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

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

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

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

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

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

(Vellinger *et al*., 2012). It has been used for sublethal testing, such as growth (Maltby *et al*., 2002), reproduction (Welton and Clarke, 1980) activity (Gerhardt *et al*., 1994), and acute endpoints (Güven *et al*., 1999). Furthermore, *G. pulex* is known to be sensitive to a range of stresses (Felten *et al*., 2008). Triclosan was specifically chosen as, like many other personal care products which are of growing concern to regulators, it is a polar compound with ionic characteristics, with a pKa in the range where environmentally relevant pH values significant affect its behaviour and bioavailability.

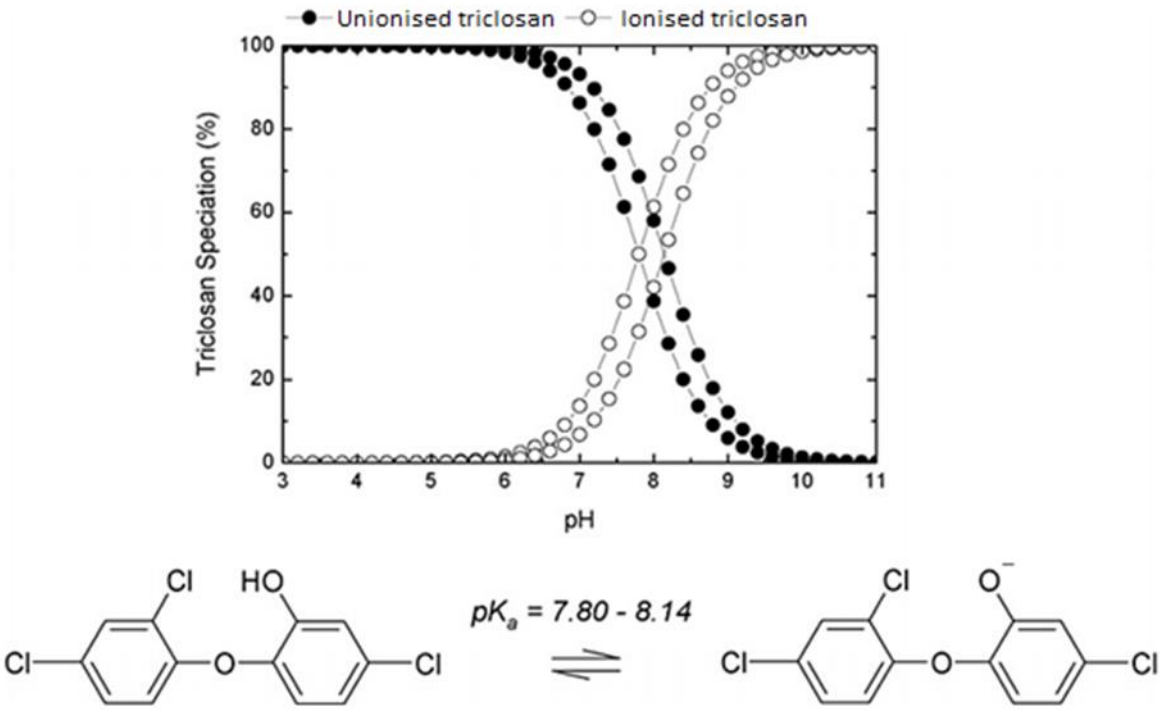

Figure 1. Speciation of triclosan as a function of the solution pH. Calculation based on pKa values of 7.80 and 8.14 (Nghiem and Coleman, 2008 (Edited)).

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

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

The concentrations of zinc in the positive control test and calcium within the stock tanks were determined using an ICP-OES instrument (Thermo Scientific, iCAP 7400) calibrated between 0 and 50 mg L^{-1} and utilising procedural blanks and three replicate samples.

2.4.2 High-Performance Liquid Chromatography (HPLC)

Triclosan was determined using a Shimadzu LC20AD liquid chromatograph, Shimadzu SIL20A HT Autosampler, Shimadzu SPD20A UV-vis spectrophotometer (230nm). A Phenomenex C18 4.6X150 mm reversed-phase column with guard column was used for all determinations. Isocratic elution using 70:30 acetonitrile (HPLC grade, 99.99%) and Milli-Q water mobile phase was used at a 1ml min^{-1} flow rate. Calibration was achieved from the peak areas of triplicate determinations standards made up in methanol (0, 0.5, 1, 3, 5, 10 mg L^{-1}). Standards prepared in varying HA and effluent solutions confirmed a lack of matrix interference.

2.5 Statistical analysis

Observed responses for *G. pulex* immobilisation at measured concentrations were used to model predicted dose response curves and 95% confidence intervals using a Probit Analysis (sigmoidal function) within SigmaPlot® 12.5. The process was undertaken for each test repeat for both 24 and 48 hour results and the modelled data output was used to derive mean test EC10, EC20 and EC50 values. Minitab® statistical software was used to undertake a One-Way Analysis of Variance (ANOVA) with grouping Information using the Tukey post hoc test to identify statistical significance between results. This statistical method was used after confirming normal data distribution using the Anderson-Darling Normality test for all EC values being tested for all testing conditions. Supporting statistical values (DF, SS, MS, F and P) derived during the ANOVA analysis are presented in Table A3 within the ESI.

3 RESULTS

Daily measurements of environmental parameters, and weekly measurements of nutrient levels and hardness (based on Ca content) of the holding tanks collected during the study

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

3.1 Analytical data

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

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

3.2 Toxicity data

3.2.1 Zinc positive control results

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

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).				

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

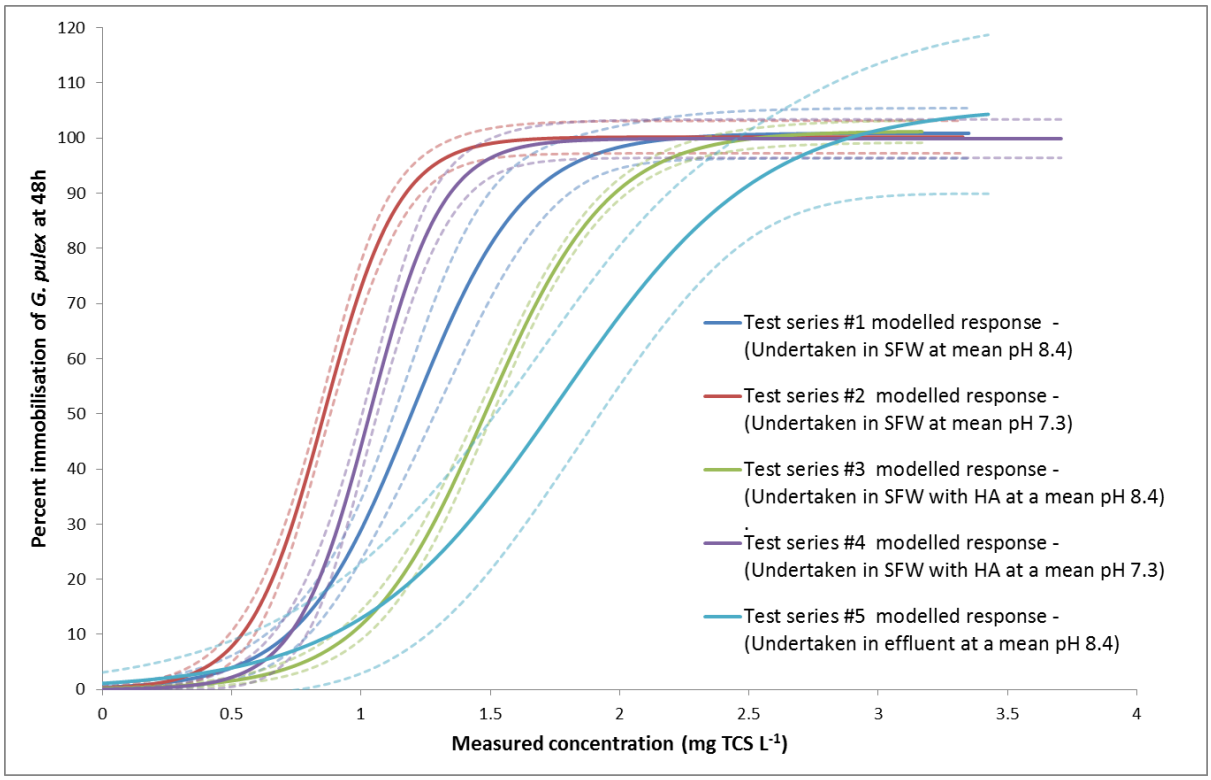

Figure 2. Mean modelled toxicological results and their 95% confidence Intervals (CI) (dashed line) from SigmaPlot® based on data from four repeats of five tests for triclosan to *G. pulex*.

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

3.2.3 Effect of pH

Test series #1, undertaken in SFW at a mean pH of 8.4, was calculated to have an EC50(48h) of 1.22 mg L^{-1}. This was almost 50% higher than the mean EC50(48h) during Test series #2, also undertaken in SFW but at a lower mean pH (7.3), which was calculated as 0.82 mg L^{-1}. The difference between these mean EC50 values was found to be 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

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

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

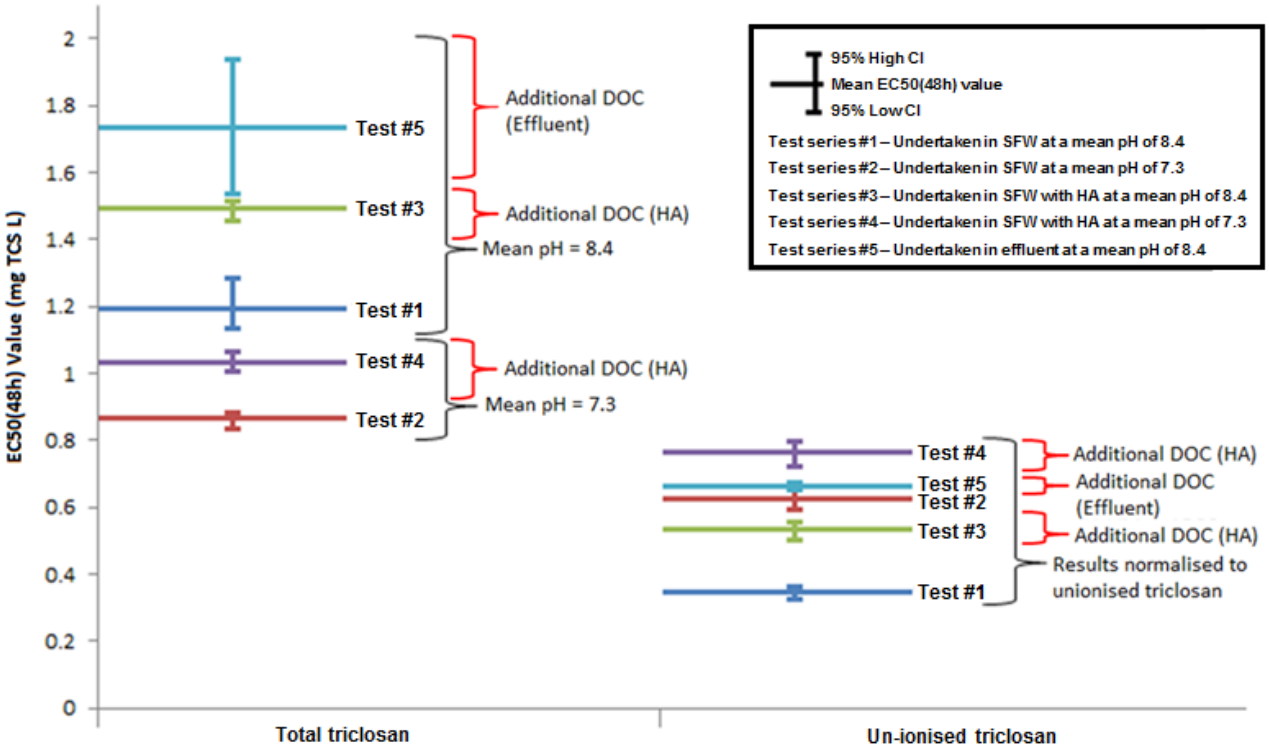

Figure 3. Comparison of variation between EC50(48h) values calculated using both total and un-ionised triclosan concentrations for each of the different test conditions (error bars = 95% confidence intervals)

3.2.5 Effect of triclosan speciation

The percentage of ionised and un-ionised triclosan in a solution can be calculated by using the pKa (taken as 8.00) and the measured pH. This is significant as, for example, with one pH unit change between pH values 7-9 a 40% change in triclosan species is observed (Figure 1). Consequently, the concentration of un-ionised triclosan (most toxic species) was calculated for all experimental data and the EC50s were recalculated (Figure 3, Figure A3 and Table A9 of ESI). These EC values and associated 95% CI were not calculated for each 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

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

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

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	49.7 (11.8)
EC20	0.62	0.91	46.8	
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	80.6 (3.95)
EC20	0.75	1.36	81.3	
EC10	0.63	1.16	84.1	

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

Although normalisation of EC50 for un-ionised triclosan reduces the variance between EC values for similar test conditions (e.g. SFW with and without added HA) it does not eliminate it (Figure 3). This suggests that the varying toxicity between tests is not purely a result of pH. This would be expected for tests containing HA as these chemicals would also behave 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.

436 **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

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

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

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

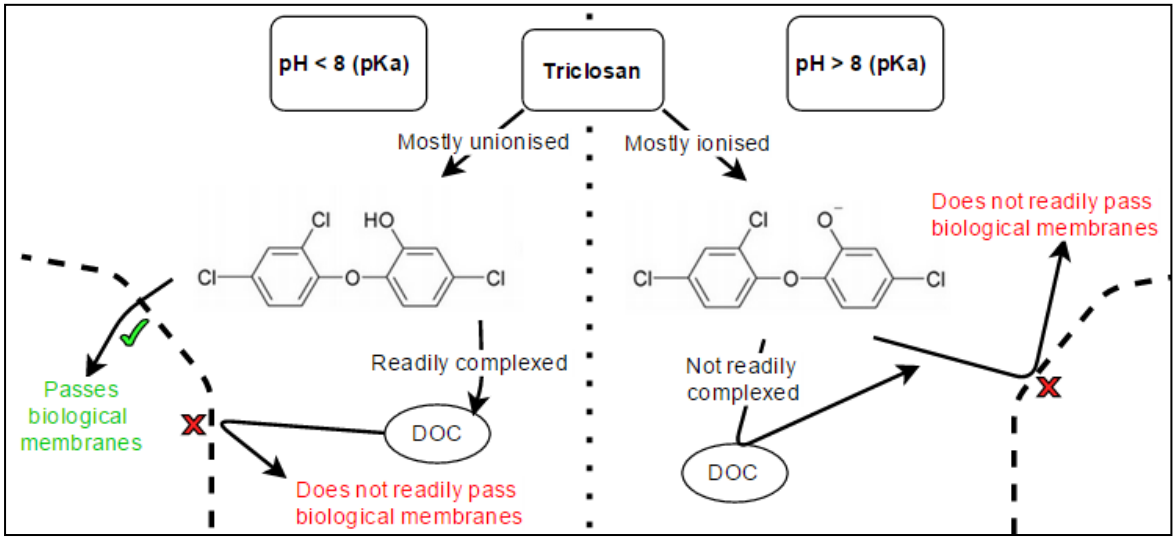

Figure 4. A conceptual diagram displaying the effect of pH and DOC on the bioavailability of triclosan in solution

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

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

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

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

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

4.4 Importance of results and environmental relevance

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

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

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

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

5 CONCLUSIONS

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

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

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

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

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

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

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

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717 ***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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719 ***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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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**	**-**	**-**	**-**	**-**

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 $CaCO_3$)	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

Table A5. Percentage immobilisation of *G. pulex* at 24 and 48 hours for the zinc 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.

Table A6. EC values calculated for the zinc positive control test using observed immobilisation result for *G. pulex* and measured zinc 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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750 **Table A7. Mean percentage *G. pulex* immobilisation during 24 hour exposure to triclosan**
751 **calculated from four repeat tests (n = 4 for all test results) (NB. Measured test**
752 **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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755 **Table A8. Mean percentage *G. pulex* immobilisation during 48 hour exposure to triclosan**
756 **calculated from four repeat tests (n = 4 for all test results) (NB. Measured test**
757 **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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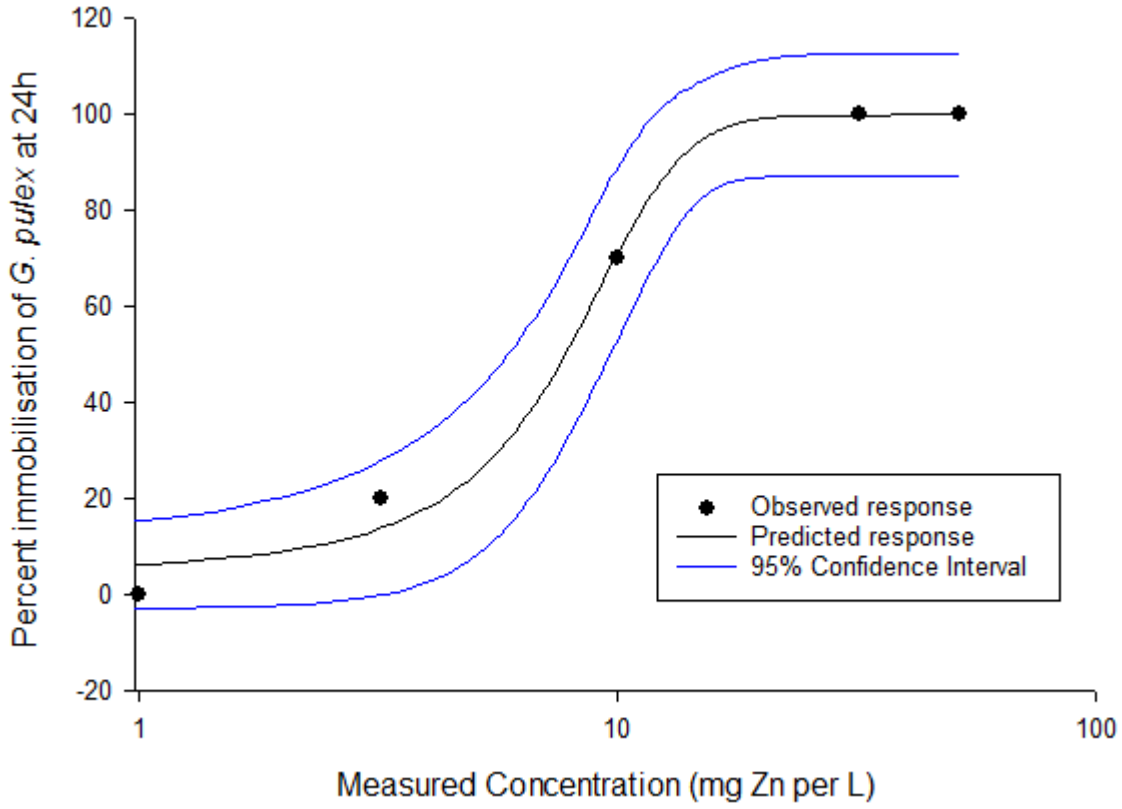

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785 **Figure A1. Graph created in SigmaPlot® showing toxicological results at**
786 **24hours for zinc to G. pulex during the positive control study.**

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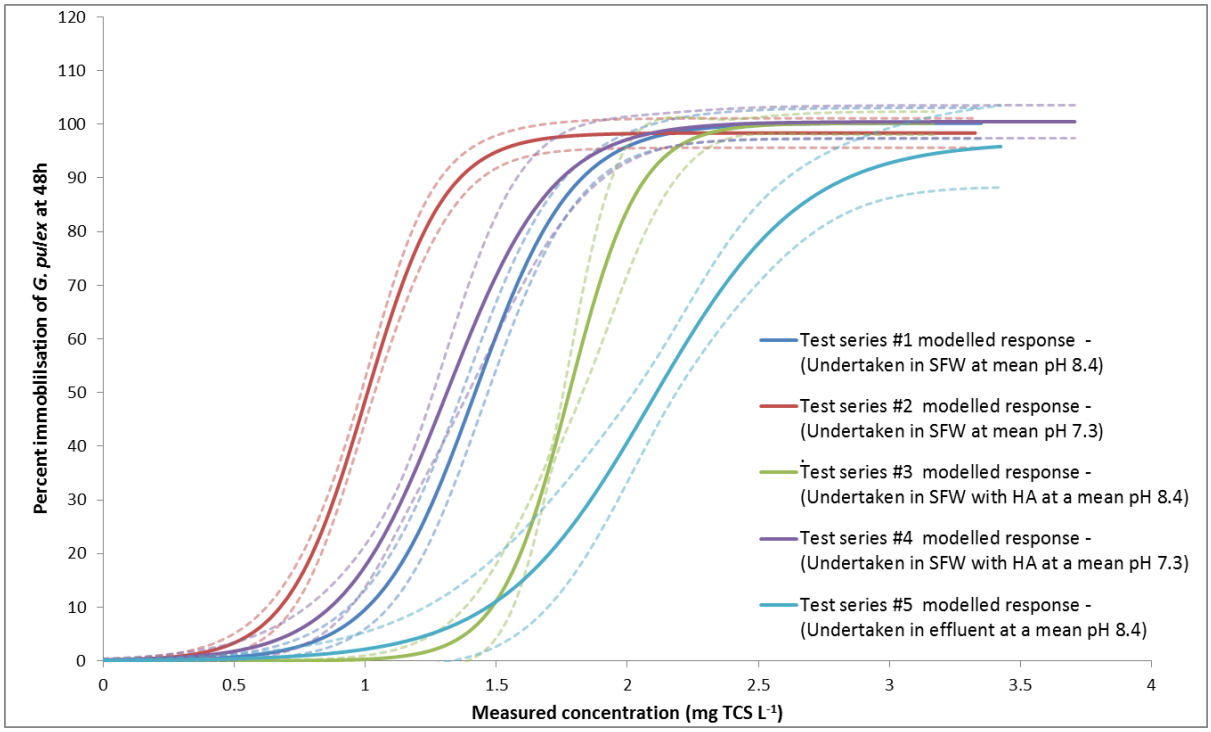

Figure A2. Mean modelled toxicological results and their 95% confidence Intervals (CI) (dashed line) from SigmaPlot® based on data from four repeats of five tests at 24 hours for triclosan to G. pulex, using mean measured triclosan concentration and mean observed immobilisation.

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798 **Table A9. Mean EC50(48h) values for triclosan to *G. pulex* during all tests, calculated using**
799 **mean observed immobilisation from four repeat experiments and both the measure total**
800 **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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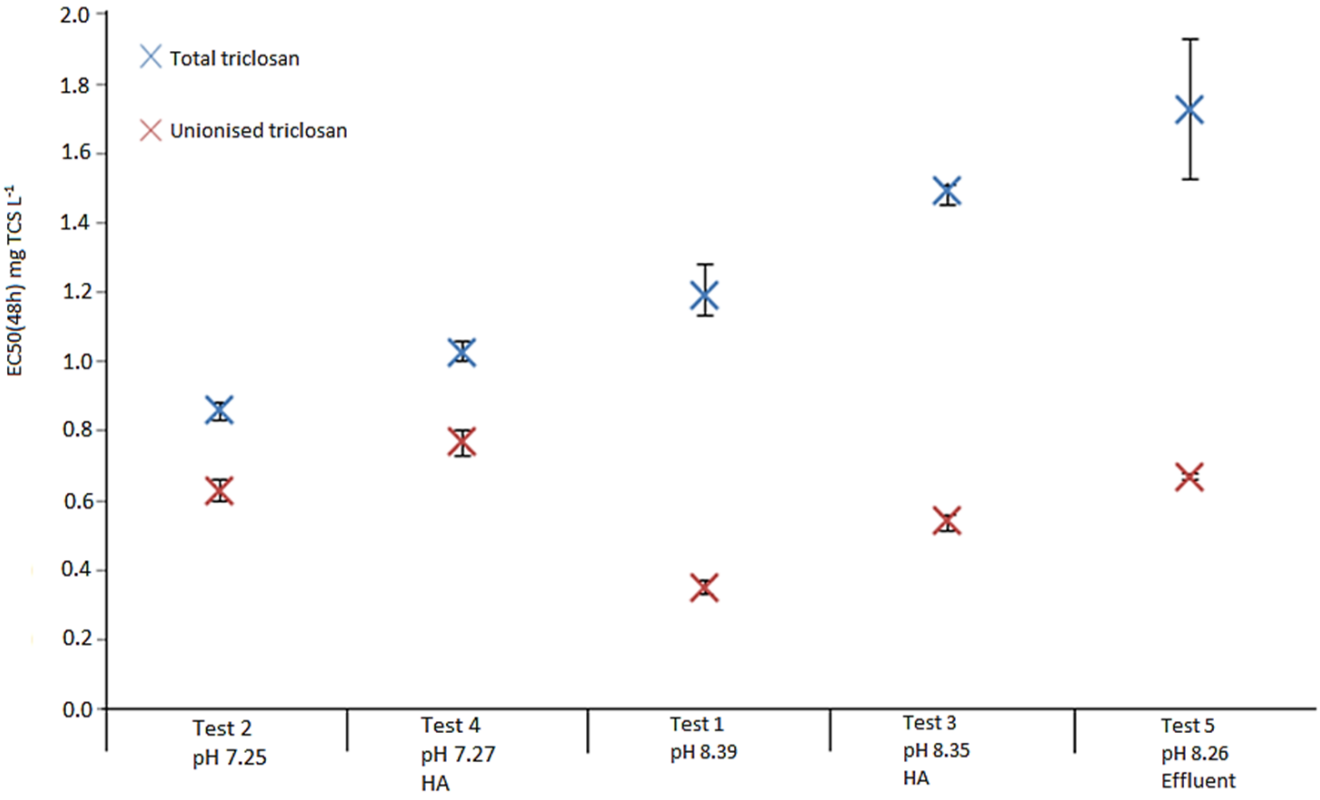

Figure A3. Mean EC50(48h) values for total triclosan and unionised triclosan for each of the different test conditions (error bars = 95% confidence intervals)