Influence of pH on the toxicity of ionisable pharmaceuticals and personal care products to freshwater invertebrates

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Highlights

- Enrofloxacin and triclosan toxicity assessed at different pH conditions
- Toxicity values differed up to a factor of three under different pH conditions
- The efficiency of three pH-dependent toxicity models was evaluated
- Models that only consider the neutral chemical form showed the best fit
- Models allow the inclusion of spatial pH variations into risk assessment

Abstract

The majority of pharmaceuticals and personal health-care products are ionisable molecules at environmentally relevant pHs. The ionization state of these molecules in freshwater ecosystems may influence their toxicity potential to aquatic organisms. In this study we evaluated to what extent varying pH conditions may influence the toxicity of the antibiotic enrofloxacin (ENR) and the personal care product ingredient triclosan (TCS) to three freshwater invertebrates: the ephemeropteran *Cloeon dipterum*, the amphipod *Gammarus pulex* and the snail *Physella acuta*. Acute toxicity tests were performed by adjusting the water pH to four nominal levels: 6.5, 7.0, 7.5 and 8.0. Furthermore, we tested the efficiency of three toxicity models with different assumptions regarding the uptake and toxicity potential of ionisable chemicals with the experimental data produced in this study. The results of the toxicity tests indicate that pH fluctuations of only 1.5 units can influence EC50-48h and EC50-96h values by a factor of 1.4-2.7. Overall, the model that only focuses on the fraction of neutral chemical and the model that takes into account ion-trapping of the test molecules showed the best performance, although present limitations to perform risk assessments across a wide pH range (i.e., well above or below the substance pKa). Under such conditions, the model that takes into account the toxicity of the neutral and the ionized chemical form is preferred. The results of this study show that pH fluctuations can have a considerable influence on toxicity threshold, and should therefore be taken into account for the risk assessment of ionisable pharmaceuticals and personal health-care products. Based on our results, an assessment factor of at least three should be used to account for toxicity differences between standard laboratory and field pH conditions. The models evaluated here can be used to perform refined risk assessments by taking into account the influence of temporal and spatial pH fluctuations on aquatic toxicity.

Keywords: ionisable compounds, pH-related toxicity, freshwater invertebrates, pharmaceuticals, personal care products

1. Introduction

Residues of pharmaceuticals and chemicals contained in personal health care products (PHCPs), have been monitored in a wide range of aquatic ecosystems across the world (Boxall et al. 2004; Kümerer et al. 2009; Ankley et al. 2007; Boxall et al. 2012). Although monitored concentrations are generally low (i.e., ng/L to µg/L range), some of these chemicals are continuously emitted (Monteiro and Boxall 2010), and might pose risks for aquatic organisms (Brown et al. 2007; Bringolf et al. 2010; Kidd et al. 2014). More than 80% of the available pharmaceuticals and PHCPs are known to be ionisable substances at environmentally relevant pH conditions (Manallack et al. 2007). Some studies have demonstrated that changes in water pH can influence the bioavailability, uptake and toxicity of ionisable pharmaceuticals to aquatic model organisms, where ionisable substances are generally more bioaccumulative and toxic in their neutral than in their charged form (Valenti et al. 2009; Kim et al. 2010; Rendal et al. 2011a; Meredith-Williams et al. 2012; Karlsson et al. 2017).

The three main processes that influence the behavior of ionisable compounds with changing pHs are: i) the reduction in lipophilicity when a neutral compound becomes ionized, which limits uptake and toxicity, ii) electrical attraction, which influences the uptake of cations in negatively charged cells, and iii) the ion trap effect, which depends on the pH gradient between the exposure medium and inside the organism's body, and the differences in dissociation of the chemicals in these two compartments (Rendal et al. 2011b). Bioaccumulation and toxicity predictive models used for the ecological risk assessment of pharmaceuticals and PHCPs are generally based on the hydrophobic nature of chemicals and may therefore provide less accurate predictions when applied for ionisable substances. Some studies have proposed alternative bioaccumulation modelling approaches based on the pH-corrected octanol/water partition coefficient or the pH-corrected liposome/water partition coefficients to predict the bioaccumulation of ionisable substances in aquatic organisms (Paterson and Metcalfe 2008; Fu et al. 2009; Meredith-Williams et al. 2012). For example, Karlsson et al. (2017) presented a combined experimental and modeling approach to characterize the uptake of three ionisable chemicals to the annelid *Lumbriculus variegatus* over time at different pH conditions in contaminated water and sediment exposure scenarios. Taking into account the range of water pHs measured in European streams, Karlsson et al. (2017) estimated

that uptake of highly ionisable substances may vary by a factor of more than 3000 depending on the pH conditions, which may have severe consequences for the bioaccumulation and ecotoxicological potential of these substances.

Several authors have proposed toxicity models of different complexity to predict toxicity variation of pharmaceuticals regarding fluctuating pH values. Boström and Berglund (2015) proposed a simple model to predict acute toxicity to *D. magna* based on the fraction of neutral chemical and assuming that only this fraction is active. Neuwoehner and Escher (2011) tested the pH-dependent toxicity of five basic pharmaceuticals on the green algae *Scenedesmus vacuolatus* and developed two mechanistic models that take into account the differences in toxicity related to the neutral and the charged chemical form. The first model assumes that the neutral and the charged form of the chemical are biologically active but have different toxicities, and that the effect of the two forms can be predicted based on the concentration addition model. The second model is based on the ion trap effect and assumes a preferential uptake of the neutral form of the chemical followed by a fast intracellular dissociation. Recently, Baumer et al. (2017) tested the three afore-mentioned models for 42 pharmaceuticals with a pH gradient of 5.5 to 9, using the bioluminescence inhibition test with the bacterium *Aliivibrio fischeri*. These authors concluded that neither the model that neglects uptake of the charged fraction, nor the model that accounts for equal uptake between the charged and uncharged fraction fully explain the observed results. Probably the actual processes interfering with the compound's toxicity are in between the two assumptions proposed by these models. On the other hand, the model that takes into account ion trapping improved predictions for some pharmaceuticals and pH values, but not for all (Baumer et al. 2017).

The quantitative estimation of the pH-dependency of effects of pharmaceuticals and PHCPs chemicals on aquatic organisms is important for several reasons. First, to provide recommendations on worst-case pH values (or ranges) to be used in further toxicity testing. Second to assess their toxicity taking into account daily pH fluctuations of freshwater ecosystems. And third, to make risk extrapolations across different aquatic ecosystems with substantial pH differences (e.g. oligotrophic vs eutrophic). To date, the available models for assessing pH-dependent toxicity have been mainly evaluated with microorganisms and *D. magna*, while there is little or no information

regarding their predictive power for non-standard test invertebrates and other higher aquatic organisms. This leaves a margin of uncertainty on the suitability of the proposed modelling tools for making risk predictions for species with different biological traits, which should be further studied and incorporated into future hazard and risk assessments.

The main objectives of the present study were to assess the toxicity of a pharmaceutical and a PHCP ingredient to three aquatic invertebrates under a gradient of environmentally relevant pH conditions, and to evaluate the suitability of the aforementioned pH-dependent toxicity models for them. The selected compounds were enrofloxacin (ENR) and triclosan (TCS). ENR is a fluoroquinolone antibiotic which is frequently used as veterinary medicine in livestock and aquaculture production (Boxall et al. 2003; Rico et al. 2014; Sun et al. 2016). It can be considered as a weak acid or a weak base due to its dual pKa value (pKa_1=6.06; pKa_2=7.70) and has a relatively low bioaccumulation potential (log Kow=0.39; Table S1). TCS is an antimicrobial compound used as component of a wide range of PHCPs such as body soaps and toothpastes (Singer et al. 2002; Tsai et al. 2008). It is a weak acid (pKa=8.14) with relatively high hydrophobic characteristics (log Kow=4.76; Table S1). Some studies have shown high dissociation properties and varied toxicity exerted by these chemicals to aquatic standard test organisms depending on the tested pH (Kim et al. 2010; Khatikarn et al. 2016; Li et al. 2018). In this study we extend these evaluations with non-standard test organisms and provide some recommendations on the extrapolation factors needed to account for toxicity differences between standard laboratory and varying pH conditions usually observed in the field.

2. Materials and methods

2.1 Study chemicals

ENR (active ingredient ≥ 98%) and TCS (active ingredient ≥ 97%) were purchased from Sigma Aldrich (St Louis USA). Separate stock solutions of ENR (50 g/L) and TCS (2 g/L) were prepared by diluting the pure substances in Milli-Q water with the help of NaOH, and were stored at -20°C until their use in the experiments.

2.2 Test organisms

The toxicity of ENR and TCS was evaluated on three invertebrate species: the amphipod crustacean *Gammarus pulex*, the insect nymphs of *Cloeon dipterum* and the freshwater snail *Physella acuta*. *G. pulex* were collected from an uncontaminated stream in Heelsum, the Netherlands. *C. dipterum* and *P. acuta* were collected from the outdoor mesocosms of the Sinderhoeve research station (Renkum, the Netherlands, www.sinderhoeve.org). The collected organisms were acclimatized to the laboratory conditions for at least 48 h prior to the start of the experiments. For this, organisms were kept in plastic buckets filled with uncontaminated groundwater, using a constant temperature of 20°C and a light:dark regime of 12:12 h.

Prior to the experiments the water content, the lipid content and the internal pH of the test organisms was evaluated (Table 1). The first two parameters were measured to characterize the test organisms, while the internal pH was used for the modeling calculations. The water content was calculated as the difference between the wet weight of the animals measured alive (after external water elimination with a paper tissue) and the dry weight measured after water evaporation in the oven (105 °C) for 24 h (APHA, 2005). The lipid content was determined using an adaptation of the method described by Folch et al. (1957). Briefly, dried individuals were weighed and introduced into a chloroform and methanol (2:1) solution. The sample was homogenized using an orbital shaker at 20°C and then centrifuged for 20 min at 1400 rpm. The supernatant was transferred into a new centrifuge tube. The sample volume was measured and water was added (20% of the sample volume). Next, the centrifuge tubes containing the sample were vortexed for 30 s to separate the water from the lipid layer of the sample. The lipid phase was transferred into a pre-weighed vial and the excess solvent contained in this sample was evaporated under a nitrogen stream. After evaporation, the vials were weighed again and the total lipid content of the sample was determined to calculate the lipid content of the aquatic organisms. The internal pH of the test organisms was determined according to the method described by Sommer et al. (2000). The internal pH was measured using an ion-selective pH sensor (unisensor), which contained a reference sensor and a measuring micro sensor. Before measurements, measuring and reference micro sensors were both calibrated with pH 4 and 7. After this, we inserted

both micro sensors into one organism of *P. acuta*. The same technique could not be applied to *G. pulex* and *C. dipterum* due to their small size as compared to *P. acuta*. For *G. pulex* and *C. dipterum*, three individual organisms were put together and smashed in 2 mL of Milli-Q water. Then, both micro sensors were inserted into the solution formed and the pH was read from this sample.

2.3 Toxicity experiments

Toxicity experiments were performed following a 4 x 6 factorial design, with 4 different pHs (6.5, 7, 7.5 and 8), one control and 5 chemical concentrations. The pHs were considered environmentally relevant, and were selected taking into account the dissociation constant of the test chemicals and the pH tolerance range of the test organisms based on preliminary tests. The test concentrations were decided according to the outcomes of previously performed toxicity range-finding tests (Table S2). The toxicity experiments were carried out in triplicate using glass beakers containing 500 mL of exposure media (groundwater) and 10 individuals per test unit, except for the *P. acuta* with ENR, for which 8 individuals were used. The experiments lasted for 96 h and the pH of the exposure media was measured and adjusted every 24 h by titration with 0.1 M hydrochloride acid (HCl) in the 6.5, 7 and 7.5 pH levels, and with 0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer in the 8 pH level.

The experiments were performed following some general recommendations provided in the Organisation for Economic Co-operation and Development (OECD): test guideline No. 202 (OECD, 2004). For example, experiments were only considered as valid when the immobility did not exceed 10% during the experimental period in the chemical controls. The chosen temperature and light:dark regime was 20°C and 12:12h, respectively. The beakers of the *G. pule*x experiment contained a stainless steel mesh that was used as distraction material to prevent cannibalism among them. Temperature, conductivity and dissolved oxygen concentration in the exposure media were measured at the beginning and at the end of the toxicity experiment (Table S3). Immobilization was used as evaluation endpoint, which can be considered a proxy of mortality and is commonly used to assess effects on small organisms, for which it is difficult to distinguish between immobile and dead ones. The number of immobile animals was counted in each replicate at 48 h and 96 h after the start of the exposure period. *G. pulex*

and *C. dipterum* individuals were counted as immobile when they showed inability to move after a tactile stimulus provided with a glass Pasteur pipette. *P. acuta* individuals were considered as immobile when no reaction was observed after tactile stimuli of the soft body for three times with a glass Pasteur pipette or when they were turned upside down.

2.4 Chemical analyses

ENR and TCS concentrations were measured in the test medium at 2 h and 96 h after the application of the test compounds to verify the nominal concentrations and to assess the dissipation of the test compounds (Table S4). Water samples were filtered through a 0.22-µm cellulose acetate membrane. Next, the sample was diluted by adding 200 µL of acetonitrile to 800 µL of test medium sample in glass amber vials. The samples taken for the analysis of TCS were centrifuged at 4500 rpm for 20-30 min. Finally, 1 mL of the supernatant was transferred to 2 mL-amber glass vials using a glass Pasteur pipette.

Chemical quantification was performed by injecting the amber glass vials into a triple quadrupole LC/MS system equipped with an ESI+. A full description of the equipment and conditions used for the analysis of ENR and TCS are provided in the Supporting Information (see also Tables S5 and S6). Additional tests were performed to evaluate the recovery of ENR and TCS from the test medium, using a concentration of 1 mg/L of ENR and 634 µg/L of TCS, which are in the low-to-middle range of the concentrations used in the toxicity tests. The mean recovery rates for ENR and TCS from the water medium ranged between 64% and 98%, and between 108% and 141%, respectively (Table S7).

2.5 Toxicity models

Model 1: Only the neutral chemical form is active

The model considers the speciation of compounds in the exposure medium, and assumes that the neutral chemical form is taken up faster than the charged, so that the charged form does not contribute at all to the observed effect and can be neglected (Boström and Berglund 2015). The fractions of neutral molecules are calculated based

on the Henderson-Hasselbach equation according to:

$$\alpha_N = \left(\frac{1}{1+10^{pKa_1-pH}+10^{pH-pKa_2}}\right) \text{ for ENR} \quad \text{(eq. 1)}$$

$$\alpha_N = \left(\frac{1}{1+10^{pH-pKa}}\right) \quad \text{for TCS,} \quad \text{(eq. 2)}$$

For ENR, we used pKa_1= 6.06 and pKa_2=7.7 (Kim et al. 2010); for TCS, we used pKa=8.14 (Aldous et al. 2012).

The EC50 (pH) at a given water pH value is defined as:

$$EC_{50}(pH) = \frac{1}{\alpha_N} \cdot EC_{50}(neutral) \quad \text{(eq. 3)}$$

where α_N refers to the fraction of neutral or uncharged chemical, and EC50 (neutral) refers to the EC50 of the neutral chemical form. Hence, the slope coefficient ($1/\alpha_N$) is calculated and used as independent variable in a linear regression, and the EC50 (neutral) is determined from the regression slope coefficient.

Model 2: Both chemical forms are active and act additively

The model assumes that both the neutral and the charged forms are biologically active but with different effect concentrations, EC50 (neutral) and EC50 (charged), and that the neutral and the charged concentration act additively in the mixture, i.e., using the concentration addition model (Neuwoehner and Escher 2011). The EC50 at a given pH is defined as:

$$\frac{1}{EC_{50}(pH)} = \left(\frac{1}{EC_{50}(neutral)} - \frac{1}{EC_{50}(charged)}\right) \cdot \alpha_N + \frac{1}{EC_{50}(charged)} \quad \text{(eq. 4)}$$

Hence, the fraction of neutral chemical (α_N,) is used as independent variable in a linear regression, and the EC (neutral) and EC50 (charged) are determined from the slope and intercept regression coefficients. For simplicity, we assume that the cationic chemical form (in the case of ENR) does not contribute to the overall effect and consider only the anionic form.

Model 3: Only the neutral chemical fraction is active and results in an ion-trap effect

Similarly to model 1, this model assumes that the uptake of neutral chemical form by the aquatic organisms is much faster than that of the charged one, and therefore assumes permeability of the neutral chemical form only. Moreover it considers dissociation of the chemical inside the organisms due to a difference between the pH of the exposure medium and the internal pH of the organisms, leading to an ion trap effect. According to Büttner and Büttner (1980), the relationship between the internal concentration of the neutral chemical form and the external concentration can be formulated as:

$$C_{int,neutral} = C_{ext,neutral} \cdot \frac{1+10^{pH_{int}-pKa}}{1+10^{pH_{ext}-pKa}} = C_{ext,neutral} \cdot BCF_N \qquad \text{(eq. 5)}$$

where $C_{int,neutral}$ refers to the internal concentration of the neutral chemical form, $C_{ext,neutral}$ the external concentration of the neutral chemical form, and BCF_N to the bioconcentration factor calculated for the neutral chemical.

Then, the following equation can be derived to estimate the EC50 at a given pH:

$$EC_{50}(pH) = \frac{1}{BCF_N} \cdot EC_{50}(neutral, int\ pH) \qquad \text{(eq. 6)}$$

where the independent variable ($1/BCF_N$) is plotted in a linear regression form, and the EC50 (neutral, int pH) is determined from the slope regression coefficient.

2.5 Data analyses

The immobility data obtained from the toxicity experiments were used to calculate EC50 (immobility) values, and their 95% confidence intervals, after an exposure period of 48 h and 96 h. The calculations were performed using a log-logistic regression model as described by Rubach et al. (2011), and using the GenStat 11th edition software (VSN International Ltd., Oxford, UK). All calculations were done on the basis of the average measured exposure concentrations during the experimental period. Models 1-3 were implemented in Mathematica 12.0 (Wolfram Research) and fitted to experimental data. Linear regression coefficients (R^2) and Pearson p-values were calculated using the

method “LinearModelFit”, and were used as indicators of correspondence between the calculated experimental data and the fitted models.

3. Results and discussion

3.1 Invertebrate’s sensitivity at different pH levels

Toxicity tests were performed to evaluate the sensitivity of the three invertebrate species to ENR and TCS at four different nominal pH levels. Differences between the measured pH values and the nominal pH in the test medium of the toxicity experiments were generally within 0.2 units, with few exceptions going up to 0.3 units (Table 2). This indicates the pH was succesfully controlled in the different treatments. No immobility was recorded in the controls of the ENR experiments, while in the test units without TCS addition some immobolity was observed only for *G. pulex* and *P. acuta*, reaching maximum values of 7% and 10%, respectively. No clear relationship was observed between the pH in the chemical controls and the observed immobility. This supports the assumption that any potential differences of the toxicity of the chemicals is related to their dissociation at different pH conditions, and not to an influence of the pH on the fitness of the test organisms. The observed immobility could have been caused by some damage due to the manipulation of the organisms when setting up the experiments, and was considered acceptable since it was within or close to the maximum treshold (10%) established by the OECD guideline (OECD, 2004).

Measured concentrations of ENR in the three toxicity experiments were within 67% and 130% of the nominal concentrations at the start of the experiment (2h after the application) and were kept relatively constant during the experimental period. Measured concentrations of TCS at the start of the experiment were within 77-132% of the nominal concentrations in the three tests. TCS, however, showed a faster dissipation rate as compared to ENR with concentrations becoming 30% of the initial measured concentrations at the end of the 96h exposure period. The dissipation was taken into account in the EC50 calculations (by using the average measured concentrations), and was not found to be pH-dependent. According to Aranami and Readman (2007), the fast water dissipation of this compound is explained by its photolytic nature, its high sorption capacity to organic matter, and to a lower extent by hydrolisis. Given the test

conditions in our study (i.e., no sediment and low density of living organisms), photolysis and hydrolisis are the most likely degradation routes, however this was not assessed experimentally.

The tested aquatic organisms were clearly more sensitive to TCS than to ENR, with EC50's differing by about 2-3 orders of magnitude. This can be partly related to differences in the bioacumulative potential of both molecules, with TCS having a Kow that is about four orders of magnitude larger than that of ENR (see Table S1). The EC50-48h values for ENR to *G. pulex*, *C. dipterum* and *P. acuta* at different pH conditions were 36-58, 27-70 and 115-206 mg/L, respectively; while those for TCS were 0.19-0.55, 0.26-0.51 and 0.51-1.29 mg/L, respectively. The EC50-96h values for ENR to *G. pulex*, *C. dipterum* and *P. acuta* at different pH conditions were 16-24, 21-29 and 80-143 mg/L, respectively; while those for TCS were 0.06-0.1, 0.09-0.24 and 0.29-0.70 mg/L, respectively (see Table 2). Overall, *G. pulex* and *C. dipterum* showed a higher sensitivity to both chemicals as compared to *P. acuta*, which may be related to some differences in the water and lipid content (Table 1), but also to different morphological and physiological traits influencing toxicokinetics of the tested molecules in the organisms (Rubach et al. 2012; Rico et al. 2015).

The sensitivity of the tested species to ENR is similar to that reported by other studies performed with standard and non-standard invertebrate species. For example, Park and Choi (2008) reported an EC50-48h for *D. manga* of 56.7 mg/L, and Williams et al. (1992) reported an EC50-48h (mortality and morbidity) for larvae of the shrimp *Litopenaeus vannamei* of 29.4 mg/L. In another study, Rico et al. (2014) described the sensitivity of five invertebrate species collected from tropical ecosystems and reported a toxicity range of 202-520 mg/L (EC50-48h). This range is slightly above the values found in our study. However, in their tests, pH values increased well above 7. The sensitivity of the tested species to TCS is also in the range of that reported by other authors. Orvos et al. (2002) report an EC50-48h for *D. magna* of 0.36 mg/L, while Khatikarn et al. (2016) describes an acute sensitivity range (EC50-48h and 96h values) for non-standard tropical and temperate invertebrate species between 0.07 and 2.9 mg/L.

Based on the measured pH values, the dissociation percentage of ENR in the different treatmens aproximately varied from 24% to 64% (Table 2). The fraction of neutral

chemical form in the pH 8 treatment of the ENR toxicity tests was aproximately 2 times lower than that in the pH 6.5 treatment. Accordingly, ENR EC50-96h values at pH 8 were 2, 1.4 and 1.8 times higher than those calculated at pH 6.5 for *G. pulex*, *C. dipterum* and *P. acuta*, respectively (Table 2). Hence, the neutral chemical fraction difference and the EC50-96h differences between pH 8 and 6.5 were very similar for ENR. This supports that the toxicity of this compound is closely related to the fraction of neutral chemical. Our findings are in line with the study by Kim et al., (2010), who reported an increase in the toxicity of ENR (EC50-48h, immobilisation) to *Daphnia magna* of 1.7 with a pH difference of 1.8 units.

The dissociation of TCS in the tested pH range was a bit lower than for ENR, and ranged from 3% to 35%, approximately (Table 2). The fraction of neutral chemical in the pH 8 treatment of the TCS toxicity tests was about 1.5 times lower than that in the 6.5 pH treatment. The TCS EC50-96h values for *C. dipterum* and *P. acuta* at pH 8 were 2.7 and 2.1 times higher than those calculated at pH 6.5. For *G. pulex*, TCS EC50-96h values were low and showed less marked differences; however EC50-48h values showed the same trend as for the other invertebrates, with a toxicity value that was 1.5 times higher in the pH 8 treatment as compared to the 6.5 treatment (Table 2). The later results are similar to those reported by Rowett et al. (2016), which show an increase of 1.6 times in the EC50-48h of TCS to *G. pulex* when the pH increased in a similar pH range (7.3 to 8.4). In contrast, for *C. dipterum* and *P. acuta* the toxicity of TCS showed a sligthly larger variation than expected regarding the change in the fraction of neutral chemical. Li et al. (2018) also reported large pH-dependent effects of TCS to *Daphnia magna*, with an increase of almost 4-fold when the pH increased from 5 to 9. Karlsson et al. (2017) found that the uptake rates of the neutral and ionized form of TCS to the freshwater worm *Lumbriculus variegatus* were very similar, and Erickson et al. (2006) presented similar conclusions for chlorinated phenols uptake in fish gills at different pH values. These studies sugest that the uptake of the ionized form of TCS could have been as fast as for the unionized form, and therefore toxicity would possibly depend less on pH values. Our observations do not confirm these results, neither do other authors that have reported toxicity test results with algae (Roberts et al. 2014; Khatikarn et al. 2016), *D. magna* (Li et al. 2018) or fish embryos (Klüver et al. 2019). Another explanation for such large pH-dependent toxicity effect may be related to ion trapping in the lowest tested pH, altough differences between the organism pH and the medium pH were not

436 considerably large for *C. dipterum* and *P. acuta* (Table 1 and 2).
437
438 **3.2 pH-dependent toxicity models**
439
440 Model 1 showed a good representation of the variability in the pH-variable toxicity
441 values for both tested compounds (Fig. 1 and 2, Table 3), with R^2 values above 94%
442 and 85% for ENR and TCS, repectively, and significant Pearson correlations (p-values
443 < 0.05). This was expected, as differences in toxicity are related to the changes in the
444 ionization fraction of the evaluated substances. However, Model 1 is rather
445 counterintuitive, as fully charged chemicals have also shown to display toxicity (Escher
446 et al. 2017), so it is questionable whether it will provide accurate results in wider pH
447 ranges that result in a broad spread on the fraction of neutral chemical. Cases of poor
448 fitting of this model with experimental data for aquatic organisms are reported by
449 Boström and Berglund (2015) and Baumer et al. (2017) for several acids and bases
450 tested with a wider pH range.
451
452 From a theoretical point of view, Model 2 would be the preferred option as compared
453 to Model 1 since it assumes that both the charged and the neutral chemical forms are
454 active, and altough have different toxic potency, they act additively. However, Model 2
455 showed the poorest fit for ENR and TCS, with Pearson correlation p-values above 0.05
456 (Table 3). Baumer et al. (2017) argue that this model should be preferably tested for
457 compounds that allow a wide range of speciation at environmental pH values, covering
458 a neutral chemical fraction of 0.1 to 0.9. This was not the case in our study, partly
459 because preliminary tests showed unacceptable effects in the test organisms beyond the
460 tested pH range. Baumer et al. (2017) found that the ratio between the EC50s (charged)
461 and EC50s (neutral) for several ionisable compounds varied up to four ordes of
462 mangitude. In our study, differences between the charged and neutral EC50 values
463 varied between the tested invertebrate species and were up to two orders of magnitude
464 in the case of ENR EC50-48h *P. acuta* (see Table 3). The latter confirms that for ENR
465 the EC50s (neutral) is more toxic than the EC50s (charged). In the case of TCS, most
466 calculated EC50 (charged) values were negative. This problem is related to the
467 unstability of the model when the intercept, the inverse of the EC50 (charged), is very
468 low. This problem has been earlier reported by Baumer et al. (2017), and yields
469 meaningless extrapolated EC50 values for the neutral and the charged chemical forms.

Therefore we can conclude that for TCS, Model 2 was not a suitable option.

Model 3 showed a very good performance, with calculated R^2 values at 96h that were above 90% for ENR and TCS, and significant Pearson correlation p-values (Fig. 1 and 2; Table 3). This model is similar to Model 1, in the sense that only takes into account transport of the neutral chemical form, but considers ion trapping inside the organism. As previously mentioned, ion trapping may have ocurred to some extent in the lowest pH treatment, particularly to *G. pulex*, which shows the largest difference between the internal pH and the exposure medium pH (Table 1). In fact, this invertebrate species shows also the largest difference between the EC50 internal (calculated for Model 3) and the EC50 neutral (calculated for Model 1; Table 3), both for ENR and TCS. However, these results must be interpreted taking into account that only a narrow pH range could be tested, the internal pH values of the tested organisms were close to neutrality, and the variability in the EC50 values was comparatively large. This explains why the results of Model 3 are very similar to those provided by Model 1 (Table 3).

4. Conclusions

This study supports the need to take into account the variability in pH conditions of aquatic ecosystems for the risk assessment of ionizable pharmaceuticals and PHCPs. It shows that the toxicity of ENR and TCS to freshwater organisms may differ by almost a factor of three under changed pH of the exposure medium and dissociation of the test compounds. The sensitivity of the invertebrate species included in this study and the pH-dependent toxicity found for ENR and TCS is similar to that described in other studies with standard test species. Our study suggests that at least an assessment factor of three is needed to cover pH differences between the ones used in the laboratory tests (usually 7-8) and other environmentally relevant pHs for preliminary risk assessment studies. Moreover, this study shows the efficiency of three models that can be used to extrapolate toxicity values under different pH conditions. Out of the three evaluated models, the model that takes into account uptake of only the unionized fraction of the chemical (Model 1) and the model that takes into uptake of the unionized fraction with ion trapping inside the organism (Model 3) showed the best performance, altough these models are known to be less suitable to extrapolate toxicity to wide pH ranges (i.e., well beyond the pKa value of the evaluated substance). For such purposes, the model that

takes into account toxicity produced by the neutral as well as the ionized fraction of the test chemical (Model 2) may be preferred, although it shows some practical limitations and requires further validation with aquatic organisms. The models described in this study can be considered as useful tools for assessing chemical risks taking into account daily pH fluctuations and pH variation across water bodies at the landscape scale, and therefore contribute to improve the risk assessment of ionizable pharmaceuticals and PHCPs for freshwater ecosystems.

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Conflict of interest

The authors declare no conflicts of interest.

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Tables

Table 1. Water content, lipid content and internal pH of the tested organisms (mean ± SD).

Species	Water content (%) (n=30)	Internal pH (n=5)	Lipid content (%) (n=4)
G. pulex	80.9±3.36	7.91±0.20	1.37 ± 0.21
C. dipterum	42.0±14.1	7.10±0.08	6.22 ± 0.25
P. acuta	87.7±4.40	6.97±0.26	1.98 ± 0.06

693 **Table 2**. EC50 values for enrofloxacin (ENR) and triclosan (TCS) on the three test invertebrate species at different pH conditions. The measured
694 pH conditions in the test medium are provided together with the calculated fraction of neutral chemical (α_N).
695

			48 h			96 h		
Chemical	Species	Nominal pH	Measured pH (mean ± SD)	α_N	EC50 (mg/L) (95% CI)	Measured pH (mean ± SD)	α_N	EC50 (mg/L) (95% CI)
ENR	*G. pulex*	6.5	6.75±0.04	0.76	35.5 (29.4-42.8)	6.65±0.04	0.74	16.3 (NC)
		7.0	7.12±0.02	0.74	42.1 (33.9-52.4)	7.1±0.02	0.74	15.6 (11.9-20.5)
		7.5	7.49±0.02	0.61	55.1 (NC)	7.49±0.01	0.60	22.1 (17.8-27.4)
		8.0	7.88±0.04	0.40	58.2 (48.1-70.5)	7.91±0.03	0.38	24.3 (NC)
	C. dipterum	6.5	6.72±0.04	0.76	26.7 (19.9-35.9)	6.68±0.03	0.75	21.4 (15.8-29.1)
		7.0	7.16±0.03	0.73	34.6 (27.0-44.4)	7.13±0.02	0.74	26.9 (21.4-33.8)
		7.5	7.54±0.02	0.58	34.4 (26.8-44.1)	7.53±0.01	0.58	26.8 (21.2-34.0)
		8.0	7.94±0.02	0.36	69.5 (58.4-82.7)	7.95±0.02	0.36	29.2 (22.6-37.7)
	P. acuta	6.5	6.68±0.04	0.75	115 (NC)	6.63±0.05	0.74	79.7 (68.6-92.6)
		7.0	7.28±0.06	0.69	133 (110-160)	7.20±0.04	0.72	112 (91.6-137)
		7.5	7.50±0.04	0.60	192 (154-239)	7.50±0.03	0.60	121 (99.3-148)
		8.0	7.88±0.06	0.39	206 (163-259)	7.92±0.04	0.37	143 (116-176)
TCS	*G. pulex*	6.5	6.74±0.03	0.96	0.36 (0.26-0.50)	6.64±0.02	0.97	0.08 (0.05-0.11)
		7.0	7.04±0.05	0.93	0.19 (0.11-0.33)	7.06±0.03	0.92	0.09 (0.06-0.13)
		7.5	7.49±0.02	0.82	0.25 (NC)	7.49±0.02	0.82	0.10 (NC)
		8.0	7.85±0.02	0.66	0.55 (0.54-0.56)	7.89±0.01	0.64	0.06 (0.03-0.11)
	C. dipterum	6.5	6.70±0.06	0.96	0.26 (0.18-0.38)	6.69±0.04	0.97	0.09 (0.06-0.15)
		7.0	7.15±0.02	0.91	0.45 (0.37-0.54)	7.15±0.01	0.91	0.09 (0.06-0.14)
		7.5	7.54±0.01	0.80	0.49 (0.37-0.65)	7.54±0.01	0.80	0.10 (0.06-0.19)
		8.0	7.91±0.01	0.63	0.51 (0.40-0.65)	7.93±0.01	0.62	0.24 (0.18-0.31)
	P. acuta	6.5	6.62±0.05	0.97	0.51 (0.49-0.55)	6.62±0.03	0.97	0.33 (0.24-0.45)
		7.0	7.07±0.04	0.92	0.75 (0.61-0.94)	7.05±0.04	0.92	0.45 (0.40-0.50)
		7.5	7.42±0.02	0.84	1.29 (NC)	7.43±0.01	0.84	0.29 (NC)
		8.0	7.78±0.02	0.70	0.55 (0.55-0.56)	7.83±0.01	0.67	0.70 (0.66-0.73)

696 NC: could not be calculated.
697

Table 3. Regression coefficients (R^2) and calculated Pearson correlation p-values (between brackets) of the single model fits, followed by the calculated model parameters. EC50 (neu): EC50 calculated for the neutral chemical form. EC50 (charged): EC50 calculated for the charged chemical form. EC50 (internal): EC50 calculated taking into account the internal pH. All EC50 values are in mg/L.

Chemical	Exposure time	Model	*G. pulex*	*C. dipterum*	*P. acuta*
ENR	48 h	1	0.97 (0.002) EC50(neu)=27.0	0.99 (<0.001) EC50(neu)=23.5	0.98 (0.001) EC50(neu)=126
		2	0.74 (0.14) EC50(neu)=31.6; EC50(charged)=184	0.85 (0.07) EC50(neu)=21.4; EC50(charged)=-405	0.77 (0.12) EC50(neu)=93.9; EC50(charged)=4891
		3	0.97 (0.003) EC50(internal)=72.1	0.99 (<0.001) EC50(internal)=30.5	0.97 (0.002) EC50(internal)=154
	96 h	1	0.98 (0.002) EC50(neu)=10.8	0.94 (0.007) EC50(neu)=13.4	0.97 (0.002) EC50(neu)=61.9
		2	0.82 (0.09) EC50(neu)=13.1; EC50(charged)=16.5	0.49 (0.30) EC50(neu)=21.3 ; EC50(charged)=48.0	0.60 (0.22) EC50(neu)=74.1; EC50(charged)=90.7
		3	0.96 (0.003) EC50(internal)=28.9	0.91 (0.01) EC50(internal)=17.2	0.96 (0.003) EC50(internal)=76.1
TCS	48 h	1	0.92 (0.009) EC50(neu)=0.29	0.98 (0.001) EC50(neu)=0.34	0.85 (0.03) EC50(neu)=0.64
		2	0.34 (0.41) EC50(neu)=0.23; EC50(charged)=-0.52	0.52 (0.28) EC50(neu)=0.30; EC50(charged)=-0.90	<0.001 (0.99) EC50(neu)=0.68; EC50(charged)=0.69
		3	0.93 (0.01) EC50(internal)=0.45	0.98 (0.001) EC50(internal)=0.37	0.85 (0.03) EC50(internal)=0.69
	96 h	1	0.99 (<0.001) EC50(neu)=0.08	0.93 (0.008) EC50(neu)=0.11	0.94 (0.006) EC50(neu)=0.38
		2	0.89 (0.21) EC50(neu)=0.08; EC50(charged)=-0.18	0.91 (0.05) EC50(neu)=0.08; EC50(charged)=-0.14	0.39 (0.38) EC50(neu)=0.31; EC50(charged)=-0.82
		3	0.99 (<0.001) EC50(internal)=0.12	0.93 (0.009) EC50(internal)=0.12	0.94 (0.006) EC50(internal)=0.40

Figures

Figure 1. Comparison of EC50-96h values for enrofloxacin with the calculated parameters of Model 1, 2 and 3. Comparisons for the EC50-48h values are provided in Fig. S1.

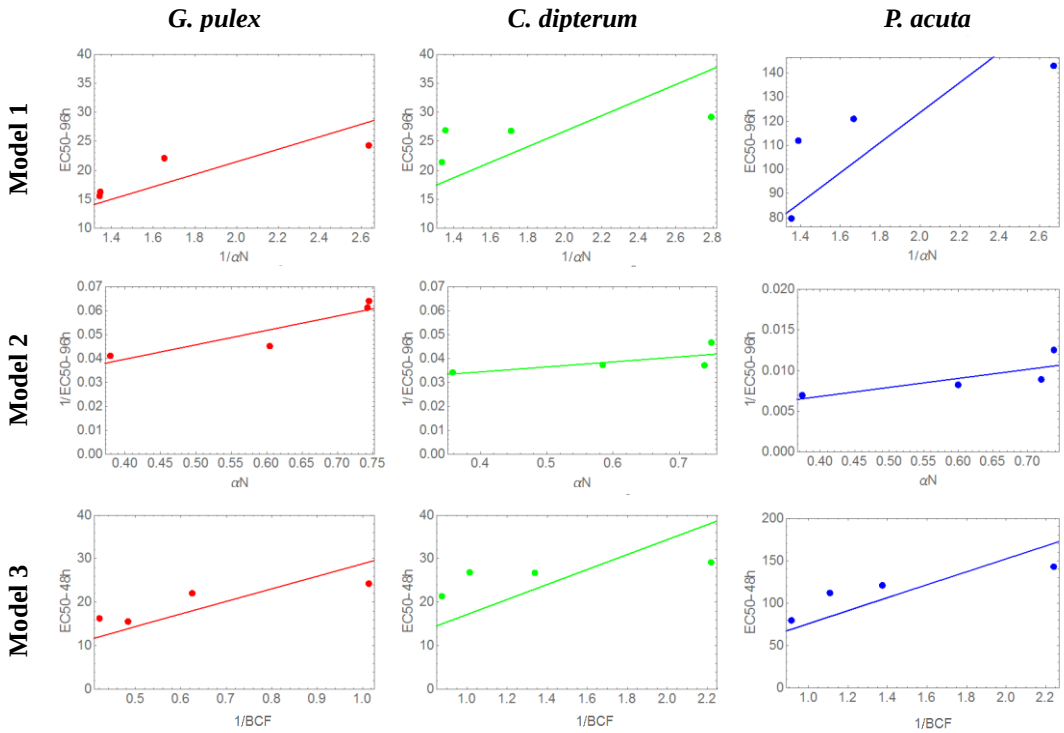

Figure 2. Comparison of EC50-96h values for triclosan with the calculated parameters of Model 1, 2 and 3. Comparisons for the EC50-48h values are provided in Fig. S2. For *G. pulex*, the EC50 value for pH=8 was not included in the modelling calculations.