mgl^{-1})	Test series #1 percentage mortality		Test series #2 percentage mortality		Test series #3 percentage mortality		Test series #4 percentage mortality		Test series #5 percentage mortality	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0.0	0.0	2.5	5.0	0.0	0.0	2.5	5.0	0.0	0.0
0.032	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.100	5.0	5.8	0.0	0.0	0.0	0.0	2.5	5.0	0.0	0.0
0.320	2.5	5.0	0.0	0.0	0.0	0.0	5.0	10.0	0.0	0.0

0.560	5.0	5.8	20.0	8.2	0.0	0.0	2.5	5.0	2.5	0.0
0.800	17.5	5.0	40.0	18.3	5.0	5.8	37.5	9.6	12.2	5.0
1.00	25.0	5.8	82.5	5.0	7.5	9.6	75.0	17.3	17.5	5.0
1.80	95.0	5.8	100.0	0.0	72.5	5.0	100.0	0.0	60.0	8.2
2.60	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0
3.20	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0	100.0	0.0

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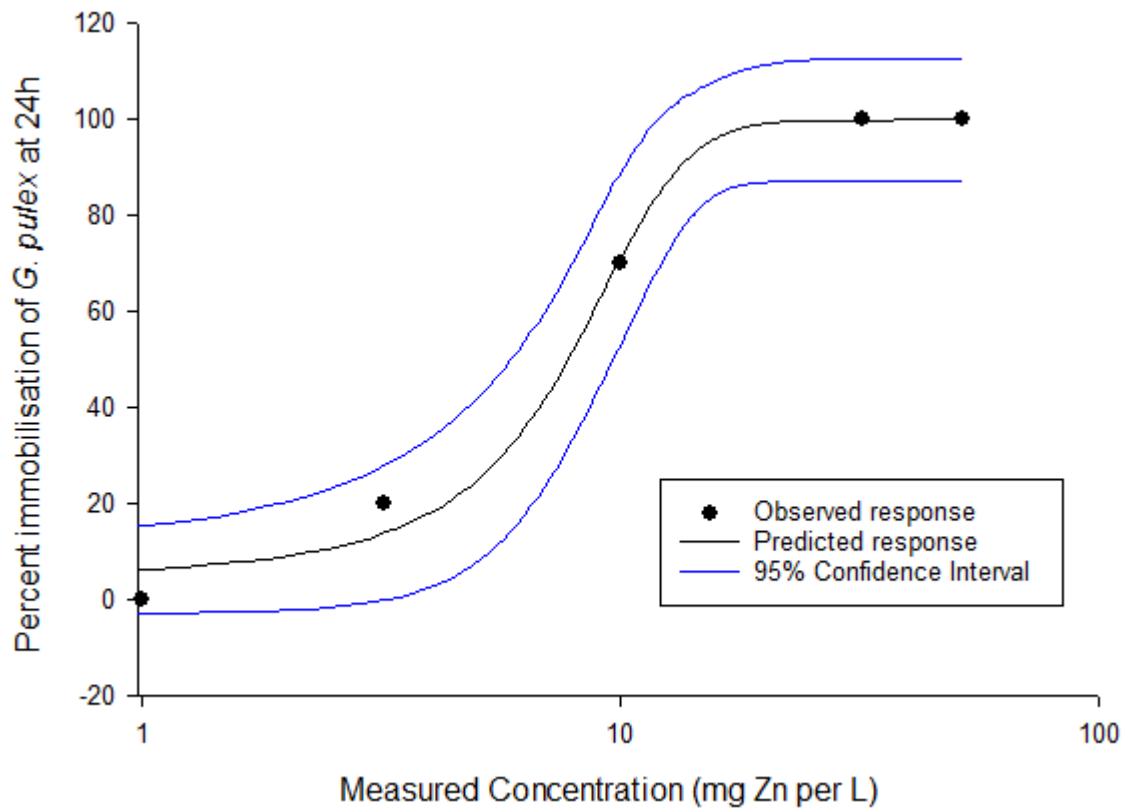768

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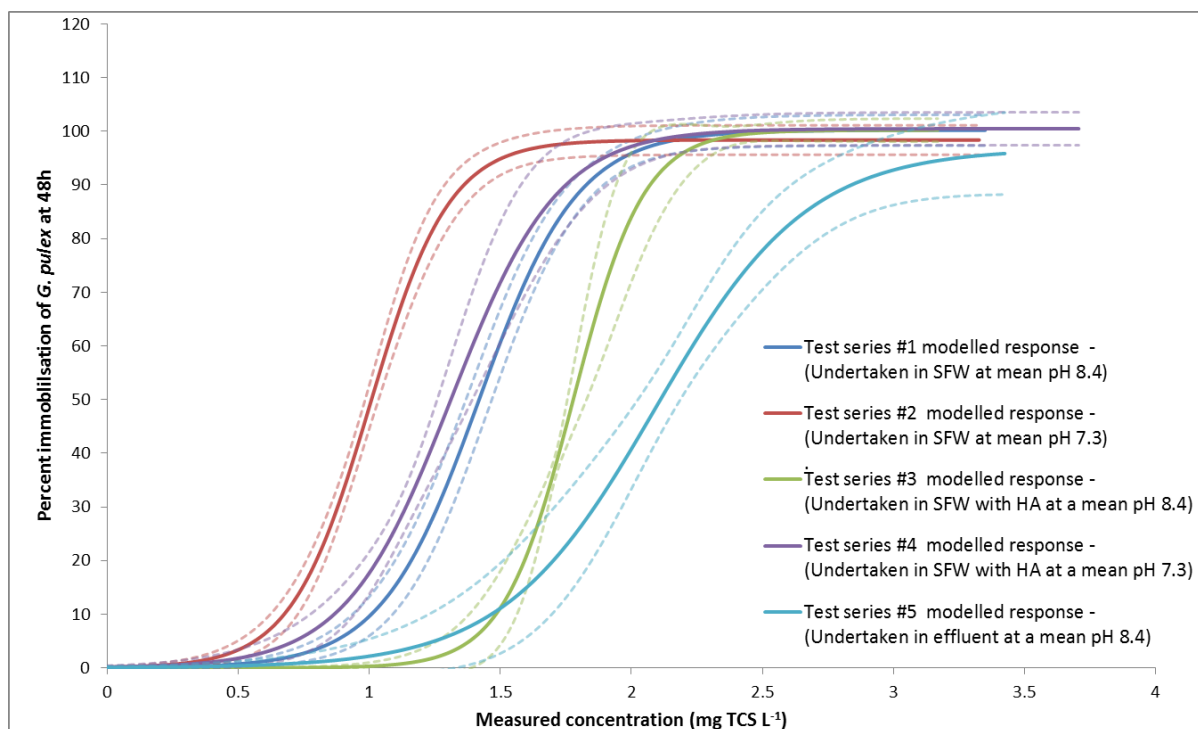785 **Figure A1. Graph created in SigmaPlot® showing toxicological results at**
786 **24hours for zinc to G. pulex during the positive control study.**

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792 **Figure A2. Mean modelled toxicological results and their 95% confidence Intervals (CI) (dashed line)**
793 **from SigmaPlot® based on data from four repeats of five tests at 24 hours for triclosan to G. pulex,**
794 **using mean measured triclosan concentration and mean observed immobilisation.**

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Table A9. Mean EC50(48h) values for triclosan to *G. pulex* during all tests, calculated using mean observed immobilisation from four repeat experiments and both the measure total triclosan and calculated unionised triclosan concentrations.

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| Test series	Total triclosan EC50 (mgl^{-1}) (±95% CI)	Unionised triclosan EC50 (mgl^{-1}) (±95% CI)
#1	1.19 (1.13–1.28)	0.35 (0.33-0.37)
Mean measured concentrations: 0.0, 0.102, 0.295, 0.598, 0.787, 0.990, 1.738, 2.57, 3.35mg TCS l^{-1} **Mean unionised calculated concentrations:** 0.0, 0.008, 0.030, 0.085, 0.17, 0.23, 0.29, 0.50, 0.75, 0.97mg l^{-1} **Mean environmental parameters (SD)**: pH 8.39 (±0.08) (N= 120); Conductivity (mS) 0.0006 (±0.0007) (N= 120); Temperature (^{o}C) 14.9 (±0.28) (N= 120); DO (%) 81.9 (±3.95) (N= 120); Salinity (PPT) 0 (±0) (N= 120).		
#2	0.86 (0.83–0.88)	0.63 (0.60-0.66)
Mean measured concentrations: 0.000, 0.033, 0.107, 0.35, 0.62, 0.81, 1.07, 2.16, 2.71, 3.33mg TCS l^{-1} **Mean unionised calculated concentrations:** 0.0, 0.091, 0.291, 0.440, 0.616, 0.744, 1.454, 1.992, 2.691mg l^{-1} **Mean environmental parameters (SD)**: pH 7.25 (±0.18) (N= 120); Conductivity (mS) 0.0008 (± 0.0001) (N= 120); Temperature (^{o}C) 14.8 (±0.2) (N= 120); DO (%) 77.1 (±4.6) (N= 120); Salinity (PPT) 0 (±0) (N= 120).		
#3	1.49 (1.45–1.51)	0.54 (0.51-0.56)
Mean measured concentrations: 0.0, 0.033, 0.107, 0.34, 0.52, 0.75, 0.85, 1.7, 2.35, 3.17mg TCS l^{-1} **Mean unionised calculated concentrations:** 0.0, 0.008, 0.024, 0.112, 0.19, 0.24, 0.31, 0.62, 0.75, 1.06mg l^{-1} **Mean environmental parameters (SD)**: pH 8.35 (±0.09) (N= 120); Conductivity (0.0003mS) (±0.0005) (N= 120); Temperature (^{o}C) 14.6 (±0.4) (N= 120); DO (%) 80.0 (±4.4) (N= 120); Salinity (PPT) 0 (±0) (N= 120).		
#4	1.03 (1.00–1.06)	0.77 (0.73-0.80)
Mean total measured concentrations: 0.0, 0.037, 0.11, 0.39, 0.65, 0.95, 1.196, 2.16, 3.1, 3.71mg TCS l^{-1} **Mean unionised calculated concentrations:** 0.0, 0.028, 0.090, 0.30, 0.53, 0.69, 0.90, 1.82 2.3, 2.8mg l^{-1} **Mean environmental parameters (SD)**: pH 7.27 (±0.20) (N= 120); Conductivity (mS) 0.0009 (±0.0008) (N= 120); Temperature (^{o}C) 14.7 (± 0.3) (N= 120); DO (%) 79.0 (±4.8) (N= 120); Salinity (PPT) 0 (±0) (N= 120).		
#5	1.73 (1.53–1.93)	0.67 (0.66-0.68)
Mean measured concentrations: 0, 0.027, 0.077, 0.362, 0.618, 0.788, 0.992, 2.018, 2.421, 3.425mg TCS l^{-1} **Mean unionised calculated concentrations:** 0.0, 0.013, 0.039, 0.14, 0.23, 0.34, 0.42, 0.77, 1.1, 1.3mg l^{-1} **Mean environmental parameters (SD)**: pH 8.26 (±0.16) (N= 120); Conductivity 0.0008 (±0.0009) (N= 120); Temperature (^{o}C) 14.7 (±0.02) (N= 120); DO (%) 74.4 (±6.8) (N= 120); Salinity (PPT) 0 (±0) (N= 120).		

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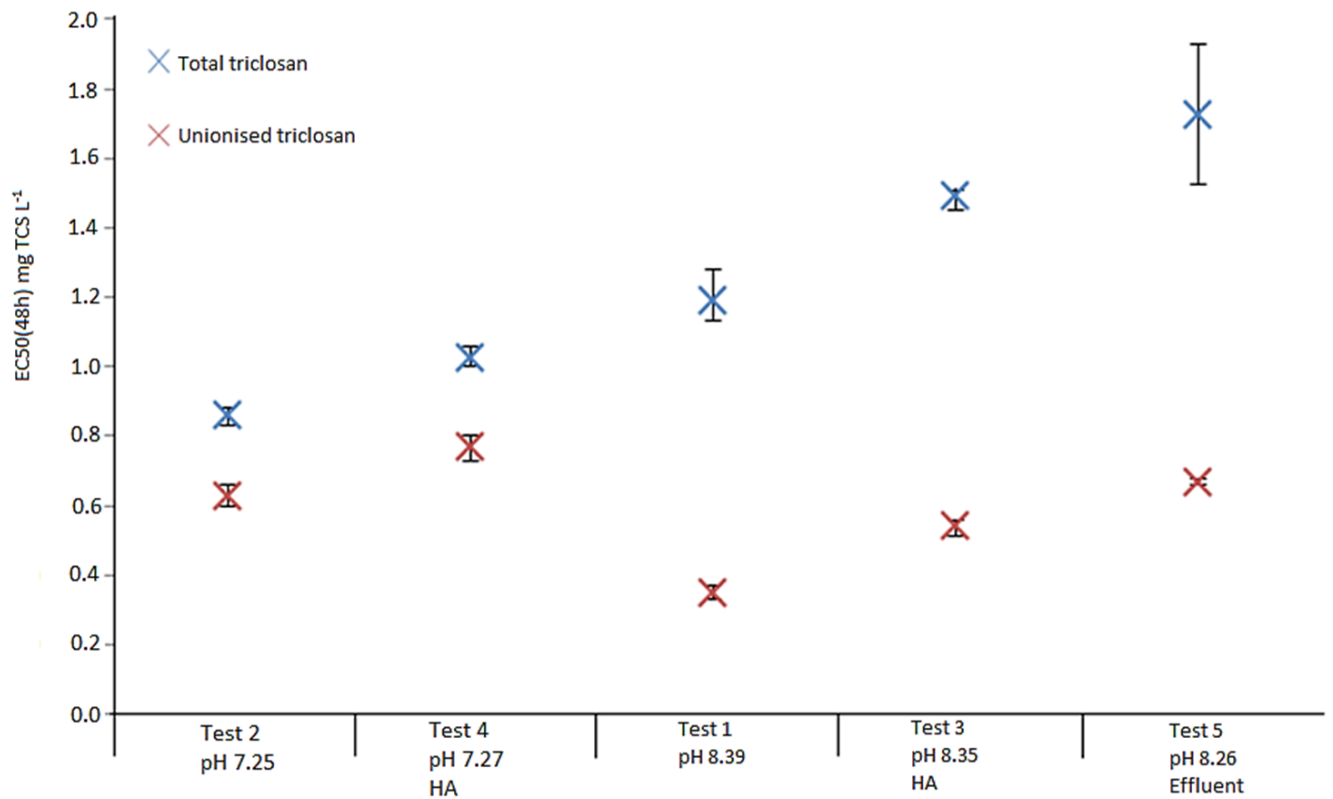
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808 **Figure A3. Mean EC50(48h) values for total triclosan and unionised triclosan for each of**
809 **the different test conditions (error bars = 95% confidence intervals)**

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