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The impacts of pH on the toxicity of mixtures of antidepressants to *Daphnia magna*

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Abstract

Selective Serotonin Reuptake Inhibitors (SSRI) including Citalopram (CTP), Fluoxetine (FLX) and Sertraline (SRT), Selective Norepinephrine Reuptake Inhibitor (SNRI) including Duloxetine (DUL), and Tricyclic Antidepressant (TCA) including Dosulepin (DOS) have been widely prescribed in antidepressants. While these pharmaceuticals have been frequently detected in surface waters, sediments and biota, extremely limited information is available on their *in vivo* toxicity, particularly in invertebrates. In the present study the individual and mixture neurotoxicity of antidepressants and its underlying mechanisms were investigated acutely and chronically at different pH levels (5.5, 6.0, 7.0, 7.5, 8.0, and 9.0) using *Daphnia magna*. A model was designed to calculate the expected EC50. The total amount (mg) of each antidepressant dispensed from 2009 to 2018 was calculated using dataset provided from National Health Service (NHS). It was grouped in three, A (2009-2011), B (2012-2014), and C (2015-2018), based on the change (%) of prescription. Concentration Addition (CA) model was used to test the mixture toxicity. Daphnia neonates were exposed to various expected concentrations of mixture A, B or C to determine the acute and chronic toxicity in various pH. The toxicity of antidepressants was increased acutely and chronically with increasing pH conditions. SSRIs commonly resulted reproduction enhancement and decrease in growth effects while reproduction inhibition and no effect on growth were determined with SNRI or TCA exposure on *D. magna*. Moreover, the dry mass of the daphnids was decreased as the size decreases. Our observation clearly indicates that survival, reproduction, growth, and dry mass performance in aquatic invertebrate could be affected by trace level exposure to studied antidepressants and the toxicity increases as pH increases. Our mixture results also clearly reflected the change of the antidepressant concentration in last decade in the UK. Hence, consequences of greater diverse class of antidepressants exposure on aquatic invertebrate warrant further investigation.

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DECLARATION

I, Jegak Seo, declare that the work submitted in this dissertation is the result of my own work and investigation and all the sources I have used have been indicated by means of completed references.

Signed: Jegak Seo

Date: 17/01/2020

Chapter 1

General Introduction

1. Literature review and introduction

Usage, pathway and occurrence of pharmaceuticals

Pharmaceuticals include prescription drugs, over-the-counter remedies, and synthetic or natural chemicals that can be found in veterinary medicine (WHO, 2012). Pharmaceuticals contain active ingredients that have pharmacological effects and are designed to greatly benefit society (WHO 2012). However, in recent years, the occurrence and effects of pharmaceuticals in the natural environment have received increasing attention from the research community because they are highly biologically active compounds (Brown et al., 2007). Concentrations of pharmaceuticals in sewage treatment plant effluents, surface waters, groundwaters, sea water and some drinking waters have been increasing dramatically due to growing medicine consumption by the population over the last few decades (Tahrani et al., 2015).

Burns et al. (2017) identified 1,912 active pharmaceuticals ingredients (APIs) that are registered for human use which are sold in the UK. These compounds are prescribed by hospital clinicians or physicians, or may be obtained from pharmacies or shops (WHO, 2003). In the UK, pharmaceuticals are legislated under the Medicines Act 1968 which defines three legal categories of medicines, namely: general sales list (GSL) medicines, pharmacy medicines (P), and prescription-only medicines (POMs). GSL medicines may be sold from a wide range of shops such as newsagents, supermarkets and petrol stations. Normally, only products with reduced concentrations of active ingredients are sold *via* this route compared to those sold by a pharmacy. POMs can only be obtained with a prescription, which is usually obtained from a General Practitioner (GP) or dentist, but in some cases can be obtained from a nurse, pharmacist or other healthcare professional (WHO, 2003). Some POMs, such as morphine, pethidine and methadone, are further classified as controlled drugs. These medicines may be misused or sold illegally, so there are stricter legal controls on their supply (Peate and Hamilton, 2013).

Following use by a patient, most pharmaceuticals are excreted in the urine or faeces as either the free parent drug, the drug conjugated with other substances to increase solubility, or as metabolites (Ghibellini et al., 2006). In higher income countries, following excretion, the drugs and their transformation products will then enter the sewage system, which is the primary route of entry into the environment. In the sewage system, pharmaceuticals may be further degraded or removed, but some residues can remain and will therefore be present, in trace levels, in the effluent from the wastewater treatment plants. Pharmaceuticals are also widely used in animal husbandry and fish farming and can reach the land in slurries from intensive animal rearing, or directly from grazing animals (Boxall and Long, 2005). Inputs from manufacturing plants are also possible and can result in high localised concentrations of pharmaceutical in regions of drug production and formulation

(Fick et al., 2009; Kessler, 2010).

Over the past decade, research into environmental impact of pharmaceuticals has increased with the expansion of the European and UK pharmaceutical market (EEA, 2010). Moreover, the frequency of pharmaceutical detection in the aquatic environment has been increasing due to advances in analytical technology (Gaw et al., 2014). Different types of pharmaceuticals, including B-blockers, hormones and antidepressants, are detected in the environment at low concentrations (Küster and Adler, 2014). Concentrations in the 10s-100s of µg/L of medicinal products have been detected in wastewater treatment plants (WWTPs) effluents with slightly lower concentrations (in the ng/L to µg/L range) typically being found in surface water bodies (Zhang et al., 2016; Radjenovic et al., 2006). The main contributor to these observed concentrations is thought to be from emissions of municipal wastewater effluents (WHO, 2012; Shraim et al., 2017).

Current known effect of pharmaceuticals

There are major concerns over the detection of pharmaceuticals due to the evidence that these molecules may adversely affect aquatic life. Although pharmaceuticals are detected in the freshwater environment at relatively low concentrations, they can be bioaccumulated into organisms resulting in high tissue concentrations. For example, when goldfish (*Carassius auratus*) were exposed to gemfibrozil at an environmentally relevant concentration over 14 days, a plasma bio-concentration factor of 113 was obtained (Mimeault et al., 2005). Another study reported the uptake and depuration of pharmaceuticals in reclaimed water by mosquito fish (*Gambusia holbrooki*) and the accumulation of fluoxetine in snails with the bioaccumulation factor of 3000 (Wang and Gardinali, 2013). Moreover, freshwater shrimp (*Gammarus pulex*) and water boatman (*Notonecta glauca*) were exposed to moclobemide, 5-fluorouacil, carbamazepine, diazepam, carvedilol and fluoxetine over 48 hours to determine the uptake, depuration, and bioconcentration factors (BCFs) (Meredith-Williams et al., 2012). The BCFs of freshwater shrimp were significantly higher than water boatman which means smaller aquatic species were at even higher risks (Meredith-Williams et al., 2012). Pharmaceuticals are also biologically active molecules and are designed to effect certain metabolic, enzymatic, or cell-signalling in target organisms (Osorio et al., 2016; Ramsay et al., 2018). The evolutionary conservation of these molecular targets in species in the environment means that there is a possibility that these pharmaceuticals will also affect non-target organisms (Arnold et al., 2014). This mode of action (MoA) concept can be applied to all aquatic biota that are unintentionally exposed to pharmaceuticals in their natural environment, thus raising the risk of ecotoxicological effects.

It has been confirmed in a number of preliminary studies that trace levels of human or veterinary pharmaceuticals could adversely affect various animals, plants and insects including both vertebrates and invertebrates (EEA, 2010). There are presently two well-

documented examples of pharmaceuticals adversely affecting wildlife: ethinyl estradiol (EE2) contributing to the feminization of male fish, and diclofenac killing vultures in regions of India and Pakistan (Sumpter, 2010, Swan et al., 2006). EE2's role in the feminization of male fish has been reported in many countries across the world (Sumpter, 2010). The reported effects, such as elevated plasma vitellogenin concentrations, oocytes in testes and disrupted reproductive ducts, are probably a consequence of exposure to a mixture of estrogenic chemicals, with EE2 being a major component of the mixture in many countries (Jobling et al., 2006). Laboratory experiments have convincingly shown that EE2 is very potent in fish. Concentrations as low as a few ng/litre feminize males which can then lead to reduced or no reproduction and population crashes. More importantly, concentrations of EE2 below 1 ng/L have been reported to affect fish (Rose et al., 2002). Fish appear to be the most sensitive group of aquatic organisms (Rose, Paczolt and Jones, 2013) although Vandenbergh et al. (2003) found that reproduction of the aquatic invertebrate, *Hyalella azteca* was significantly reduced when exposed to 0.1 *μ*g/L EE2 for 35 weeks. Also, mouthpart deformation has also been observed in *Chironomus riparius* after being exposed to 0.01 *μ*g/L EE2 (Watts et al., 2003).

Diclofenac, when used as a veterinary pharmaceutical, killed tens of millions of vultures in Asia (Swan et al., 2006). The mass killing of vultures by diclofenac is believed to be one of the worst incidents in which an animal population was adversely affected by veterinary pharmaceuticals. Some particular groups of animals could be extremely sensitive to trace level of pharmaceuticals which entered through unexpected routes. The drug was administered to ill livestock, especially cows, which were then left in the environment. When they died, their carcasses were consumed by scavengers such as vultures. In a period of 15 years, populations of three species of vultures declined by more than 97% to a stage where they are now classified as critically endangered (Prakash et al., 2012). Diclofenac causes acute renal failure and the vulture dies within a few days (Oaks et al., 2004). Experimental evidence has confirmed that diclofenac is the cause of this mass poisoning of wildlife (Cuthbert et al., 2014). Other non-steroidal anti-inflammatory drugs (NSAIDs) also appear to be highly toxic to birds, including groups other than raptors (Cuthbert et al., 2006). However, one NSAID, meloxicam, is apparently not toxic to birds. The good news is that diclofenac has been banned for cattle use in the region and programmes have been introduced to help the populations to recover.

Antidepressants are also a group of pharmaceuticals that could pose a potential risk to aquatic organisms based on their biological activity, widespread use or detection, and existing toxicity data (Muñoz et al., 2008; Sanderson et al., 2004; Alonso et al., 2010; Cooper et al., 2008; Donnachie et al., 2016). For example, Selective Serotonin Reuptake Inhibitors (SSRIs) and Serotonin-Norepinephrine Reuptake Inhibitors (SNRIs) inhibit the reuptake of serotonin from synaptic cleft thereby increasing the signals between neurons. Serotonin is an important neurohormone in invertebrates controlling many biological

functions including growth, immunity, reproduction, and metabolism as well as behaviour (Fong and Ford, 2014). Fong and Ford highlighted that since the MoA of antidepressants is by modulating the neurotransmitters serotonin, dopamine, and norepinephrine, aquatic invertebrates who possess transporters and receptors sensitive to activation by these pharmaceuticals are potentially affected by human antidepressants. In addition, they highlight that many SSRIs do not only act upon the reuptake proteins but also actively bind to multiple neurological receptors increasing the potential for variable downstream physiological effects (Fong and Ford, 2016). Probably the most widely studied SSRI, in terms of aquatic effects is fluoxetine. Fluoxetine has been the most studied antidepressant in terms of its aquatic toxicity to various aquatic organisms. Different adverse effects have being reported following exposure to the compound. For example, chronic effects studies for fluoxetine have indicated that exposure to the molecule can both increase or decrease reproduction of *D. magna* at the same concentrations (Campos et al., 2012 and 2016). Also, they have increased the growth rate of mollusc (Fong and Ford, 2016) after exposed to fluoxetine for 14 days. The heart rate of *D. magna* was increased by the serotonin effects after exposed to fluoxetine for 21 days (Halliwushka, 2016). Another study that exposed sertraline (SSRI) to *Daphnia magna* and *Pseudokirchneriella subcapitata* resulted in a reduction in reproduction over 21 days and the proliferation of the algae was inhibited, respectively (Christensen et al., 2007). Moreover, *Crassostrea gigas* embryo-larvae had a reduction in mean net percentages of normal development at concentration range between 200 to 400 µg/L of duloxetine (Di Poi et al., 2013).

Tricyclic antidepressants (TCAs) are known for an effective treatment for a wide range of conditions, including depression, anxiety disorder, and pain syndromes (Maubach et al., 1999). Also, they produce inactivation which occurs largely via CYP450 enzymes, by demethylation of tertiary TCAs to their secondary amine metabolites, hydroxylation, then glucuronidation and excretion in the urine. Amitriptline, nortripytline, and clomipramine are examples of TCAs. Their mechanism of activity is through the inhibition of serotonin and noradrenaline uptake in presynaptic nerve endings (Maubach et al., 1999). In addition, TCAs can also bind to many other receptors, such as muscarinic, histaminergic and alpha 1 and 2 adrenergic receptors, leading to a wide range of adverse effects (Stahl, 2013), such as sedation (Zajecka and Tummala, 2002), cardiotoxicity (Bames et al., 1968; Callaham et al, 1988), or neurotoxicity (Mannerström and Tähti, 2004). Interestingly, TCAs have been shown to be more toxic than the newer SSRIs (Hawton et al., 2010). These emerging adverse effects from the different antidepressants classes have been raising concerns which eventually have motivated our research.

The uptake and toxicity of antidepressants is also known to be affected by natural environmental conditions such as temperature, UV-lights or pH. For instance, reproduction enhancement of *D. magna* is commonly observed when exposed to SSRIs at increasing pH conditions. The ionisable compounds fully dissociate at the pH where near to their pK_a

value. Therefore, it is obvious that the adverse effects of ionisable compounds could vary depends on the pH conditions. It is also very rare to find an aquatic environment containing single antidepressants so organisms will be exposed to a mixture of these compounds. It is possible that depending on the interation, the combined effects of these mixtures could be greater the effects from exposure to single pharmaceutical exposure (Geiger et al., 2016; Affek et al., 2018; Godoy et al., 2019). The impacts of these mixtures can be affected by the ratio of the antidepressants in the mixture and the modes of action of the mixture constituents. While there is now a good body of data on the ecotoxicity of selected single antidepressants and on the effects of pH on the toxicity and uptake of these, limited data are available for many commonly used antidepressants and on the impacts of antidepressant mixtures.

1.1. Aims and objectives

The overall aim of this study was therefore to explore the ecotoxicity of mixtures of antidepressants, commonly used in the UK under different pH conditions. The study was delivered through three specific objectives:

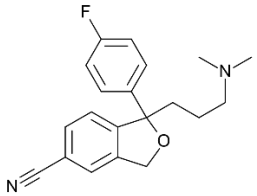
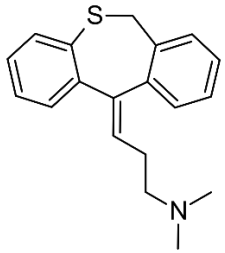
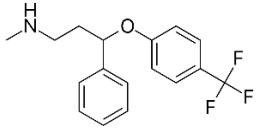
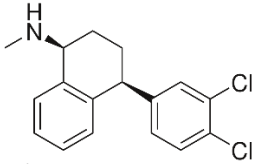
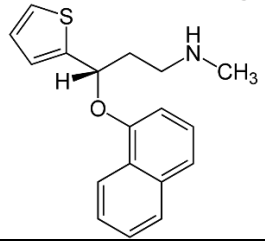
(1) to assess the effects of water pH on the acute and chronic toxicity of commonly used antidepressants (Table 1) to the aquatic invertebrate, *Daphnia magna*
(2) to explore the acute and chronic effects of mixtures of antidepressants on *D. magna* under different pH conditions; and
(3) develop a modelling approach for estimating the mixture effects of antidepressants on aquatic invertebrates under different pH conditions.

Experimental work to deliver the objectives is presented in two different chapters. The first Chapter explores how the toxicity of antidepressants is modified by pH variation. The second Chapter studied the adverse effects of the each antidepressant. A final Chapter draws on the data to explore how mixture models (Concentration Addition and Independent Action) could be used to assess the risks of antidepressants under different pH conditions.

1.2. General information about studied antidepressants in different classes

The selected antidepressants were citalopram, fluoxetine, sertraline, duloxetine, and dosulepin (Table 1). In chapter 2, study focused on the selected five antidepressants with the highest risk quotients (RQ) calculated from NHS prescription datasets from 2009 to 2018.

Table 1. Chemical information of 5 different antidepressants; SSRI= Selective serotonin reuptake inhibitor; SNRI= Serotonin and norepinephrine reuptake inhibitor; TCA= Tricyclic Antidepressant.

Chemical	CAS R N	Chemical Formula	Chemical Structure	Description	Chemical Properties
Citalopram hydrobromide	59729-33-8	C20H21FN2O		Antidepressant; SSRI; prescribed to over 21 million people globally (Croos et al., 2005)	Kow: 3.74; p*K*a: 9.78
Fluoxetine hydrochloride	54910-89-3	C17H18F3NO		Antidepressant; SSRI; prescribed to over 40 million people globally (Wenthur et al., 2013)	Kow: 4.65; p*K*a: 9.80
Sertraline hydrochloride	79617-96-2	C17H17Cl2N		Antidepressant; SSRI; prescribed to over 37 million people globally (Saleh et al., 2017).	Kow: 5.29; p*K*a: 9.85
Duloxetine hydrochloride	116539-59-4	C18H19NOS		Antidepressant; SNRI; Prescribed to over 15 million people globally (Hudson et al., 2007)	Kow: 4.29; p*K*a: 9.34
Dosulepin hydrochloride	113-53-1	C19H21NS		Antidepressant; TCA; the prescription has reduced but it is still being prescribed significantly globally with high toxicity (Petrie et al., 2015)	Kow: 4.68; p*K*a: 9.76

Citalopram (SSRI)

Citalopram is one of the most widely studied antidepressants in terms of ecotoxicity over the world because it is one of the most prescribed compounds. Although it has the lowest minimum and maximum daily dose (mg) among the three studied SSRI antidepressants, it was always highly ranked in terms of consumption (Ahlford, 2007). The amount of prescribed citalopram has led to a large concern for aquatic organisms (Kellner et al., 2015). The concentration between 10-150 ng/L of citalopram is commonly found in the surface water. However, the therapeutic levels for fish to be equal level to humans is 141 ng/L of citalopram (Fick et al., 2010). For example, citalopram was detected in the range between 4 ng/L (Ahlford, 2007) to 76 µg/L because some of the sites were located 150 m away from extreme conditions such as a sewage treatment plant in India (Fick et al., 2009). Also, the concentration was increased in aquatic environments near to hospitals. The area which has relatively high number of mentally ill patients demonstrated the greater concentration of citalopram. Most importantly, a greater predicted environmental effect concentration (PEC) of citalopram is predicted at some places (Styrishave et al., 2010). For instance, the PEC of citalopram is 0.2 µg/L which was derived from the sold amount (kg) in Stockholm in 2005 because it was the most sold SSRI antidepressant in Sweden from 1992 to 2005 (Ahlford, 2007). In Stockholm, the amount of purchased citalopram reached 13 million defined daily doses (DDD) which corresponds to 262.0 kg per year

Fluoxetine (SSRI)

One of the most commonly prescribed antidepressants is fluoxetine (Prozac) for which arguably the most ecotoxicological data exists amongst all antidepressants. Various adverse effects have been reported on *Daphnia magna* including effects on mortality, reproduction, or growth and often these studies have explored impacts of pH or effects over multiple generations (Nakamura et al., 2008; Boström and Berglund, 2015; Flaherty and Dodson, 2005; Barbosa et al., 2017). Most of the studies were designed with simple experimental designs, but some studies with combined experiment designs with inconsistency results (Mennigen et al., 2008; Weinberger and Klaper, 2014). Fluoxetine is excreted from a human body primarily via the urine, and approximately 2.0-11.0% of the administered dose is excreted as the unchanged compound (Stanley et al., 2006). Fluoxetine has been measured in surface waters at nanogram per litre level in the UK and other EU countries. Several studies have detected fluoxetine in the environment with concentrations in fresh waters ranging between 0.012 and 0.54 µg L^{-1} (Weston et al., 2001; Kolpin et al., 2002; Chen et al., 2006; Gardner et al., 2012). For example, Kolpin et al. (2002) measured fluoxetine at 0.012 µg L^{-1} downstream from wastewater treatment plants and Weston et al. (2001) observed concentrations from 0.32 to 0.54 µg L^{-1} in municipal effluent. Concentrations of fluoxetine in the estuary of Long Island Sound (New York City) have been recorded at 0.7 ± 0.3 ng L^{-1} following an approximate 80% removal rate from the wastewater treatment plant (Influent 144 ng L^{-1}; effluent 27 ng L^{-1}; Lara-Martin et al., 2014). However,

the Risk Quotient (RQ) for fluoxetine was greater than 1, indicating a potential risk to the environment for fluoxetine (Wu et al., 2017).

Sertraline (SSRI)

Sertraline was the least prescribed and purchased among all of our studied antidepressants based on the UK data. However, the amount of sertraline consumed has increased rapidly in last decade, so that it is now the most consumed pharmaceutical in England (NHS, 2019). Moreover, sales nearly doubled compared to the amount of citalopram sold in the same year at Stockholm, which corresponds to 432.0 kg per year (Ahlford, 2007). The PEC value was 0.31 µg/L in Stockholm which was almost 50% greater than the PEC of citalopram. Furthermore, the PEC value in Europe and North America was calculated to range from 0.6 to 1.2 µg/L (Ahlford, 2007). Sertraline has been detected in the surface waters of the Mississippi River with a detection frequency of 88 percent at a mean concentration of 0.04 µg/L, with concentration ranging from 0.076 to 0.11 µg/L (Kwon and Armbrust, 2008). Although it has similar PEC concentration to other SSRIs, an understanding of its impact on aquatic organisms is lacking.

Duloxetine (SNRI)

Duloxetine has been prescribed at a relatively greater amount compared to other antidepressants all over the world. Although it was prescribed more than sertraline 10 years ago, the amount of prescription has still remained the same or above. The maximum PEC value of duloxetine was expected to be 0.05 µg/L (FDA, 2009), the estimated plasma concentration in fish is 0.09 µg/L. The maximum predicted environmental effect concentration (PEC_{max}) is projected to be lower than the estimated plasma concentration in fish, however, there is no chronic ecotoxicity data that supports the PEC_{max} is safe to aquatic organisms. Although it is expected to have no significant adverse effects on aquatic organisms in past, the risk have been promoted recently to same level of fluoxetine (Janusinfo, 2015). However, there is no data about ecotoxicological effects on aquatic organisms.

Dosulepin (TCA)

Dosulepin is also known for being prescribed and sold in considerable amounts all over the world. It had a similar level of prescription to sertraline 10 years ago and more has been sold than duloxetine since 2015 (NHS, 2019). There are no relevant ecotoxicity data available on this molecule because it was not considered to have a significant adverse effects on aquatic organisms (Pharmacopoeia, 2019). However, Petrie, Barden and Kasprzyk-Hordern (2015) found that dosulepin has a high affinity to particulate matter and has been found to be within the particulate phase of influent wastewater at significant concentration (>20% of the total concentration). It is crucial to provide new toxicity datasets which considered the unmonitored routes with relevant ecotoxicological approaches.

Chapter 2

The effects of pH on the toxicity of antidepressants to *Daphnia magna*

2. Introduction

The pH conditions of the aquatic environment may alter the toxic effects of pharmaceuticals. Around 80% of all pharmaceuticals are ionisable meaning that differences in pH in aquatic systems can alter the speciation of many of these molecules (Manallack, 2007). The speciation may be predicted based on the pH of the system and the acid dissociation constant (pK_a) of the compound using the Henderson-Hasselbalch equation. Differences in speciation are known to affect the uptake of pharmaceuticals (Carter et al., 2014). For example, the toxicity of pharmaceuticals increased when they were exposed to increasing pH conditions which were similar to the pK_a of the pharmaceutical (Campos et al., 2012; Nakamura et al., 2008, Brooks et al., 2003).

In general, most of the toxicity studies only focus on exposing pharmaceuticals to single pH level. Such single exposure approach has increasingly been criticised due to its lack of regards to potential changes in toxicity at different pH conditions (Rendal et al., 2011). The studied compounds are ionisable pharmaceuticals – the pK_a value of each tested chemical were above 9.0. The greater proportion of uncharged ions from the pharmaceuticals was expected to be dissociated at pH where close to their pK_a value. We have expected higher toxicity and bioaccumulation at higher pH for bases because it is traditionally known that the ionized fraction of the electrolyte is more polar, it will typically exhibit lower permeability into membranes and fatty tissues than the neutral fraction (Rendal et al., 2011). Hence, we should take particular attention to the study compounds at different pH conditions.

A number of the studies have demonstrated the influence of aquatic pH on the uptake toxicity of ionisable pharmaceuticals (Nakamura et al., 2008; Valenti et al., 2009; Karlsson et al., 2017). For example, Valenti et al. (2009) tested the toxicity of sertraline at pH values between pH 6 and 9. Sertraline has a pK_a value of 9.47 so large differences in speciation would be expected across this pH range. Effects on mortality, growth and feeding of *Daphnia magna* were generally greater when individuals were exposed to test water with higher pH than those exposed at lower pH values. Nakamura et al. (2008) explored the effects of pH on the uptake and effects of fluoxetine on the Japanese medaka (*Oryzias latipes*). Higher bio-concentration factor values were observed at higher pH levels. Adverse chronic effects of fluoxetine were reported with contradictory results compared to the past research according to the recently published literature (Alboni et al., 2015). Karlsson et al. (2017) investigated the novel approach for characterizing pH-dependent uptake of ionisable chemicals in aquatic organisms. A 37-fold uptake difference was observed for fluoxetine between pH 5.5 and 8.5 and the uptake difference for diclofenac was 47-fold between the same pH ranges as fluoxetine. The constructed model statistically well predicted the uptake using the pK_a value and the environmental pH conditions. Therefore, our study aim to establish another model to predict the toxicity with other pharmaceuticals which are

abundant in our environment or risk to the aquatic organisms.

Reported water pH values for Europe range from 2.2 to 9.8 (Karlsson et al., 2015; Coughlan et al., 2013). Even within a country there can be large difference in pH. In Spain, for example, water pH has been reported to cover 6 pH units (Boström and Berglund, 2015). Water pH can also vary at an individual site due to the effects of photosynthesising organisms, such as algae and aquatic macrophytes, whose activity is driven by sunlight and temperature (Wurts, 2003). The effects of ionisable compounds, such as antidepressants, are therefore likely to vary significantly both spatially and temporally. However, with the exception of the studies described above, there is a serious lack of ecotoxicity data on the effects of changing pH conditions on toxicity of pharmaceuticals.

The aim of this chapter was therefore to explore the effects of water pH on the acute and chronic toxicity of a range of antidepressant compounds to aquatic invertebrates. The specific objectives of the work were to:

(1) assess the effects of water pH on the acute and chronic toxicity of antidepressants to the aquatic invertebrates, *Daphnia magna*

(2) establish a model that estimates the toxicity of each antidepressant to aquatic invertebrates at increasing pH levels with the difference in uptake between non-ionised and ionised compounds using the data obtained from objective 1 and the Henderson-Hasselbalch equation.

The study compounds (Table 1) used have widely been prescribed as antidepressants and have frequently been detected in surface water (Ebele et al., 2017). As these molecules are generally basic in nature, effects are likely to be influenced by the pH of the system.

2.1. Material and methods

Prioritisation of tested pharmaceuticals

The study compounds were selected from previous studies that have prioritized pharmaceuticals in terms of their potential environmental risk (Guo et al., 2016; Burns et al., 2017). The study chemicals, citalopram, doselupin, duloxetine, fluoxetine and sertraline, were purchased from Sigma-Aldrich (St. Louis, MO, USA), all with purities ≥ 98%. All other chemicals used in the present study were analytical or reagent grade.

Maintenance of *Daphnia magna* culture

D. magna were originally obtained from FERA Science (York, UK) and maintained in the Environmental Toxicology Laboratory at the University of York. The *D. magna* culture was maintained at 20 ± 1°C in six 2 L glass beakers containing 2 L of ADaM media (Klüttgen et al., 1994) following protocols developed by the US EPA (US EPA, 2002). The average dissolved oxygen concentration in media was maintained at > 3 mg/L, based on OECD guidelines (OECD, 2008). Cultures were maintained under a white fluorescent light (12.1 µmol/m^2/s) with a 16:8 h light:dark photoperiod. *D. magna* were fed with YCT (1:1:1 mixture of Yeast, Ceropyl® and Tetramin®) and algae (*Chlorella vulgaris*). A standard *Daphnia* reference toxicity test was conducted every two weeks by exposing organisms to NaCl for 48h to assess changes in organism sensitivity and assure the quality of the antidepressant toxicity tests (US EPA, 2002; see Supplementary data).

Maintenance of *Chlorella vulgaris* culture

Chlorella vulgaris were grown in Kuhl's medium in 2 L glass flasks at 23ºC and 6000-10000 lux. All of the glass flasks were sterilized and sealed with cotton wools at the flask opening. Discontinuous large-scale culturing was used for the main culture flasks. Semi-continuous small-scale culture was used for the sub-cultures in 200 mL glass flasks. Algae were harvested and centrifuged at 3000 RPM for 3 minutes and stored at 4ºC to stop growth. The culture flasks were refilled with new media and algae after the old culture was successfully done. Harvested algae was not kept for longer than 3 weeks. Cell counts of the algae were taken every 6-8 days to record cell growth using an absorbance spectrophotometer until the satisfied cell density of $30x10^6$ cells/mL was reached (Park and Choi, 2008).

D. magna acute and chronic tests: individual antidepressants

The acute (48 h) and chronic (21 d) toxicity of the antidepressants to *D. magna* was explored at pH values of 5.5, 6.0, 7.0, 7.5, 8.0, and 9.0. The pH levels were maintained using 2 mM of Phosphate buffer (pH 5.5-6.0) or Tris-HCl buffer (pH 7.0-9.0). During the tests, the pH was monitored and recorded every 24 h to ensure that the target values were maintained.

Measured pH values did not vary by more than ±0.1 pH unit through this study (Appendix 2). The test solutions were renewed every 48h or individually when the pH values did not meet the target pH in 21d chronic tests.

The 48h acute toxicity test of each molecule with *D. magna* were carried according to the OECD Guideline 202 (OECD, 2008). Prior to the 48h acute toxicity tests, a range-finding (US EPA, 2002) study, for each molecule at each different pH, was conducted to determine the appropriate concentration ranges for the definitive acute toxicity tests. Four replicates of five *Daphnia magna* neonates (<24hr) were exposed to 0.1, 1.0, and 10.0 mg/L of each antidepressant at each pH value. Each replicate was contained in 40 ml of test medium in a 50 ml glass beaker. Immobilization was the endpoint of the test and it was determined if no movement was observed for 15 s after gentle shaking of the test vessel. All exposures were done at 20 ± 1°C using a 16:8 h photoperiod. Water quality parameters including pH, temperature, and dissolved oxygen of the test medium were measured before and after the 48h exposure. The test solutions were not replaced during the 48h static non-renewal test. Definitive tests used the same conditions and test design but various concentration of the antidepressants were used, i.e. citalopram = 0, 0.31, 0.63, 1.25, 2.5, 5.0, or 10.0 mg/L; fluoxetine = 0, 0.04, 0.08, 0.16, 0.31, 0.63, or 1.25 mg/L; sertraline = 0, 0.02, 0.04, 0.08, 0.16, 0.31, 0.63, or 1.25 mg/L; duloxetine = 0, 0.31, 0.63, 1.25, 2.5, 5.0, or 10.0 mg/L; and dosulepin = 0, 0.04, 0.08, 0.16, 0.31, 0.63, or 1.25 mg/L.

The 21d chronic toxicity tests were also performed to determine the effects of the antidepressants on the survival of original neonates, number of young per female, number of brood, time to first reproduction, growth and population growth rate (PGR) using the methods described in OECD Guideline 211 (OECD, 2008). The test concentrations for each molecules at different pH levels were determined based on the LC50 data from the acute toxicity tests. The LC50 being set as the highest concentration for serial concentration exposure according to the US EPA Guideline (2002). Definitive tests used the same conditions and test design but various concentration of the antidepressants were used, i.e. citalopram = 0, solvent control, 7.5, 15, 30, 60, 120, or 240 µg/L; fluoxetine 0, solvent control, 2.5, 5, 10, 20, 40, or 80 µg/L; sertraline = 0, solvent control, 1.0, 2.0, 4.0, 8.0, 16, or 32 µg/L; duloxetine = 0, solvent control, 12.5, 25, 50, 100, 200, or 400 µg/L; and dosulepin 0, solvent control, 2.5, 5, 10, 20, 40, or 80 µg/L. Ten replicates were prepared for each concentration/pH combination containing one neonate each (< 24h old).

One extra test vessel was prepared for each test treatment to measure the pH consistency. The organisms were exposed over 21d at 20 ± 1°C with a 16:8 h photoperiod. Test solutions were renewed every 48 h. Water quality parameters such as pH, temperature, and dissolved oxygen were measured in test media before and after the 48 h exposure. *D. magna* were fed daily with 300 µL YCT (1:1:1 mixture of Yeast, Ceropyl® and Tetramin®) and 300 µL algae per each organism. Lastly, 10 ml of new (0h) and old (48h) test samples were removed from the 50 ml beakers and collected in 15 ml vials for chemical

analysis on every third changing phases. The number of immobilized daphnids were counted to calculate EC50 of acute tests using the Probit analysis in SPSS.

Modelling ionization vs. acute toxicity of each antidepressant

Nearly full dissociation were determined at pH in basic condition as all of the tested molecules have relatively high pK_a value of greater than 9.0. The distribution of ionic speciation of each molecule was calculated as a function of medium pH following (Zarfl et al., 2008) and is depicted in Appendix 1. The estimated and measured toxicity of each antidepressant were compared to the ionic dissociation curves at increasing pH. The following steps were used to determine the estimated antidepressant acute toxicity at studied pH ranges between 5.5 and 9.0.

Firstly, the fraction of neutral and charged ions at pH 0 to 14 were calculated by the pH medium and different pK_a of each antidepressant using equation 1 and 2 (Karlsson et al., 2017), respectively.

$$\log f_{neutral}(\mathrm{pH}) = \log\left(\frac{1}{1 + 10^{(\mathrm{pH\ medium} - \mathrm{p}Ka)}}\right) \quad (1)$$

$$\log f_{charged}(\mathrm{pH}) = 1 - \log f_{neutral}(\mathrm{pH}) \quad (2)$$

Secondly, the predicted $LC50_{neutral}$ or $_{charged}$ were calculated by the measured acute LC50 and calculated fraction of neutral or charged ions using equation 3 and 4, respectively.

$$\mathrm{Predicted}\ \log \mathrm{LC50}_{neutral} = \log \mathrm{LC50}_{measured} + \log f_{neutral} \quad (3)$$

$$\mathrm{Predicted}\ \log \mathrm{LC50}_{charged} = \log \mathrm{LC50}_{measured} + \log f_{charged} \quad (4)$$

Lastly, the predicted LC50 was obtained by the predicted $LC50_{neutral}$ and $_{charged}$ and fraction of neutral and charged ions at pH range between 5.5 and 9.0 (equation 5).

$$\mathrm{Predicted\ LC50(pH)} = \left(\mathrm{Predicted\ LC50}_{neutral} \times f_{neutral}(\mathrm{pH})\right) + \left(\mathrm{Predicted\ LC50}_{charged} \times f_{charged}(\mathrm{pH})\right) \quad (5)$$

The predicted and measured LC50 were plotted over the ionic dissociation graph demonstrated by the Henderson-Hsselbalch equation for each antidepressant. Thus, the relationship between the toxicity changes and ionization curves at increasing pH was determined. Also, the sensitivity of each pharmaceutical at increasing pH was determined by comparing the difference between the measured and predicted toxicity with the fractions of ions.

Chemical analysis

Chemical analysis was conducted to determine the actual exposure concentrations for each test solution. Prior to analysis, the solid-phase extraction (SPE) was conducted on 6 mL OASIS Hydrophilic lipophilic balance SPE cartridges (Waters). 5 mL of methanol was passed through and followed by 10 mL of HPLC graded water for the preconditioning. The thawed samples were then loaded on the SPE cartridge at a rate of 10 to 20 mL/min using a vacuum manifold (Supelco-Visiprep) for extraction, after which the cartridges were rinsed with 10 mL of 5% methanol in HPLC graded water and then dried under air for 30 min. Cartridges were then eluted with 2.5 mL methanol followed by 1.0 mL of 2% NH_4OH in methanol. Eluates were dried under a gentle nitrogen stream using a concentrator (DB-3A; Techne) at 30^{o}C. The dried extract was reconstituted into 1.0 mL of water and stored in a freezer at -20^{o}C prior to analysis.

Cleaned-up extracts were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a High-performance Liquid Chromatography (HPLC) system with an Agilent 1260 Infinity II Multiple Wavelength Detector (High-speed UV detection) and a Zorbax Eclipse Plus-C18 Column. The column temperature was maintained at 25^{o}C and the pressure was set to 119.63 bars. Mobile phases A and B were 1mM of acetonitrile and 0.25 mM of ammonium acetate, and the flow rate was 1.5 mL/min. The gradient elution program was set to run 55% of acetonitrile and 45% of ammonium acetate simultaneously for 12 minutes per samples. The injected sample volume was 10 µL and detected at wavelength of 230 and 250nm. The performance of the analytical methods (precision, accuracy, limit of detection [LOD], and limit of quantification [LOQ]) were calculated (A-Khazrajy and Boxall, 2017; Appendix 3). Recoveries for the test pharmaceuticals ranged from 80.0% to 117.3%.

Statistical evaluation

Data analysis was performed using Sigma-plot software and SPSS for Windows version 24.0. In general, mortality in 48h acute tests and reproduction and growth toxicity data in 21d chronic tests were expressed as mean ± standard deviation. The concentration-response curves were fitted using the Probit regression model using SPSS. Statistical analysis was used to compare the significant difference between treatments and controls between each different pH unit using SPSS. The homogeneity of variance was checked before one-way ANOVA. One-way analysis of variance (ANOVA) was used with Tukey's post-hoc test with a p value <0.05 when necessary. Regression coefficients were used to determine significance by comparing estimated toxicity and measured toxicity in the models.

2.2. Results

There was no significant change in pH over the exposure periods for any of the treatments ($p > 0.05$). The pH measurements over 48h for acute tests and 21 days for chronic tests are provided in Appendix 2. The measured concentration of all of the tested samples were above limit of detection and limit of quantification except the control and solvent control samples (Appendix 3). Measured concentrations were at least 80% of nominal concentrations (Appendix 3). The toxicity of each antidepressant was increased at increasing pH conditions.

Daphnia magna 48h single acute tests

In the 48h acute tests no mortality was observed in the control treatments. The concentration-response curve for all antidepressants at each pH are provided in Appendix 4. As we expected, the concentration response curve shifted to left and the curves were steeper as the pH level increased. EC50 values ranged from 4.45–1.34 mg/L (pH 5.5-9.0) for citalopram, 1.07–0.26 mg/L (pH 6.0-9.0) for fluoxetine, 0.87-0.16 mg/L (pH 7.0-9.0) for sertraline, 4.22-1.47 mg/L (pH 7.0-9.0) for duloxetine, and 1.10-0.27 mg/L (pH 5.5-9.0) for dosulepin (Figure 1).

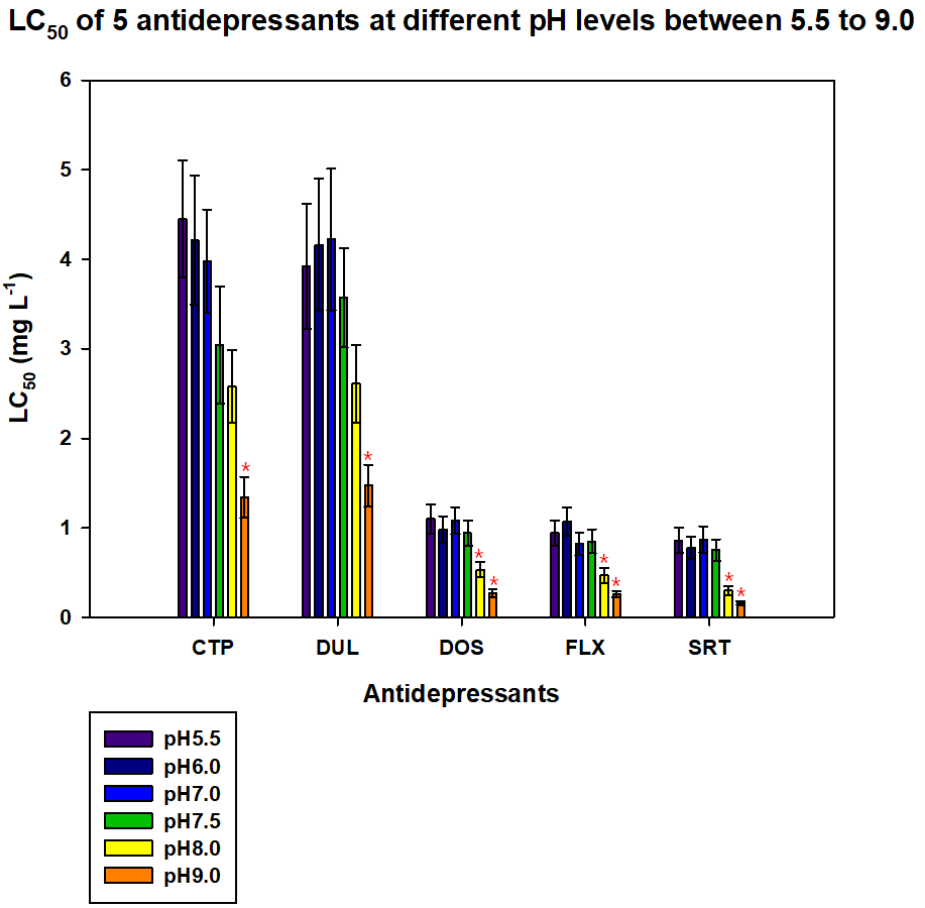


Figure 1. 48h LC50 (±95% Confidence Intervals) values for five antidepressants: citalopram (CTP), duloxetine (DUL), dosulepin (DOS), fluoxetine (FLX), and sertraline (SRT) following exposure of *D. magna* (< 24h) at six different pH 5.5, 6.0, 7.0, 7.5, 8.0, and 9.0. The asterisk (*) denotes that LC50 significantly decreased from the lower pH level (mean±S.D).

For all antidepressants, LC50 obtained at higher pH was compared to lower pH value. There was no significant difference in LC50 values obtained at pH 5.5, 6.0, 7.0 and 7.5 ($p > 0.001$). Significant differences were observed with LC50 of CTP (pH 9.0), DUL (pH 9.0), DOS (pH 8.0 or 9.0), FLX (pH 8.0 or 9.0), and SRT (pH 8.0 or 9.0) ($p < 0.001$). In summary, the stronger acute toxicity was observed at higher pH condition for all studied antidepressants.

Daphnia magna 21d single chronic test

Control mortality at all pH values did not exceed 20% at the end of the test. The mean number of live offspring produced per surviving parent animal was ≥ 60 in all the control treatments. The study therefore met the OECD acceptability criteria. There was no significant concentration effect on immobilization across the concentrations tested and pH levels for all compounds (Supplementary data). In general, SSRIs demonstrated reproduction enhancement (%) and SNRI or TCA compounds showed reproduction inhibition effects (%). The total number of reproduction of each test was used to determine reproduction enhancement or inhibition (Supplementary data).

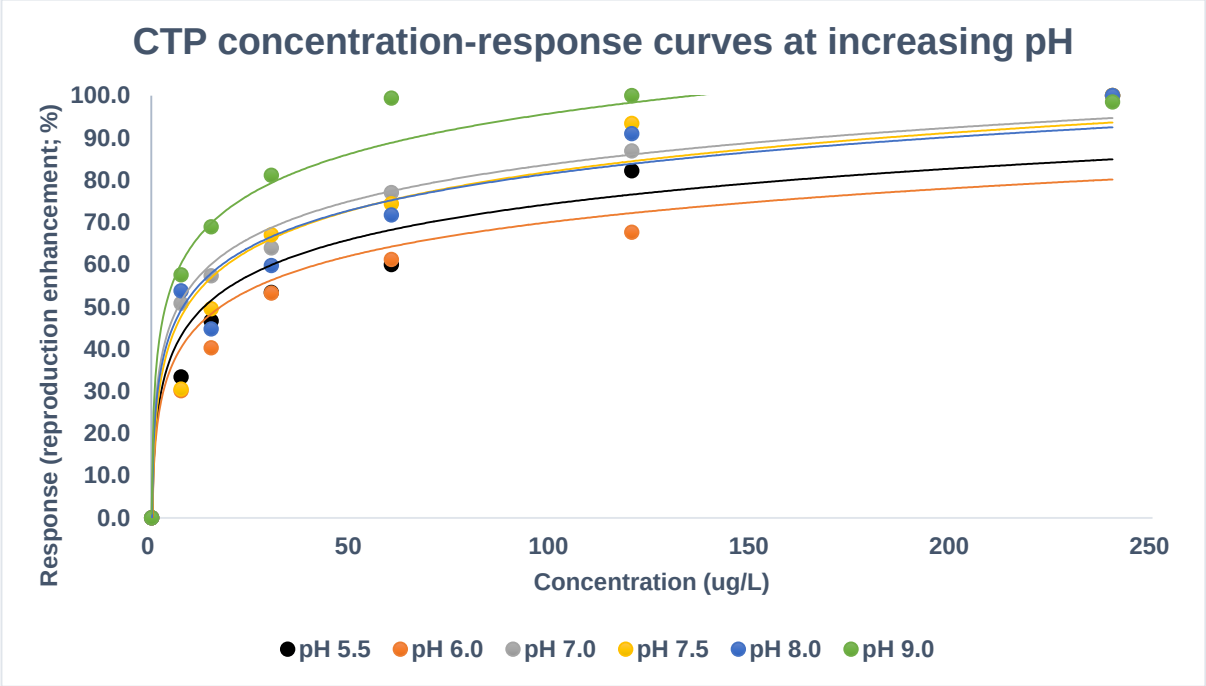


Figure 2a. Effects on *Daphnia magna* reproduction after 21d exposure to CTP at different pH levels ($p < 0.05$, d.f. = 4).

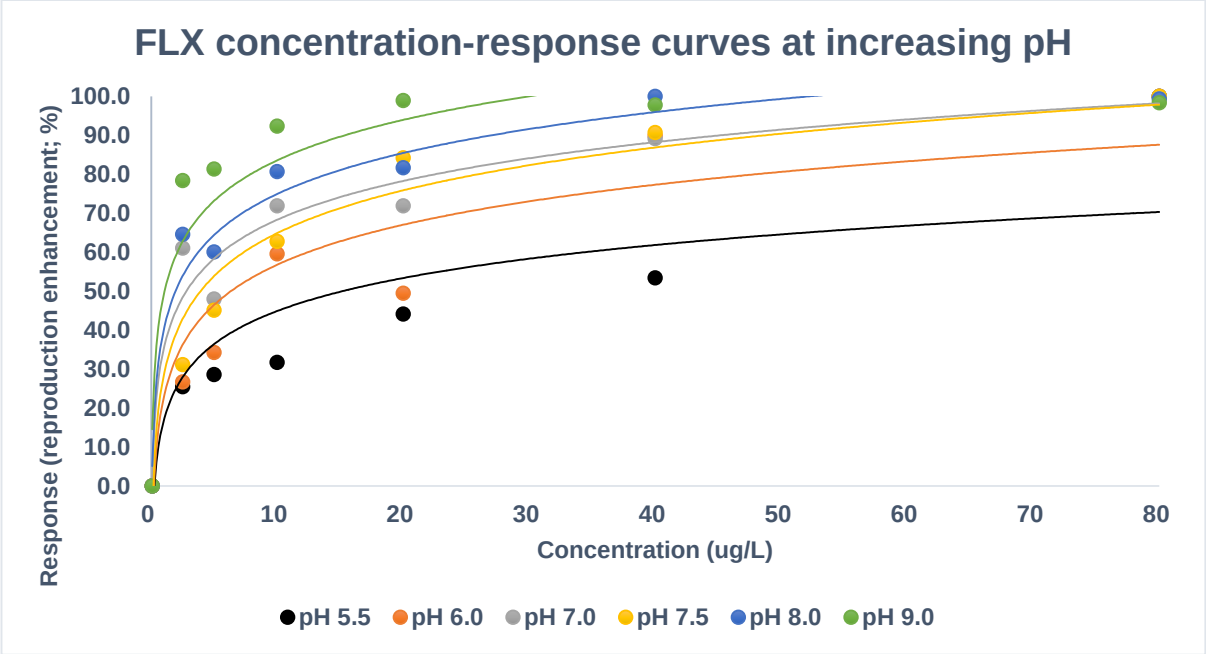


Figure 2b. Effects on *Daphnia magna* reproduction after 21d exposure to FLX at different pH levels ($p < 0.05$, d.f. = 4).

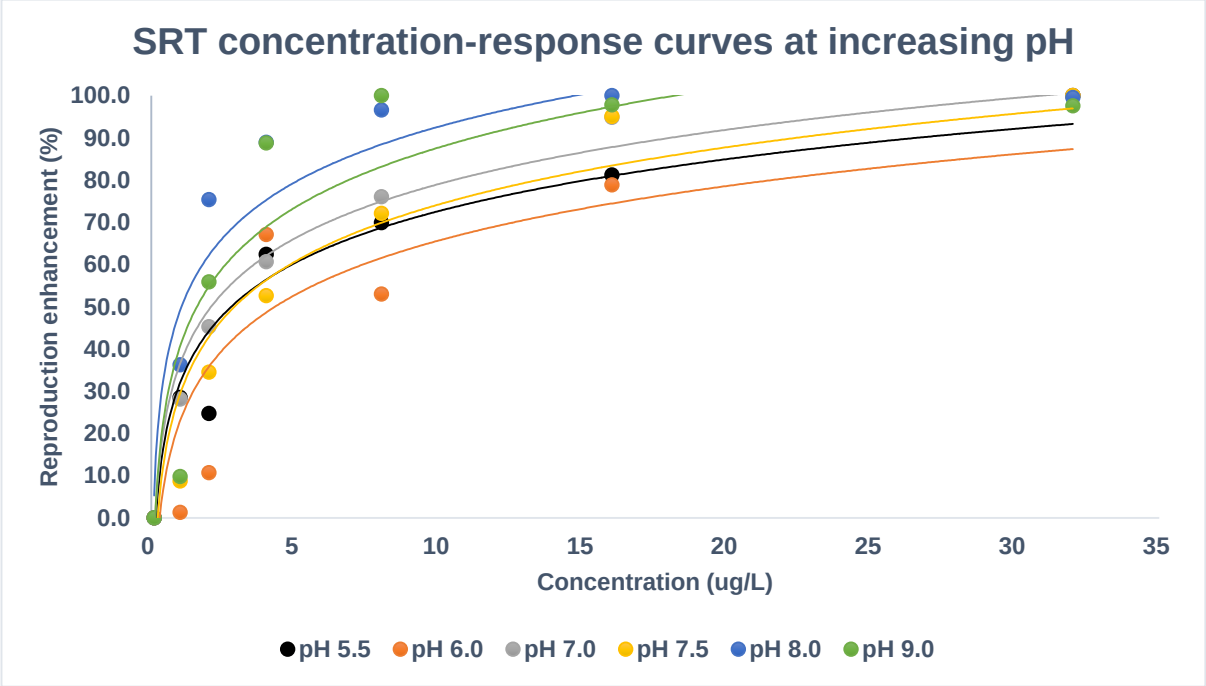


Figure 2c. Effects on *Daphnia magna* reproduction after 21d exposure to SRT at different pH levels ($p < 0.05$, d.f. = 4).

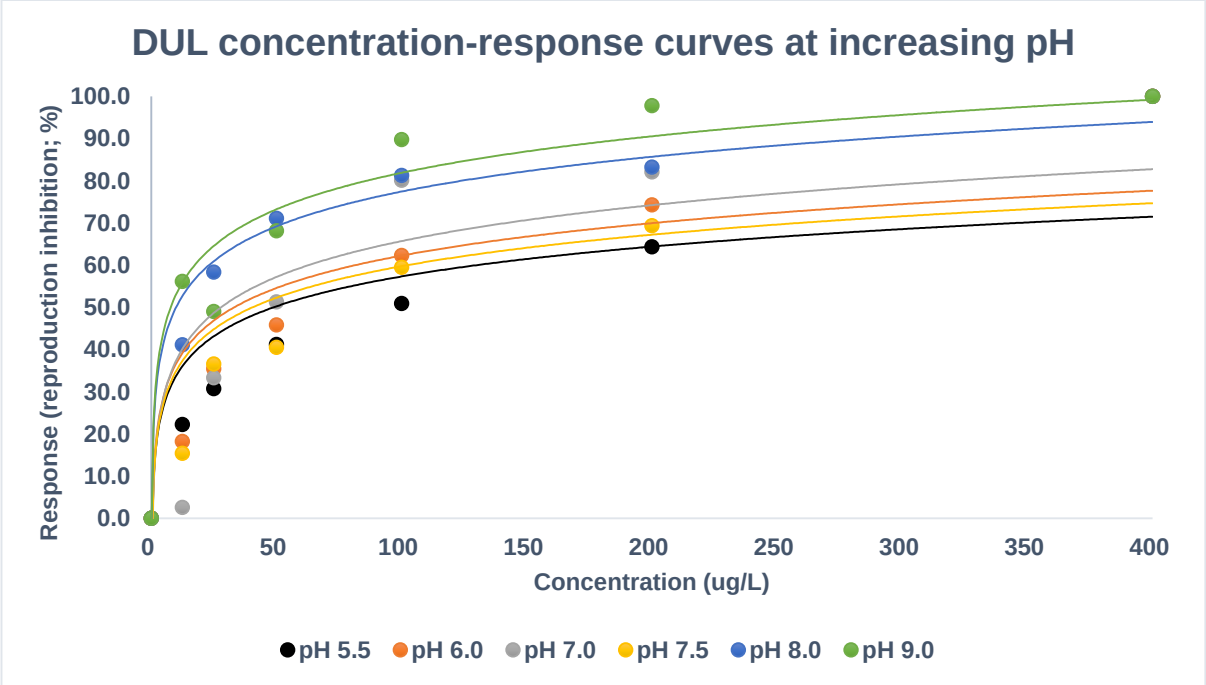


Figure 2d. Effects on *Daphnia magna* reproduction after 21d exposure to DUL at different pH levels ($p < 0.05$, d.f. = 4).

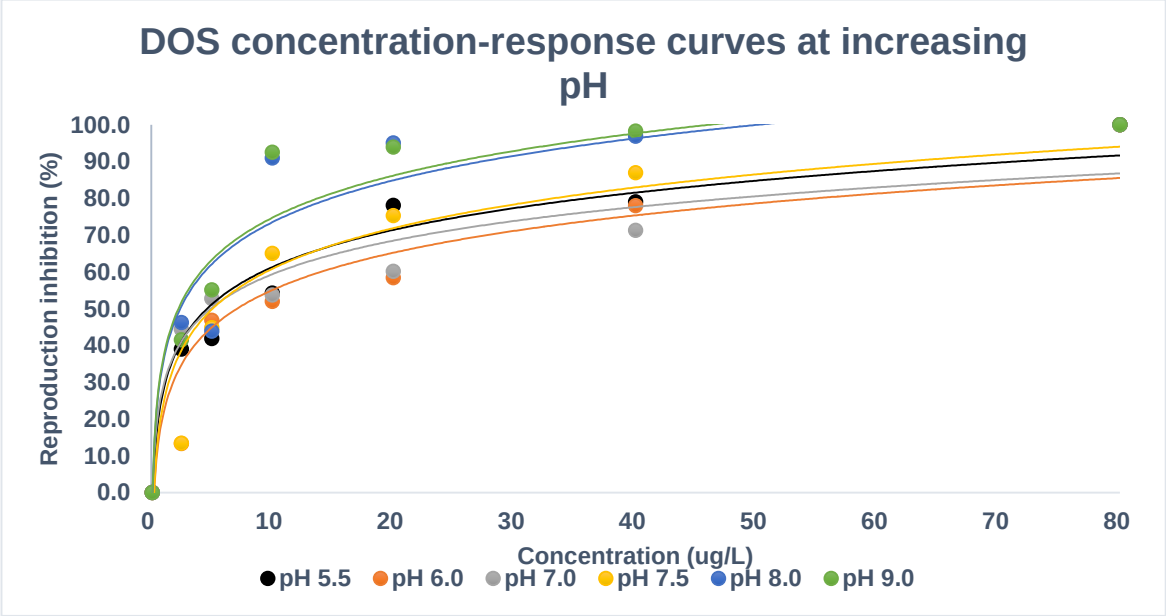


Figure 2e. Effects on *Daphnia magna* reproduction after 21d exposure to DOS at different pH levels ($p < 0.05$, d.f. = 4).

Table 2a. EC50 (µg/L) for reproduction enhancement of each SSRIs at pH between 5.5 to 9.0

pH	EC50 (µg/L) (reproduction enhancement)		
	CTP	FLX	SRT
5.5	15.8	15.2	2.78
6.0	48.1	6.34	4.29
7.0	6.69	2.80	2.08
7.5	8.89	3.92	2.89
8.0	7.93	1.93	1.05
9.0	3.04	0.83	1.59

Table 2b. EC50 (µg/L) for reproduction inhibition of SNRI and TCA at pH between 5.5 to 9.0

pH	EC50 (µg/L) (reproduction inhibition)	
	DUL	DOS
5.5	104.0	4.61
6.0	55.9	7.07
7.0	27.9	4.91
7.5	44.0	5.07
8.0	9.98	2.39
9.0	7.75	2.24

For the SSRIs, generally an increase in reproduction was observed in *D. magna* exposed to the test chemicals (Fig 2a, 2b and 2c; Table 2a). The reproduction was generally increased at greater than pH 7.0 alkali conditions compared to control groups ($p < 0.05$).

The number of young were increased nearly 61.6 % in average when exposed to SSRIs. However, the reproduction inhibition was observed over the SNRI and TCA exposure on *D. magna* at increasing pH conditions (Fig. 2d and 2e; Table 2b). The reproduction was significantly reduced when exposed with DUL from 2.5 to 80 mg/L at pH 8.0. Interestingly, the significant reduction was all found at alkali conditions. For instance, the significant reproduction inhibition was observed with 12.5 to 400 mg/L of DOS at pH 7.5 to pH 9.0. Also, the reproduction was decreased nearly 66.7% and 60.4% as maximum with DUL and DOS exposure, respectively.

Moreover, growth of parent daphnids were measured after 21d exposure. Individual survived parent daphnids were employed to measure the length from the top of the eye to the base of the tail spine using a microscope ocular micrometre.

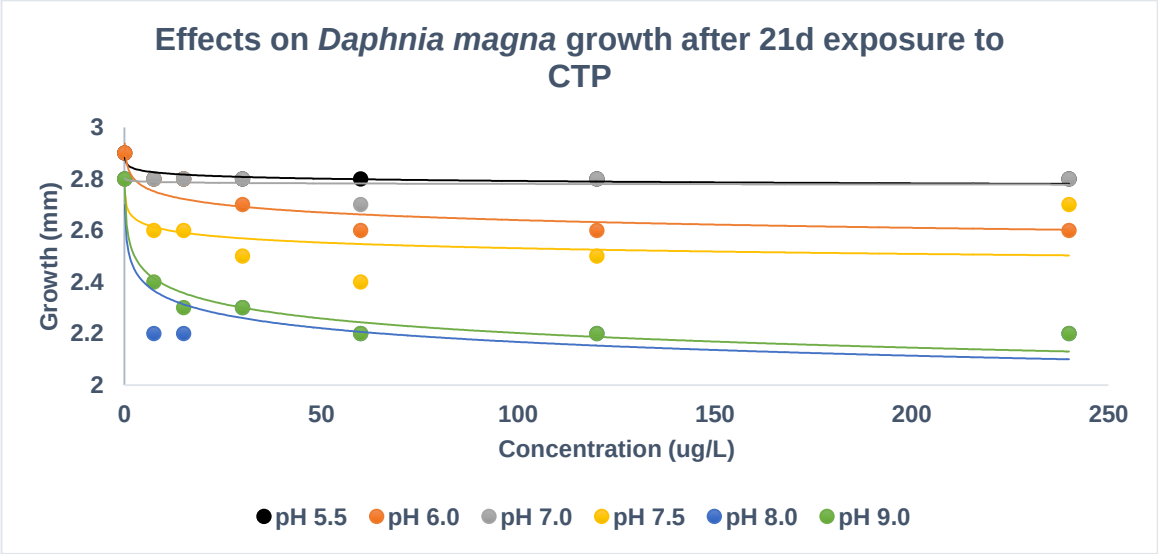


Figure 3a. Effects on *Daphnia magna* growth after 21d exposure to CTP at different pHs.

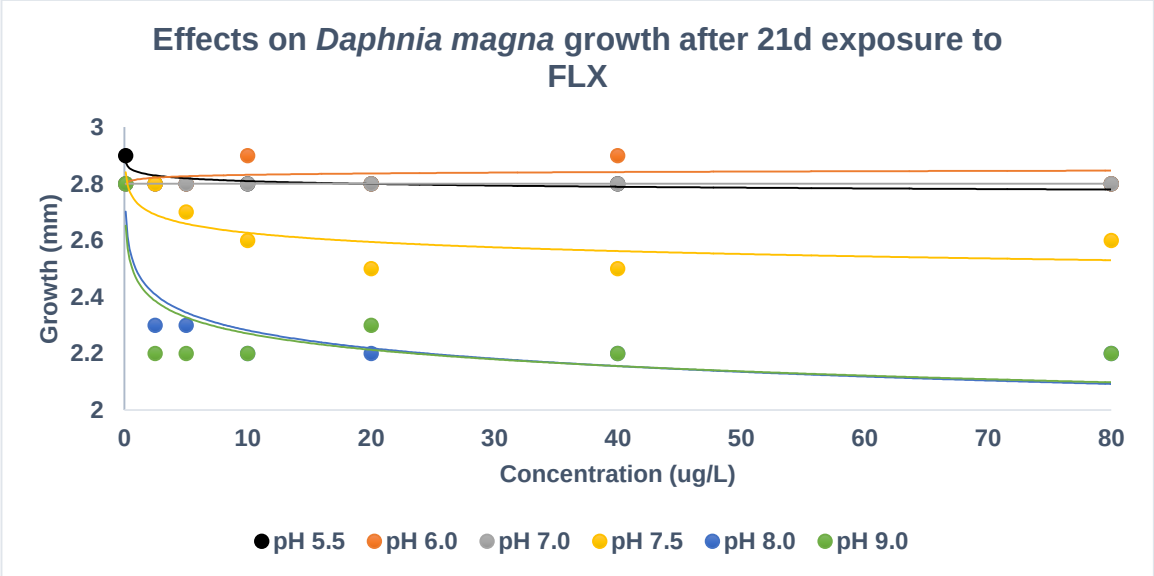


Figure 3b. Effects on *Daphnia magna* growth after 21d exposure to FLX at different pHs.

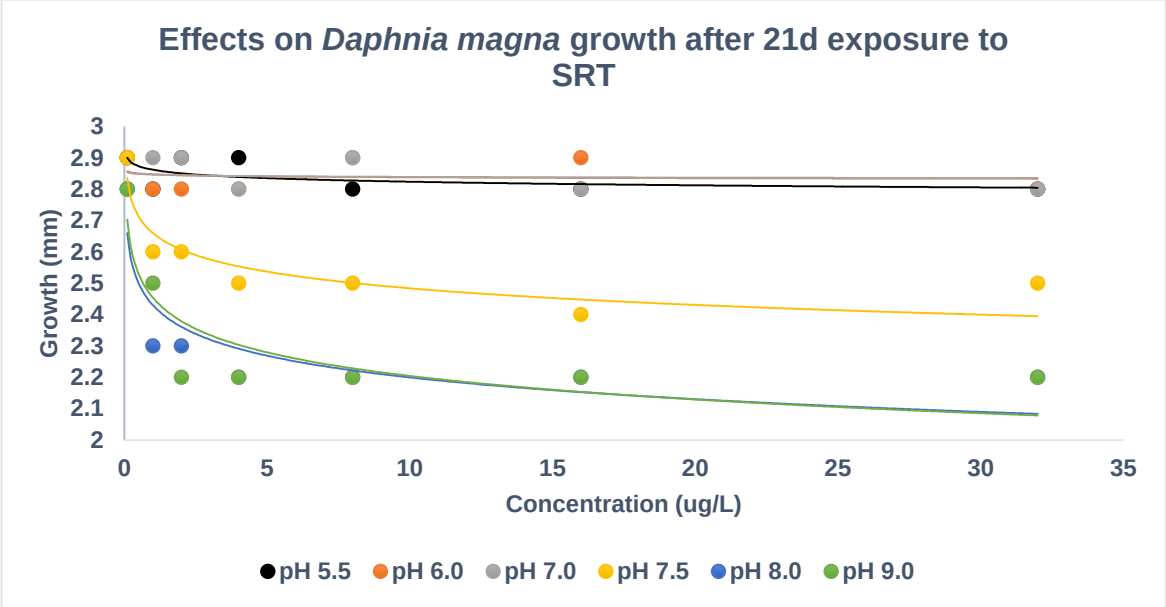


Figure 3c. Effects on *Daphnia magna* growth after 21d exposure to SRT at different pHs.

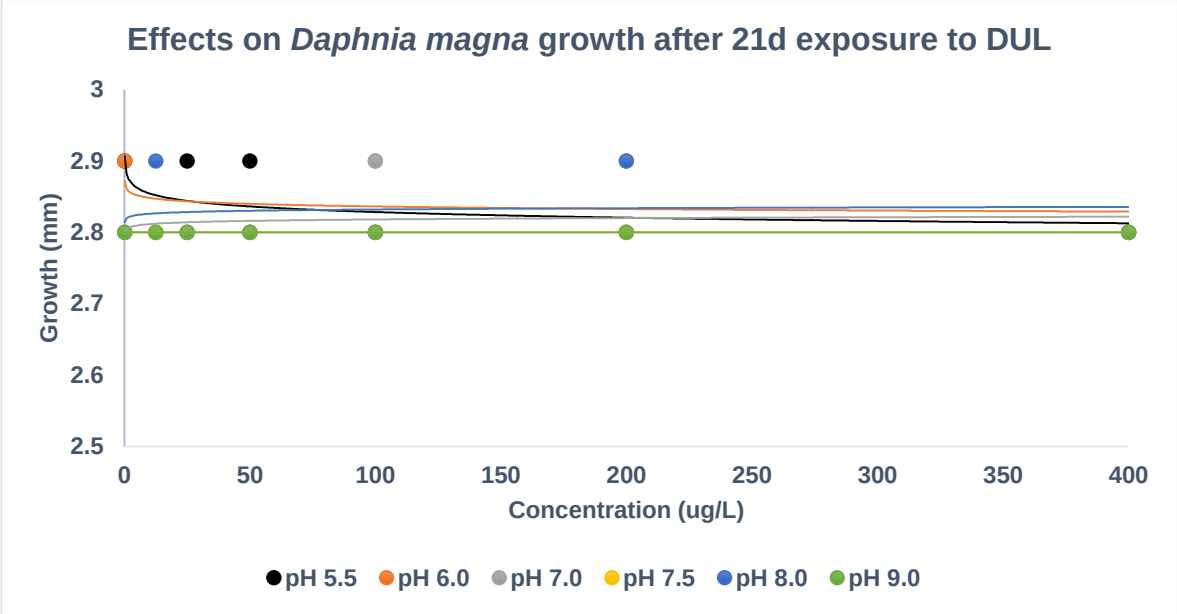


Figure 3d. Effects on *Daphnia magna* growth after 21d exposure to DUL at different pHs.

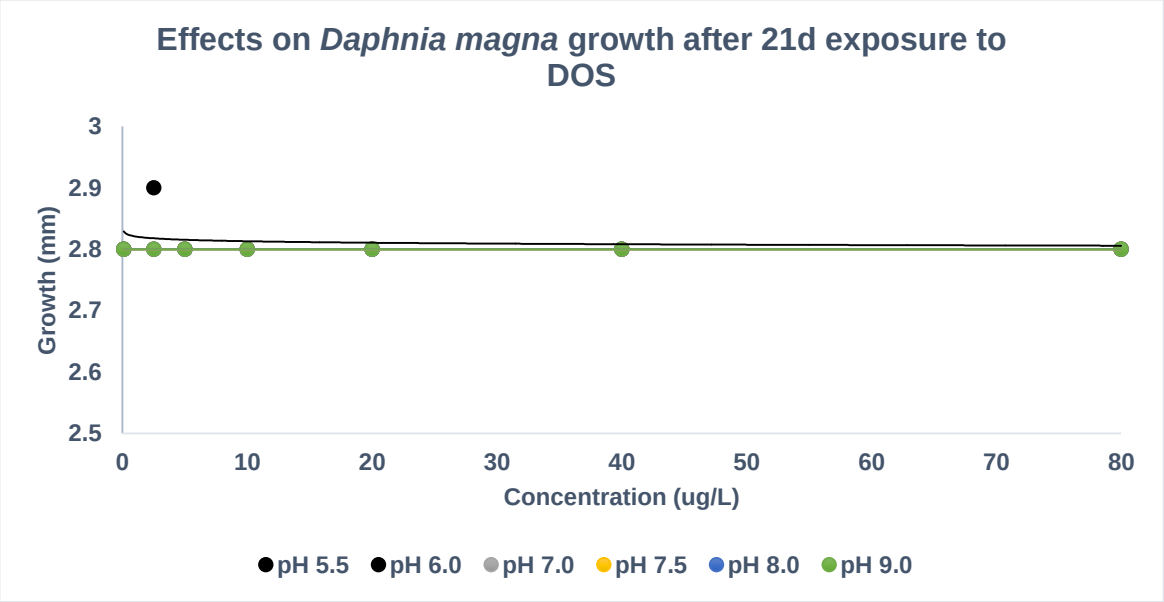


Figure 3e. Effects on *Daphnia magna* growth after 21d exposure to DOS at different pHs.

The growth of *D. magna* was significantly decreased with CTP or FLX exposure at pH8.0 and 9.0 (Fig. 3a and b). Also, the decrease trend was observed when *D. magna* was exposed to SRT (Fig. 3c). However, the growth was not affected by DUL or DOS exposure over all pH levels (Fig. 3d and e). Interestingly, the first reproduction was observed earlier with SSRI treatments but it was postponed with SNRI and TCA treatments (Supplementary data).

Model of ionization vs. acute toxicity

In general, the toxicity of each pharmaceutical was increased with the increasing fraction of neutral ions at increasing pH (Fig. 2). No significant difference between the measured LC50 and predicted LC50 between pH 5.5 and 7.5. However, the greater toxicity was observed compared to the predicted toxicity at pH 8.0 and 9.0. R^2 values between the measured and predicted toxicity for CTP and DUL were > 0.95; the measured toxicities were well-fitted to the predicted toxicities. FLX and DOS also had a good shape of fitness, R^2 > 0.90; the SRT toxicity estimation was relatively poor compared to other antidepressant, R^2 > 0.85. In other words, the sensitivity of each pharmaceutical at increasing pH could be arranged in SRT > DOS > FLX > DUL > CTP (greatest to weakest) which was very similar to the toxicity order of the acute toxicity tests results. These results demonstrate that the ionisable pharmaceuticals toxicity could be more sensitive at same pH medium.

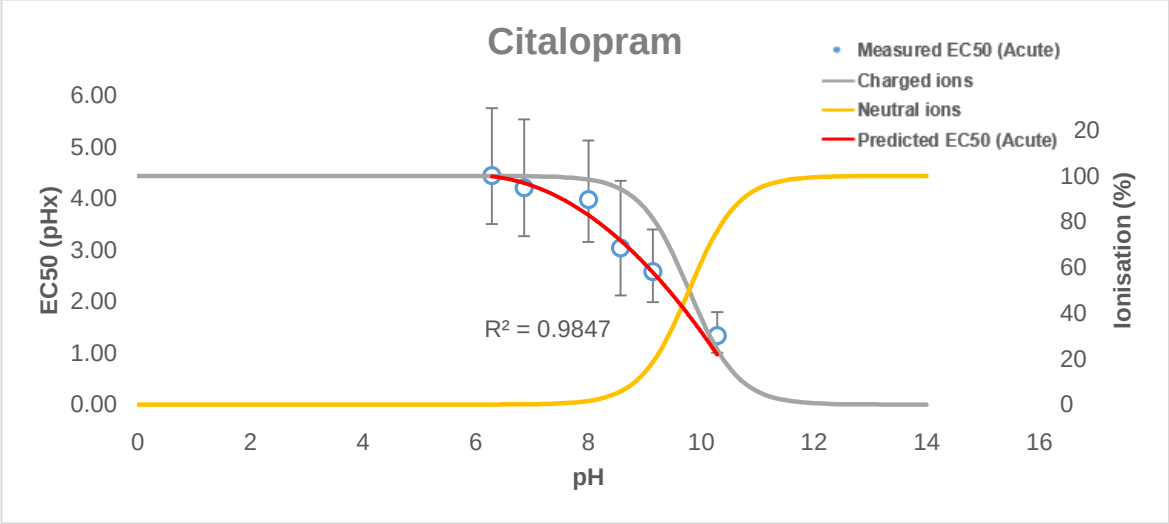


Figure 4a. A model of predicting LC50 of 48h acute toxicity of CTP at increasing pH 5.5, 6.0, 7.0, 7.5, 8.0 and 9.0 using fraction of ionization (%) and measured LC50. R^2 value between the measured and estimated LC50 was calculated for the estimation.

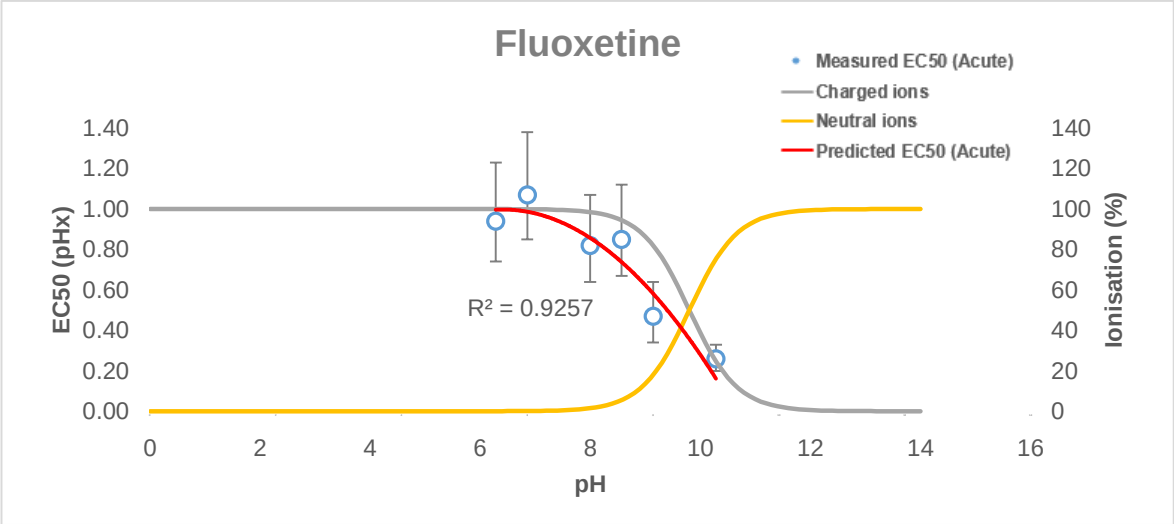


Figure 4b. A model of predicting LC50 of 48h acute toxicity of FLX at increasing pH 5.5, 6.0, 7.0, 7.5, 8.0 and 9.0 using fraction of ionization (%) and measured LC50. R^2 value between the measured and estimated LC50 was calculated for the estimation.

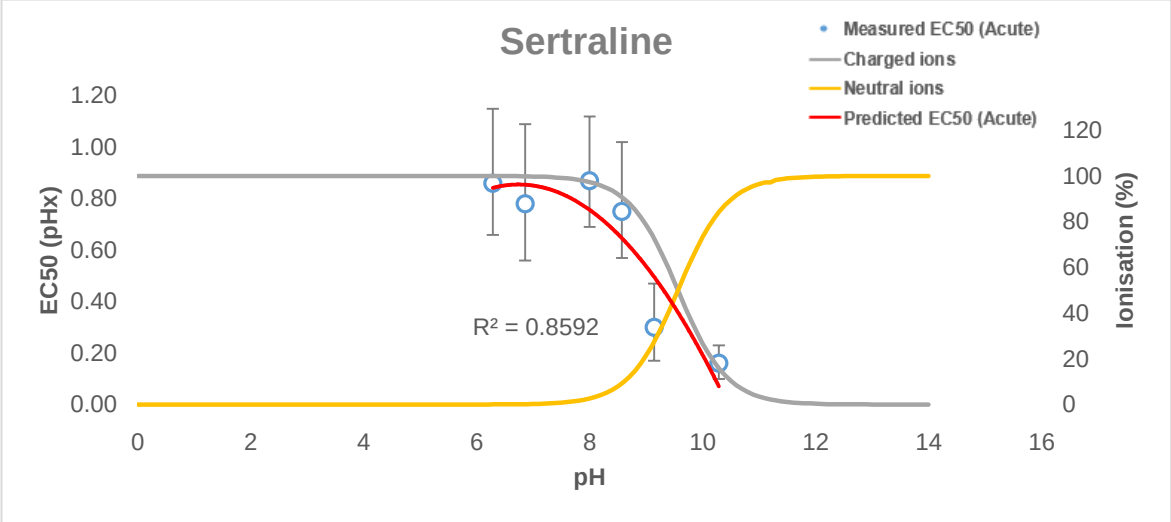


Figure 4c. A model of predicting LC50 of 48h acute toxicity of SRT at increasing pH 5.5, 6.0, 7.0, 7.5, 8.0 and 9.0 using fraction of ionization (%) and measured LC50. R^2 value between the measured and estimated LC50 was calculated for the estimation.

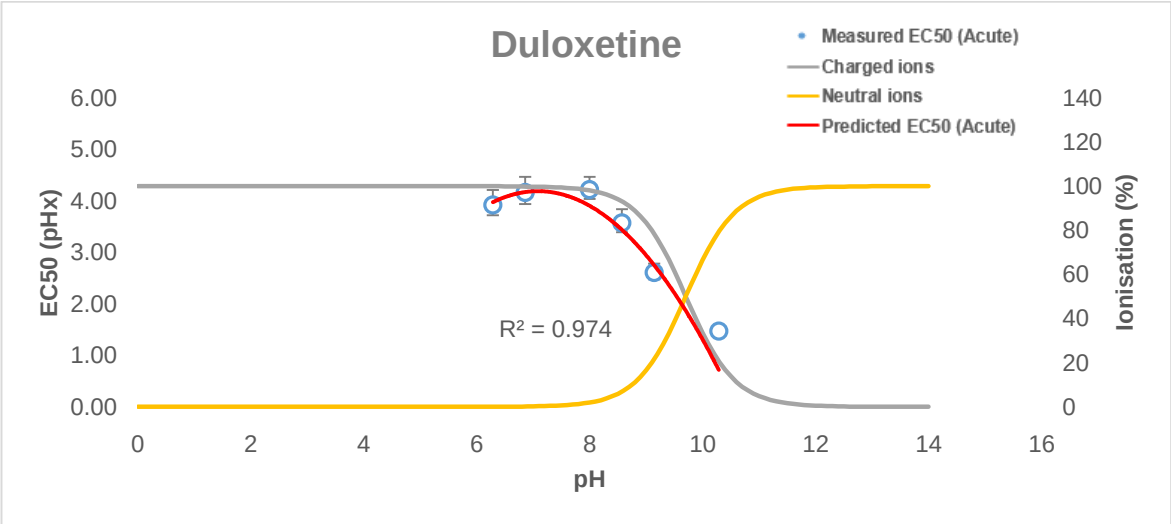


Figure 4d. A model of predicting LC50 of 48h acute toxicity of DUL at increasing pH 5.5, 6.0, 7.0, 7.5, 8.0 and 9.0 using fraction of ionization (%) and measured LC50. R^2 value between the measured and estimated LC50 was calculated for the estimation.

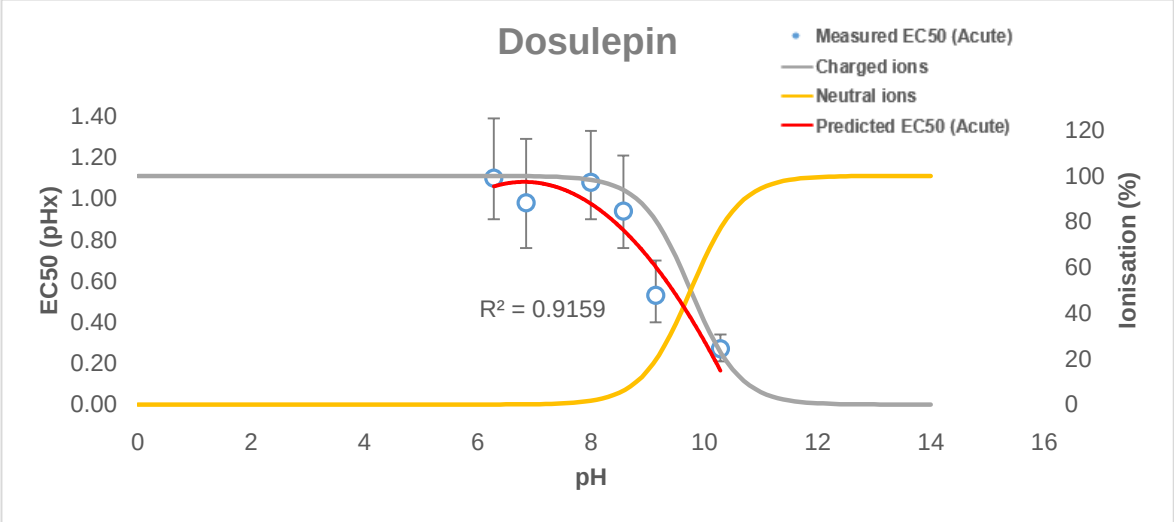


Figure 4e. A model of predicting LC50 of 48h acute toxicity of DOS at increasing pH 5.5, 6.0, 7.0, 7.5, 8.0 and 9.0 using fraction of ionization (%) and measured LC50. R^2 value between the measured and estimated LC50 was calculated for the estimation.

2.3. Discussion

Five antidepressants (CTP, FLX, SRT, DUL, and DOS) were tested in terms of their acute and chronic toxicity to *Daphnia magna* under different pH conditions.

In 48h acute study, our observation clearly indicates that survival performance in aquatic invertebrate could be adversely affected by in order of SRT > FLX > DOS > DUL > CTP (greatest to weakest). Minguez et al. (2014) found that SRT was the most toxic and CTP the least toxic in common SSRIs to *D. magna*. Although CTP is same antidepressant class with SRT and FLX, the general acute toxicity appeared at significantly different concentration in all pHs. It has been suggested that the animals are more sensitive to the specific chemical than to the antidepressant class in similar study (Di Poi et al., 2017). Unfortunately, there was no literature available to compare the acute toxicity for DOS and DUL.

In 21d chronic study, reproduction enhancement was observed with SSRIs exposure to *D. magna* and the greater enhancement was observed at increasing pH. In previous studies, there are few studies made about the effect of SSRIs to reproduction of *D. magna* and the results are inconsistent in the reproduction on aquatic organism (Brooks et al., 2003). For example, low dose of FLX stimulate offspring production in *D. magna* and *C. dubia* at 36 and 50 µg/L, respectively, whereas higher dose of FLX inhibited reproduction (Campos et al., 2012 and 2016), but there are some studies reporting opposite effects (Halliwushka, 2016). In addition, a FLX concentration range between 0 to 100 µg/L showed increased number of offspring. However, decreased number of offspring was observed at concentrations over 100 µg/L (Weinberger and Klaper, 2014). Henry and Black (2007) determined the mortality and reproduction by exposing CTP and SRT at relatively high concentration levels (0, 0.04, 0.40, 0.80, 4.00 and 8.00 mg/L; 0, 0.0009, 0.009, 0.045, 0.089, 0.447, 0.894 mg/L) and short-term of period, 7 or 8 days compared to our study. The concentrations ranges were similar to our acute test concentration range. The reproduction inhibition was observed with high mortality at greater CTP concentrations in 7-8 d exposure (Henry and Black, 2007). Previous studies presented opposite effects at higher concentration compare to our study. Therefore, our study on CTP and SRT is novel dataset that demonstrates the effects on reproduction at very low concentrations (µg/L) at various range of pH over 21 days. On the other hand, reproduction inhibition was observed with SNRI and TCA exposure on *D. magna* over 21 days and the greater inhibition effect was observed at increasing pH. Although the toxicity data for duloxetine and dosulepin were extremely difficult to find (Petrie et al., 2015), they are emerging toxicants which have recently been considered for toxicity tests from 2015 at Stockholm (Ahlford, 2007). Growth was decreased with SSRIs exposure during the chronic tests, but SNRI and TCA had no effects on *D. magna* growth level after 21 days chronic exposure. Exposing different concentrations of antidepressants with pH variation on *D. magna* has not been studied yet. However, there are some studies that explain how different antidepressant classes affect

the growth of aquatic invertebrates (Brooks et al., 2003; Flaherty and Dodson, 2005; Campos et al., 2012).

Lastly, we have modelled to estimate the toxicity using our acute toxicity data and ionic dissociation curves for each antidepressant. A number of pH-dependent toxicity studies on aquatic organisms have demonstrated that uptake and toxicity of ionisable pharmaceuticals can be very sensitive to changes in pH of the environment (Nakamura et al., 2008; Kim et al., 2010; Valenti et al., 2009; Rendal et al., 2011). Therefore, our model determines the relationship between the fractions of ionic species and toxicity of antidepressant at increasing pH condition. Also, we can see the sensitivity of each antidepressant at increasing pH condition by contrasting the measured and estimated toxicity and fraction of ionic species at specific pH level.

Effect of pH on each antidepressants on survival of *D. magna*

The mortality of *D. magna* was expected to increase with each antidepressant exposure at increasing pH because pH variation alters the toxicity of ionisable pharmaceuticals. Also, the previously published studies reported increased toxicity on aquatic communities as pH increases (Nakamura et al., 2008; Flaherty and Dodson, 2005; Barbosa et al., 2017). Our analysis of LC50 at different experimental pHs (5.5, 6.0, 7.0, 7.5, 8.0 and 9.0) showed greater toxicity with a greater fraction of the uncharged chemical form (Appendix 1 and 2). This corresponds to previously published data that looked at each pharmaceutical and *D. magna* mortality and reported significantly increased acute toxicity with increasing pH values (Campos et al., 2016). However, the different mechanisms of antidepressants needs to be discussed prior to the effect of pH uptake, because the lethal mechanisms were enhanced at increasing pH medium.

During the SSRI exposure, oxygen consumption was increased with previously reported research (Campos et al., 2012). Increased pH could influence the concentration of serotonin and dopamine by greater toxicity of SSRI. Although our study have not measured the oxygen consumption, there is an evidence that monoamine neurotransmitters (serotonin and dopamine) control the secretion of peptide hormones in malacostracan crustaceans (Fingerman, 1985). These hormones regulate carbohydrate metabolism, synthesis or secretion of ecdysteroids and terpenoids, while significantly increased oxygen demand was observed at 70 h exposure time with a relatively low FLX concentration (Campos et al., 2012). Therefore, the differences in uptake at increasing pH condition enhanced the oxygen demand with higher acute concentrations of antidepressants.

When *D. magna* was exposed to SNRI, 5-HT and norepinephrine (NE) receptors are blocked which increase the concentration of 5-HT and NE. One immune cell type that has been extensively studied for its effects with NE is T-lymphocytes (Case et al., 2016). Early *in vitro* work with T-lymphocytes demonstrated that NE decreased the amount of pro-

inflammatory cytokine production through an inhibition of interleukin 2 (IL-2) (Strell et al., 2009). However, the down regulation of IL-2 caused to increase the concentration of reactive oxygen species (ROS) as it is one of the key component for the ROS production (Novak and Rothenberg, 1990), and the increased ROS caused the greater mortality on *Daphnia magna* (Novak and Rothenberg, 1990). NE appeared to produce inhibitory effect through a β2 adrenergic receptor-mediated mechanism (Strell et al., 2009) which stimulates the immune cells activity in tissues. This inhibition slows the proliferation of T-lymphocytes which could induce the weaker immune system to increase the chance of mortality when SNRI acutely exposed.

TCA blocks 5-HT and NE transporters to increase their effectiveness in the synapse. All TCAs are rapidly absorbed by 90-95% at therapeutic plasma concentrations. Cytochrome 450 (CYP450) is a family of isozymes responsible for the biotransformation of several drugs (Sturm and Hansen, 1999) and initiating detoxification of toxic compounds mostly. They bind to membranes in *Daphnia magna* to inhibit the CYP450 enzymes. A large inactivation occurs by demethylation of tertiary TCAs to their secondary amine metabolites, hydroxylation, then glucuronidation and excretion (Gillman, 2007). Drug metabolism though the CYP450 enzyme system has emerged and determined that the occurrence of interactions between several drugs could result in toxicity, altered bioavailability, increased or decreased pharmacological effect or adverse drug reactions (Dey et al., 2015). There is a study that summarised the systematic interaction between TCAs and CYP450 enzymes, but the most inhibition was observed especially with duloxetine among the tertiary amine TCAs (Gillman, 2007). DUL caused a serious adverse interaction to CYP2C19 isoform in the enzyme, the main role for this isoform is to pump out the inhibitors and antidepressants. Furthermore, it has been studied that the mechanism of DUL induces oxidative stress (Dey et al., 2015). Hence, the concentration of ROS would have been continuously increasing to fatal points while the biotransformation was seriously inhibited via CYP450 enzyme during the duloxetine exposure.

Turning back to our results, the toxicity of all antidepressant compounds increased as water pH increases, especially at basic conditions (pH above 7.0). The calculated fraction of uncharged ions for all antidepressants had similar increasing trend which were 2.0-3.0, 8.0-10.0, 3.1-3.2 and 8.1-8.8 times greater between each pH from 5.5 to 9.0 (Appendix 1). The pH-dependent toxicity usually follows that the greater toxicity in uncharged fraction, whereas when the ionic fraction is in a great majority, toxicity may be shifted to uncharged fraction. If the toxicity was caused by the uncharged fraction only, it was expected to have very similar LC50 values over the pHs for each chemical. However, the toxicity of FLX, DOS, and SRT increased ($p < 0.001$) from pH 8.0, CTP and DUL increased ($p < 0.001$) only at pH 9.0. Hence, FLX, DOS, and SRT were much adversely interacted with *D. magna* than other 2 antidepressants at same pH level.

Our results could be explained by the K_{ow} value of each antidepressants because the K_{ow} is traditionally used to estimate uptake of neutral ions (Meredith-Williams et al., 2012). FLX, DOS and SRT, have relatively greater K_{ow} value (4.65, 4.68 and 5.29) than CTP and DUL (3.74 and 4.29) (Table 1). It has been suggested that high K_{ow} was correlated to the acute toxicities, the hydrophobic properties of compound were partly translated with their action (Minguez et al., 2014). Moreover, K_{ow} was also correlated to the bioaccumulation on aquatic organisms such as algae (*Chlorella vulgaris)* (Geiger, 2014). Antidepressants might have accumulated on their food source, algae, which is another unexpected route for adverse effect. Lastly, ion trapping might have occurred during the experiments. Elevated concentration of the charged form in the organism due to pH differences between the organism and the medium, results in two different ionic/uncharged equilibrium ratios (Simon, 1950). In our study, these unexpected cases could have affected the toxicity of each antidepressant between pH 8.0 or 9.0. Thus, daphnids exposed with SSRIs could have been a greater chance to be adversely affected through the medium itself and digesting algae which were more bioaccumulated with the chemicals.

Effect of increasing pH on each antidepressant on reproduction of *D. magna*

In SSRIs, the number of offspring at each day and total number of offspring have increased at pH increasing pH in our results (Fig. 2). The adverse effects are likely explained by the pharmacological mode of action of SSRIs of increasing serotonin postsynaptic activity to stimulate ecdystroids and juvenile hormone which are responsible for controlling oogenesis and vitellogenesis, resulting in an increased reproduction and decreased offspring size and maturation age (Fig. 3 and 4; Campos et al., 2012; Taylor et al., 2005). There is also evidence that increased serotonin activity by SSRIs stimulates ovarian and testicular development and increases size of ovaries and oocytes in decapod crustaceans (Fingerman, 1985) which explains the stimulation of fecundity in *D. magna* and *C. dubia* has resulted from increased synaptic serotonin levels. Moreover, reproduction is energetically costly, for instance the increase in *D. magna* and *C. dubia* offspring production should not necessarily be associated with the maintenance of offspring variability of fitness in some cases (Campos et al., 2012). Furthermore, the SSRIs adversely affects *D. magna* to demand greater amount of oxygen by increased aerobic respiration which caused by increased 5-HT (Campos et al., 2012). A plausible hypothesis is that SSRIs may increase oxygen consumption rates by favouring aerobic metabolism at the expenses of the anaerobic one (Campos et al., 2012). The increased offspring of zooplankton by FLX would be critical to avoid fish predation, eating in shallow waters at night and migrating to deep water. Hence, although the low dose of FLX increases offspring reproduction, the survival rate will be adversely affected. SSRIs would obviously have greater adverse effects on reproduction at increasing pH condition. Also, greater potential of bioconcentration or bioaccumulation could increase the potency of SSRIs toxicity (Nakamura et al., 2008).

The number of young per female was decreased when *D. magna* was exposed to DUL or DOS at increasing pH for 21 days (Fig. 3). In previous study, the mean number of offspring released per female daphnid in the treatments (0, 0.011, 0.037, 0.08, 0.14 and 0.26 mg/L) was also decreased at standard pH condition (EPA). However, the EC50 of our result was almost double compared to the precious study (0.28 mg/L). There are no study published about the chronic effects of DOS on *D. magna*. The concentration of ROS and the adverse effects on immune systems were increased when 5-HT and NE transporter and receptors are inhibited by individual DUL and DOS, respectively, as these are discussed at the acute toxicity. We could assume that the lowered reproductive output is evidence for higher energy needs for maintenance against the ROS production and pro-inflammatory cells, according to the principle of energy allocation (Gilbert, 2012). DOS and DUL also obviously had greater adverse effects at increasing pH condition because of the greater uptake of neutral ion fractions.

Effect of increasing pH on each antidepressants on growth of *D. magna*

The animal chronically exposed to B-blocker (SSRI) would have a reduced energy supply and must allocate the available energy between growth and reproduction. In response to the reduced growth, the animal may be forgoing reproduction in order to survive (Fig. 3). Eventually, increase in reproduction was abandoned to increase the survival rate at higher SSRI concentration. Similar trade-offs could have been observed in *D. magna* exposed to cadmium and brine shrimp *Artemia franciscana* exposed to chronic hypoxia (Campos et al., 2016).

Ecotoxicological concerns of antidepressants in perspective of PECs

Our study has conducted at acidic, neutral and alkali pH levels that cover the most of the European water pH range. In terms of EC50 for reproduction, majority of chronically exposed concentrations were close to the studied PECs of each antidepressant. Moreover, the PECs near to WWTPs, hospitals, or sewage plants were discovered to be greater than EC50. It could be discharged directly to aquatic environments by flooding. Therefore, it is urgent to monitor the antidepressants concentrations to prevent the adverse effects on aquatic organisms.

Table 3. Summary of our chronic results, predicted environmental concentration (PEC) and measured environmental concentration (MEC) in surface water.

Antidepressants	pH	EC50 for reproduction (µg/L)	$PEC_{surface-water}$ (µg/L)	$MEC_{surface-water}$ (µg/L)
Citalopram	5.5	15.8	76.0	0.01-0.15
	6.0	48.1		
	7.0	6.69		
	7.5	8.89		
	8.0	7.93		
	9.0	3.04		
Fluoxetine	5.5	15.2	0.046	0.012-0.540
	6.0	6.34		
	7.0	2.80		
	7.5	3.92		
	8.0	1.93		
	9.0	0.83		
Sertraline	5.5	2.78	0.31-1.2	0.007
	6.0	4.29		
	7.0	2.08		
	7.5	2.89		
	8.0	1.05		
	9.0	1.59		
Duloxetine	5.5	104.0	0.05	N/A
	6.0	55.9		
	7.0	27.9		
	7.5	44.0		
	8.0	9.98		
	9.0	7.75		
Dosulepin	5.5	4.61	N/A	N/A
	6.0	7.07		
	7.0	4.91		
	7.5	5.07		
	8.0	2.39		
	9.0	2.24		

Table 3 summarized the chronic results, PEC and MEC to compare and recognize the aquatic toxicity of tested antidepressants. Fick et al. (2009) reported that PEC for citalopram was 76 µg/L which was much higher than determined EC50 at every pH from our study. Although the normal range of MEC for citalopram is between 10-150 ng/L, aquatic organisms are still at high risk because EC10 would be observed at pH 9 if the normal MEC range increases by 6 times. Several studies detected fluoxetine in the aquatic environment

with concentrations in range between 0.012 and 0.54 µg/L (Weston et al., 2001; Kolpin et al., 2002; Chen et al., 2006; Gardner et al., 2012). The EC50 of fluoxetine from our results were fairly close to the detected fluoxetine concentrations over the world. From our results, the MEC of fluoxetine has already exceeded the EC_{10} of fluoxetine at every pH above 6.0. Hence, the aquatic organisms could be much adversely affected if the negligible concentration of fluoxetine increased.

Furthermore, PEC of sertraline was calculated to range from 0.31 to 1.2 µg/L in Europe and North America. The maximum PEC of sertraline was already greater than the EC50 at pH 8.0. Although sertraline results have demonstrated the urgency in the perspective of PEC, the ecotoxicity researches for sertraline are not yet done much as other pharmaceuticals. The PEC_{max} for duloxetine was 0.05 µg/L which was much lower than our EC50 at every pH level. However, the PEC_{max} was relatively close to the EC_{10} at pH 8.0 and 9.0 compared to the other EC_{10} at lower pH level. Duloxetine also requires greater ecotoxicological focus because there are extremely limited number of ecotoxicological studies about duloxetine. Dosulepin had a same endpoint as duloxetine, but the toxicity was much greater at every pH level, however, it is also difficult to find the ecotoxicological data for duloxetine.

In consequence, aquatic organisms have been already adversely affected at some sites where relatively high PEC of antidepressants. Moreover, the effects are becoming much stronger at alkali condition which could be designed by naturally or manually by humans (Wurts, 2003). Therefore, the effect of trace level of ionisable compounds in varying pH conditions are urgently to be discussed over the world.

Model estimates the toxicity of antidepressants using pK_a and pH

The figure 4 shows the LC50 estimation using the measured LC50 from the 48h toxicity tests and the fraction of ionisation (%) with studied antidepressant (equation 1). In general, the measured toxicity were well-fitted to the estimated toxicity of all studied pharmaceuticals at increasing pH ($R^2 > 0.85$). The order of estimated toxicity fitness from the least fitted to most fit were very close to the order of acute toxicity ranks from our data. This means that our acute toxicity estimation was well-estimated by comparing the measured toxicity of each antidepressant with the predicted toxicity. Also, it determines the sensitivity of each antidepressant. For example, if less fitted measured toxicity to predicted toxicity observed, we can assume that the greater sensitivity of antidepressant toxicity with the fraction of ions. The estimated toxicity were expected to be perfectly fitted to the faction of charged ions in the graphs because the toxicity was expected to be increased with increasing fraction of neutral ions. Moreover, most of literature agree that the toxicity of ionisable pharmaceuticals for aquatic test species is higher when greater proportion of the neutral species is present (Boström and Berglund, 2015; Rendal et al., 2011; Valenti et al., 2009). Our results also

followed the same trend with the literature, however, the measured LC50 of FLX, SRT, DUL and DOS at pH 9.0 were greater than the estimated LC50 values.

Our ionization modelling clearly explains that the increasing fraction of neutral ions increase the toxicity of basic pharmaceuticals, antidepressants, to aquatic invertebrates. However, the most important thing was a small change in fraction of neutral ions could enhance the toxicity because the estimated and measured toxicities were not guaranteed to be perfectly fitted to the fraction of charged ions. In other words, pharmaceuticals that classified in low or medium risk because of very low PEC, such as DOS and DUL, could also adversely affect the aquatic organisms with a negligible concentration if present in the pH condition of aquatic environment is similar to their pK_a level. Although there are no studies that explains how the toxicity become greater with the small fraction of neutral ions, we could confirm that the very small fraction of neutral ions at basic pH condition could increase the aquatic toxicity of antidepressants. Most importantly, the concentrations in antidepressant mixture have been increasing at various pH conditions from the last decade. Therefore, the toxicity of antidepressant mixtures at increasing pH conditions need to be measured.

2.4. Conclusion

Our observations clearly show that toxicity of studied antidepressants (citalopram, fluoxetine, sertraline, duloxetine and dosulepin) increase as pH increases on *Daphnia magna*. The survival performance in aquatic invertebrate could be adversely affected in the order of SRT > FLX > DOS > DUL > CTP (strongest to weakest). The EC50 of each antidepressant has changed at increasing pH levels in 48hr acute test. Reproduction enhancement with decrease on growth (mm) was determined by exposure of SSRIs. However, reproduction inhibition with no effect on growth was determined by SNRI and TCA exposure at increasing pH in 21 d chronic test. An increased reproduction in the chronic test may be determined as positive effect, but the growth of *D. magna* was reduced at increasing pH. A model was built to estimate the lethal toxicity of antidepressant at increasing pH using the pK_a and fraction of ionic species at whole range of pH. The order of estimated toxicity of antidepressants was matched with the toxicity order determined from the acute tests. Furthermore, antidepressants concentrations lower than PEC could also adversely affect the aquatic organisms if they present at pH condition that similar to their pK_a. Thus, mixture tests will be performed in next chapter to determine the effect of smaller concentration of antidepressant on *D. magna* if they present as a mixture.

Chapter 3

Effects of Antidepressant Combinations on the Mortality, Reproduction of *Daphnia magna* in UK surface waters

3. Introduction

In the natural environment, it is almost impossible to find sites contaminated with only one chemical, usually organisms are exposed to mixtures of chemicals (Heys et al., 2016). This is particularly true for surface waters, where mixtures of potentially toxic substances enter the surface waters as a result of human activities (Backhaus et al., 2004a; Verro et al., 2009). Aquatic organisms are therefore rarely exposed to individual chemicals and determining ecotoxicity of only a single pharmaceutical does not fully represent effects in the real environment which is polluted with mixtures of pharmaceuticals (Heys et al., 2016). However, chemical risk management procedures commonly rely on single compound evaluations and the determination of threshold values, like no observed effect concentrations (NOECs) or effective concentration (ECx) (Altenburger et al., 2004; Cedergreen et al., 2008; Syberg et al., 2008; Walter et al., 2002) for single substances. Studies on the toxicity of mixtures shows that components, when combined, at levels below NOECs may cause toxicity (Kortenkamp and Altenburger, 1999; Rajapakse et al., 2002; Walter et al., 2002; Altenburger et al., 2003; Vighi et al., 2003; Lydy et al., 2004; Breitholtz et al., 2008). There is still a lack of knowledge as to the underlying mechanism for such interactions (Xu and Nirmalakhandan, 1998). Therefore, the concerns regarding the use of knowledge from single substance to evaluate mixture toxicity as the mechanisms of action may be poorly understood and the interaction between chemicals hard to determine (Berenbaum 1985).

Chemicals can produce a synergism or antagonism effect compared to the expected mixture effects when they interact together. Mixed exposure tests have demonstrated that exposure to mixtures of chemicals can lead to a toxic effect higher than each chemical alone. Anderson et al. (2002) showed an increase in toxicity when the amphipod *Hyalella azteca* was exposed to three organophosphates (chlorpyrifos, methyl parathion and diazinon) in the presence of atrazine. Laetz et al. (2009) observed addition and synergism, with a greater degree of synergism at higher exposure concentrations of organophosphate and carbamates mixtures, using the *Oncorhynchus*. Nørgaard and Cedergreen (2009) showed synergism when *Daphnia magna* was exposed to prochloraz and alpha-cypermethrine. Strictly additive effects were found by Bailey et al. (1997) in experiments where *Ceriodaphnia dubia* were exposed to the organophosphates diazinon and chlorpyrifos. These studies show that there is a need for further mixture toxicity studies in order to determine the interactions between chemicals in a mixture, instead of relying on the quantification of toxicity from single test evaluations.

A mixture study is normally designed to discover adverse effects on standard endpoints including mortality, reproduction, growth, population growth rate, and first day of reproduction over 21 days exposure on *D. magna*. However, acute tests could suggest

more effective mixture evaluation. For instance, the mixture acute results may be compared between different pH levels in the same species. Moreover, binary mixture toxicity tests were the most common mixture experimental design (Di Poi et al., 2017; Mendis et al., 2018; Nys et al., 2016). Some pharmaceuticals react together in mixture state under similar or same mechanism. On the other hand, some pharmaceuticals interact using their different mechanisms, such as affecting the multiple target cells in the organisms by individual mechanisms. There are two major mixture models, concentration addition (CA) and independent action (IA), which estimate the mixture toxicity based on mechanism or endpoints of each mixture component. Therefore, this study used the mixture model to estimate the toxicity of antidepressant mixture.

Concentration Addition (CA) and Independent Action (IA)

Some researchers insist that toxicity tests for mixtures are indispensable in validating untested assumptions and simplifications (Borgert, 2004). In practice, however, conducting toxicity tests on all conceivable combinations of chemical substances is unfeasible due to the very large number of possible combinations, as well as the changeable status of chemical combinations in the environment at any time (Cassee et al., 1998; US ATSDR, 2004; Lydy et al., 2004). In addition, toxicological tests using animals are expensive, time-consuming, and raise ethical issues. Therefore, there is an essential need for appropriate mixture prediction models which use knowledge on chemicals in order to facilitate practical chemical risk assessment that satisfies the scientific, regulatory, and industrial perspectives.

Developing reliable methods for estimating mixture toxicity based on single substances is one of the main challenges in ecotoxicology (Faust and Scholze, 2004). Conventionally two predictive models: the Concentration Addition (CA) and the Indpendent Action (IA), have been used to estimate the additive toxicity of chemical mixtures based on concentration-response data of each component of the mixture. The CA (Loewe and Muischnek, 1926; formula 6) and IA (Bliss, 1939; formula 7) models are based basically on contrary assumptions: every mixture component has either similar or dissimilar mode of action (MoA) (Faust et al., 2003). The number of input parameters used in the calculation process of respective CA and IA models is the same, but the type of each parameter used in these models is different.

$$\sum_{i=1}^{n} \frac{ci}{\mathrm{ECx}i} = 1 \tag{6}$$

where *n* is the number of components in the mixture, *ci* is the concentration of the *i*th component, and ECx*i* is the concentration of the *i*th component that induces X% effect.

$$Emix = 1 - \prod_{i=1}^{n}(1 - Ei) \quad (7)$$

Where *n* is number of mixture components, *Emix* is predicted mixture effect, and *Ei* is effect of the *i*th substance.

The CA model calculates toxicity in the mixture by summation of the effective concentration parameter (e.g., EC50) of each mixture component after modifying the differences in potencies (Loewe and Muischnek, 1926; Finney, 1942; Feron and Groten, 2002). The IA model predicts mixture toxicity by summation of the responses parameter (e.g., effect (%)) of each component in a mixture based on the probability theory. The IA model does not consider the contribution of constituents existing at no-effect concentrations into the overall mixture toxicity (Bliss, 1939; Finney, 1942; Cassee et al., 1998; Feron and Groten, 2002).

The overall toxicity calculated by the CA model, especially for low mixture concentrations, differ largely from that predicted by the IA model (Drescher and Boedeker, 1995). For example, Cedergreen et al. (2008) conducted a study that tested the accuracy of the CA and IA models on binary mixtures with various MoAs (158 toxicity datasets for 98 different mixtures comprised mainly of pesticides and pharmaceuticals tested on one or more of seven test organisms). The results showed that the effects of around 20% of the mixtures were properly predicted by the IA model and 10% were correctly estimated by the CA model. Both models could equally predict the results of another 20% of the testing datasets. The toxicities of approximately half of the datasets could not be correctly estimated by either of the two models (Cedergreen et al., 2008). Although, the overall performance of the CA model was lower than that of the IA model, it has been argued that the CA model should be used as a default model from a regulatory point of view for determining aquatic toxicity of mixtures since it is usually more conservative and less data-demanding than the IA model (Arrhenius et al., 2004; Backhaus et al., 2004; Junghans et al., 2006; Cedergreen et al., 2008; Syberg et al., 2009). Moreover, the EC value calculated by the CA model is normally used to describe mixture toxicity in risk assessment rather than the effect estimate of the IA model.

There are some reports that have explored the use of these models for estimating the toxicity of mixtures of pharmaceuticals. For instance, Geiger et al. (2016) studied the mixture effects of three different antibiotics, including ibuprofen and ciprofloxacin using algae, *Chlorella vulgaris*. Ibuprofen is used as a nonsteroidal anti-flammatory drug (NSAID), known for its anti-inflammatory which is a non-selective inhibitor of cyclooxygenase, an enzyme involved in prostaglandin synthesis. Ciprofloxacin belongs to the group of fluoroquinolones, which form a major class of antibiotics. The adverse effects of both pharmaceuticals are known for inhibition of the pathways involved in photosynthetic

metabolism and finally affect the cell growth (Halling-Sorensen, 2000). Although they were from different classes, concentration addition (CA) provided better estimates of the toxicity of the mixtures compared to the independent action (IA) model which tended to underestimate the toxicity. Another study explored the effects of quaternary mixture effects of NSAIDs including diclofenac, ibuprofen, naproxen, and acetylsalicylic acid on *Daphnia magna* (Cleuvers, 2004).

Christensen et al. (2007) tested binary mixture effects with 5 SSRIs, citalopram, sertraline, fluoxetine, fluvoxamine, and paroxetine. No synergistic or antagonistic results were observed which means that CA concept was appropriate to be applied. Since the antidepressants were not frequently examined with toxicity tests relative to other pharmaceuticals or toxicants, it is difficult to find mixture chronic toxicity data (Christensen et al., 2007). Therefore, the rationale of this study is determining the effects of antidepressant mixtures that composed with negligible concentrations of individual antidepressant while each antidepressant is not toxic in concentration which found in mixtures.

Mixture concentrations were selected using the total amount (mg) of each antidepressant, dispensed or supplied by pharmacy, appliance and dispensing doctors per year from 2009 to 2018, calculated by the Prescription Cost Analysis (PCA) dataset provided from National Health Service (NHS). It is due to determine the adverse effects of antidepressant mixtures over the last decade in the UK. Three mixture groups (A, B, and C) were classified based on the change in use of antidepressants (%; above 25 or 50) over each year from 2009 to 2018. The average PEC value for each groups were calculated using the formula 8 introduced by Guo et al. (2016) (Appendix 3)

$$\mathrm{PEC_{MET}} = \frac{\mathrm{Subinhab\ x\ Fexc}}{\mathrm{WasteWinhab\ x\ Dilution}} \quad (8)$$

where $\mathrm{PEC_{MET}}$ is predicted environmental concentration for surface water assuming removal through patient metabolism (mg/L); Subinhab is substance consumed per inhabitant per day for the UK population (mg inh/d); Fexc is fraction of pharmaceutical excreted unchanged; WasteWinhab is amount of wastewater per inhabitant per day, 200 (L inh/d); Dilution is dilution factor, default value 10.

Then, the calculated $\mathrm{PEC_{MET}}$ values were used to calculate the concentration of each antidepressant in each mixture at 3 different pH levels (5.5, 7.0, 9.0) using the formula 8 (Appendix 1).

The aim of this chapter is, to produce a novel mixture chronic toxicity dataset, and determine the accuracy of CA model to review the toxicity of antidepressant mixtures in aquatic invertebrates in England in last decade. Hence, greater toxicity with mixtures of antidepressants are expected acutely and chronically on *Daphnia magna* at increasing pH conditions.

3.1. Materials and methods

The methods for maintaining *Daphnia magna* and *Chlorella vulgaris* culture, and chemical analysis were the same as those described in Chapter 2.

Test chemicals

The study compounds were same with the previous chapter that have prioritized pharmaceuticals in terms of their potential environmental risk (Guo et al., 2016; Burns et al., 2017). The study chemicals, citalopram, doselupin, duloxetine, fluoxetine and sertraline, were purchased from Sigma-Aldrich (St. Louis, MO, USA), all with purities ≥ 98%. All other chemicals used in the present study were analytical or reagent grade.

Organism culturing

D. magna were originally obtained from FERA Science (York, UK) and maintained in the Environmental Toxicology Laboratory at the University of York. The *D. magna* culture was maintained at 20 ± 1°C in six 2 L glass beakers containing 2 L of ADaM media (Klüttgen et al., 1994) following protocols developed by the US EPA (US EPA, 2002). The average dissolved oxygen concentration in media was maintained at > 3 mg/L, based on OECD guidelines (OECD, 2008). Cultures were maintained under a white fluorescent light (12.1 µmol/m^2/s) with a 16:8 h light:dark photoperiod. *D. magna* were fed with YCT (1:1:1 mixture of Yeast, Ceropyl® and Tetramin®) and algae (*Chlorella vulgaris*). A standard *Daphnia* reference toxicity test was conducted every two weeks by exposing organisms to NaCl for 48h to assess changes in organism sensitivity and assure the quality of the antidepressant toxicity tests (US EPA, 2002; see Supplementary data).

Chlorella vulgaris were grown in Kuhl's medium in 2 L glass flasks at 23°C and 6000-10000 lux. All of the glass flasks were sterilized and sealed with cotton wools at the flask opening. Discontinuous large-scale culturing was used for the main culture flasks and semi-continuous small-scale culture was carried for the sub-cultures in 200 mL glass flasks. Algae were harvested and centrifuged at 3000 RPM for 3 minutes and stored at 4°C to stop growth. The culture flasks were refilled with new media and algae after the old culture was successfully done. Harvested algae was not kept for longer than 3 weeks. Cell counts of the algae were taken every 6-8 days to record cell growth using an absorbance spectrophotometer until the satisfied cell density of $30x10^6$ cells/mL was reached.

Performance of *D. magna* acute and chronic mixture studies

The 48h acute toxicity of the mixture antidepressants to *D. magna* was examined at pH values of 5.5, 7.0, and 9.0. The 21d chronic antidepressants mixture toxicity was explored using *D. magna* at single pH 7.5. The pH levels were maintained using 2 mM of Phosphate buffer (pH 5.5-6.0) or Tris-HCl buffer (pH 7.0-9.0). The test concentrations were calculated by using the EC50 values obtained from the single toxicity tests and the PEC values of each studied pharmaceutical. The pH, dissolved oxygen (mg/L), conductivity (uS/cm) and temperature (^{o}C) were monitored and recorded every 24h to ensure that the target ranges were maintained. Measured values did not changed by more than ±0.1 pH unit through the study (Appendix 1). The test solutions were renewed every 48h or individually when the pH values did not meet the target pH in 21d chronic tests.

Table 4a. The exposed concentration of each antidepressant in different mixture at pH 5.5, 7.0, and 9.0 for 48h mixture acute tests. The concentration of each component were calculated using the CA model and the LC50 of single exposure from Chapter 2. Predicted toxic units were expected with each set of mixture concentrations at increasing pH.

Mixture	pH	Toxic Unit (TU)	Concentration (µg/L)*				
			CTP	FLX	SRT	DUL	DOS
A	5.5	0.05	35.3	21.8	9.4	2.1	8.1
		0.10	70.7	43.7	18.7	4.2	16.3
		0.50	353.4	218.4	93.6	21.1	81.4
		1.00	706.7	436.8	187.2	42.1	162.9
		1.50	1060.1	655.2	280.8	63.2	244.3
		2.00	1413.4	873.6	374.5	84.3	325.7
	7.0	0.05	30.8	19.1	8.2	1.8	7.1
		0.10	61.7	38.1	16.3	3.7	14.2
		0.50	308.4	190.6	81.7	18.4	71.1
		1.00	616.8	381.2	163.4	36.8	142.1
		1.50	925.2	571.8	245.1	55.2	213.2
		2.00	1233.5	762.4	326.8	73.5	284.3
	9.0	0.05	8.8	5.4	2.3	0.5	2.0
		0.10	17.6	10.9	4.7	1.1	4.1
		0.50	88.1	54.5	23.3	5.3	20.3
		1.00	176.3	108.9	46.7	10.5	40.6
		1.50	264.4	163.4	70	15.8	60.9
		2.00	352.5	217.9	93.4	21	81.2
B	5.5	0.05	36.8	22.7	9.7	2.2	8.5
		0.10	73.5	45.4	19.5	4.4	16.9
		0.50	367.7	227.2	97.4	21.9	84.7
		1.00	735.3	454.5	194.8	43.8	169.5
		1.50	1103	681.7	292.2	65.8	254.2
		2.00	1470.7	909	389.6	87.7	338.9
	7.0	0.05	27.7	17.1	13.3	2.7	4.2
		0.10	55.3	34.2	26.7	5.5	8.4
		0.50	276.7	171.1	133.3	27.3	41.9
		1.00	553.5	342.1	266.7	54.6	83.8
		1.50	830.2	513.2	400	81.9	125.8
		2.00	1106.9	684.2	533.3	109.3	167.7
	9.0	0.05	7.4	4.6	3.6	0.7	1.1
		0.10	14.8	9.1	7.1	1.5	2.2
		0.50	73.9	45.7	35.6	7.3	11.2
		1.00	147.9	91.4	71.2	14.6	22.4
		1.50	221.8	137.1	106.9	21.9	33.6
		2.00	295.7	182.8	142.5	29.2	44.8

	5.5	0.05	24.7	16.1	20.8	3.7	2.4
		0.10	49.3	32.3	41.5	7.3	4.9
		0.50	246.5	161.3	207.6	36.7	24.5
		1.00	493	322.6	415.2	73.5	49
		1.50	739.5	483.9	622.8	110.2	73.5
		2.00	986	645.2	830.4	146.9	98
C	7.0	0.05	22.4	14.7	18.9	3.3	2.2
		0.10	44.9	29.4	37.8	6.7	4.5
		0.50	224.3	146.8	188.9	33.4	22.3
		1.00	448.7	293.6	377.9	66.9	44.6
		1.50	673	440.4	566.8	100.3	66.9
		2.00	897.3	587.1	755.7	133.7	89.1
	9.0	0.05	5.6	3.6	4.7	0.8	0.6
		0.10	11.1	7.3	9.4	1.7	1.1
		0.50	55.6	36.4	46.8	8.3	5.5
		1.00	111.2	72.7	93.6	16.6	11
		1.50	166.7	109.1	140.4	24.8	16.6
		2.00	222.3	145.5	187.2	33.1	22.1

*Concentration of control for each treatment was 0 μg/L

In 48h acute mixture toxicity tests, the general experimental conditions were not changed from the second chapter (OECD, 2008). Four replicates of five *D. magna* neonates (<24hr) were exposed for 48h to various expected concentration of mixture A, B or C with a single combination, SSRIs+SNRI+TCA, at increasing pH 5.5, 7.0 and 9.0 (Table 4a). Each replicate was contained in 40 ml of test medium in a 50 ml glass beaker. Immobilization was the endpoint of the test and it was determined if no movement was observed for 15 s after gentle shaking of the test vessel. All exposures were done at 20 ± 1°C using a 16:8 h photoperiod. Water quality parameters including pH, temperature, and dissolved oxygen of the test medium were measured before and after the 48h exposure. The test solutions were not replaced during the 48h static non-renewal test. The number of immobilized daphnids were counted to calculate LC50 of acute tests using the Probit analysis in SPSS.

Table 4b. The exposed concentration of each antidepressant in different SSRIs mixture at pH 7.5 for 21d mixture chronic tests

Mixture	Toxic Unit (TU)	Concentration (μg/L)*		
		SSRIs		
		CTP	FLX	SRT
A	0.2	0.92	0.57	0.24
	1.0	4.59	2.84	1.22
	1.8	8.27	5.11	2.19
	2.0	9.19	5.68	2.43
B	0.2	0.73	0.45	0.35
	1.0	3.65	2.25	1.76
	1.8	6.57	4.06	3.16
	2.0	7.29	4.51	3.51
C	0.2	0.54	0.35	0.45
	1.0	2.68	1.75	2.26
	1.8	4.82	3.16	4.06
	2.0	5.36	3.51	4.51

*Concentration of control for each treatment was 0 μg/L

Table 4c. The exposed concentration of each antidepressant in different SNRI+TCA mixture at pH 7.5 for 21d mixture chronic tests

Mixture	Toxic Unit (TU)	Concentration (μg/L)*	
		SNRI	TCA
		DUL	DOS
A	0.2	0.92	0.57
	1.0	4.59	2.84
	1.8	8.27	5.11
	2.0	9.19	5.68
B	0.2	0.73	0.45
	1.0	3.65	2.25
	1.8	6.57	4.06
	2.0	7.29	4.51
C	0.2	0.54	0.35
	1.0	2.68	1.75
	1.8	4.82	3.16
	2.0	5.36	3.51

*Concentration of control for each treatment was 0 μg/L

The 21d chronic mixture toxicity tests were also demonstrated to determine the effects of the antidepressants on the survival of original neonates, number of young per female, number of brood, time to first reproduction, growth and population growth rate (PGR) using the methods described in OECD Guideline 211 (OECD, 2008). Ten replicates

were exposed to each concentration containing one neonate each (< 24h old). The 21d chronic mixture tests used the same conditions and test design but various concentration of the antidepressants were used (Table 4b and c). One extra test vessel was prepared for each test treatment to measure the pH consistency. The organisms were exposed over 21d at 20 ± 1°C with a 16:8 h photoperiod. Test solutions were renewed every 48 h. Water quality parameters such as pH, temperature, and dissolved oxygen were measured in test media before and after the 48 h exposure. *D. magna* were fed daily with 300 µL YCT and 300 µL algae per each organism. Lastly, 10 ml of test new (0h) and old (48h) samples were removed from the 50 ml beakers and collected in 15 ml vials from acute and chronic tests were for chemical analysis on every third changing phases.

Dry mass of *D. magna*

The average dry mass (µg) of *Daphnia magna* at each concentration was measured after they were exposed to each mixture for 21 days. The mass was measured with microgram sensitive balance after each species dried for 24 h at 60 ^{o}C.

Chemical analysis

Chemical analysis was conducted to determine the actual exposure concentrations for each test solution. Prior to analysis, the SPE was conducted on 6 mL OASIS Hydrophilic lipophilic balance SPE cartridges (Waters). 5 mL of methanol was passed through and followed by 10 mL of HPLC graded water for the preconditioning. The thawed samples were then loaded on the SPE cartridge at a rate of 10 to 20 mL/min using a vacuum manifold (Supelco-Visiprep) for extraction, after which the cartridges were rinsed with 10 mL of 5% methanol in HPLC graded water and then dried under air for 30 min. Cartridges were then eluted with 2.5 mL methanol followed by 1.0 mL of 2% NH_4OH in methanol. Eluates were dried under a gentle nitrogen stream using a concentrator (DB-3A; Techne) at 30^{o}C. The dried extract was reconstituted into 1.0 mL of water and stored in a freezer at -20^{o}C prior to analysis.

Cleaned-up extracts were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a High-performance Liquid Chromatography (HPLC) system with an Agilent 1260 Infinity II Multiple Wavelength Detector (High-speed UV detection) and a Zorbax Eclipse Plus-C18 Column. The column temperature was maintained at 25^{o}C and the pressure was set to 119.63 bars. Mobile phase A and B were 1mM of acetonitrile and 0.25 mM of ammonium acetate, and the flow rate was 1.5 mL/min. The gradient elution program was set to run 55% of acetonitrile and 45% of ammonium acetate simultaneously for 12 minutes per samples. Each of pharmaceutical in mixture was detected at different time (min) with different peaks. The injected sample volume was 10 µL and detected at wavelength of 230 and 250nm. The performance of the analytical method

(precision, accuracy, limit of detection [LOD], and limit of quantification [LOQ]) was followed A-Khazrajy and Boxall (2017) (Appendix 2). Recoveries for the test pharmaceuticals ranged from 83.9% to 117.9%.

Statistical evaluation

Statistical analysis was performed using SPSS (2016). Concentration-response curves were fitted using the Probit regression model using SPSS for Windows version 24.0. One-way analysis of variance (ANOVA) was used with Tukey's post-hoc test to determine the reproduction toxicity of antidepressant mixtures between the concentrations. The homogeneity of variance was checked before one-way ANOVA. The height and area peaks were calculated from HPLC (Agilent Lab Advisor Software, 2010).

Data analysis and model evaluations

The CA model was used to calculate the series of concentrations for each antidepressant in different mixtures at different pH levels (5.5, 7.0, 7.5 or 9.0) in 48h acute and 21d chronic tests. Therefore, the theoretical and measured EC50 of the mixture were compared to determine the accuracy of CA model prediction.

3.2 Results

The groups were set depend on if there are similar patterns of change in consumption compared to another group of year (fig. 5). The average consumption of CTP, FLX and DOS were decreased about 7.63, 7.53, and 39.2% from A to B. FLX and DOS were decreased similar to the next year C which are 9.3 and 43.7%. However, CTP was decreased nearly in double (14.3%) from year B to C. Interestingly, the average consumption of SRT and DUL were intensively increased relative to other antidepressants. SRT was increased about 68.4% and 49.2% over a decade. DUL was increased about 53.2 and 29.0% in B and C, respectively.

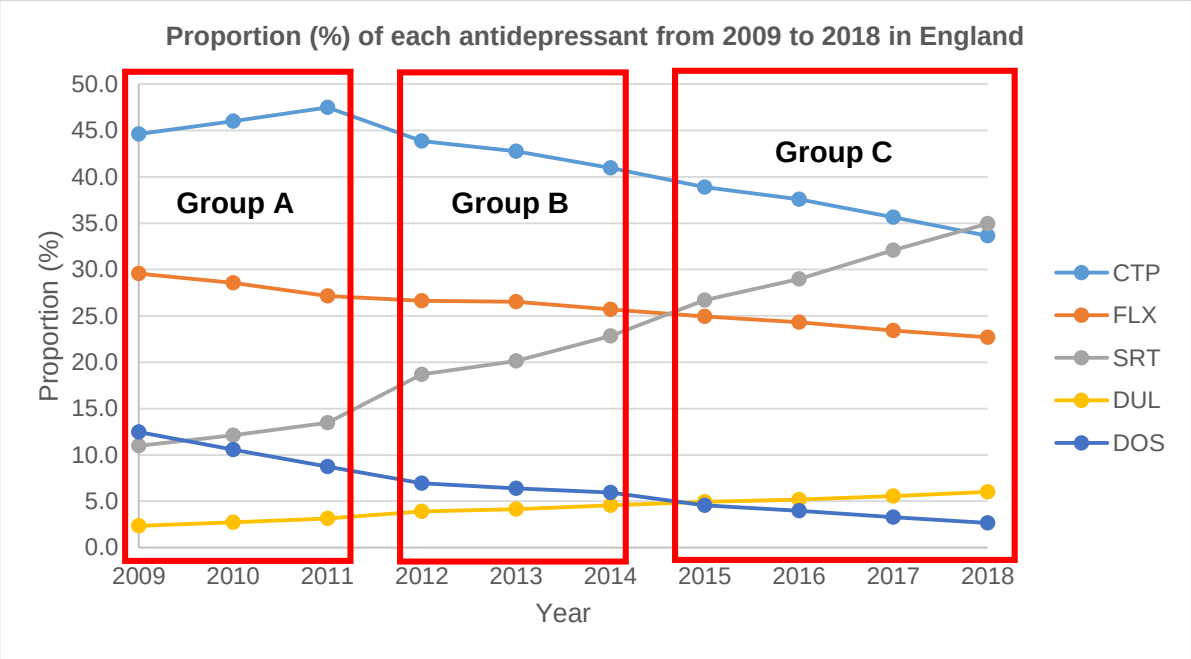
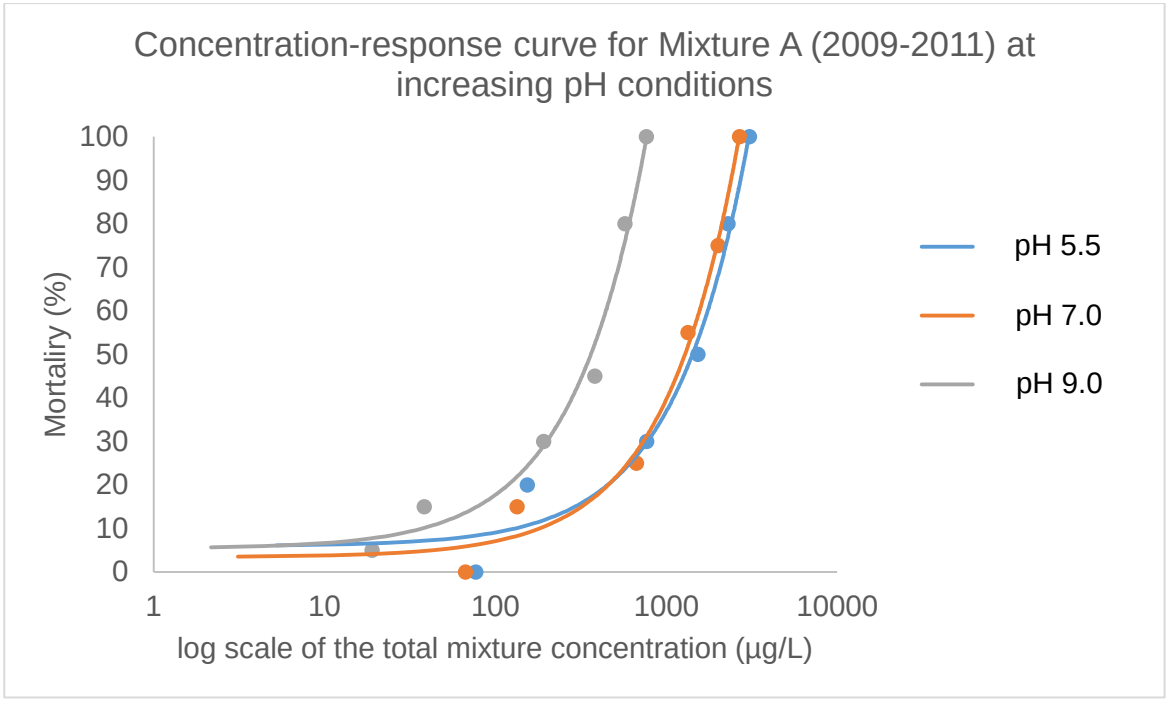


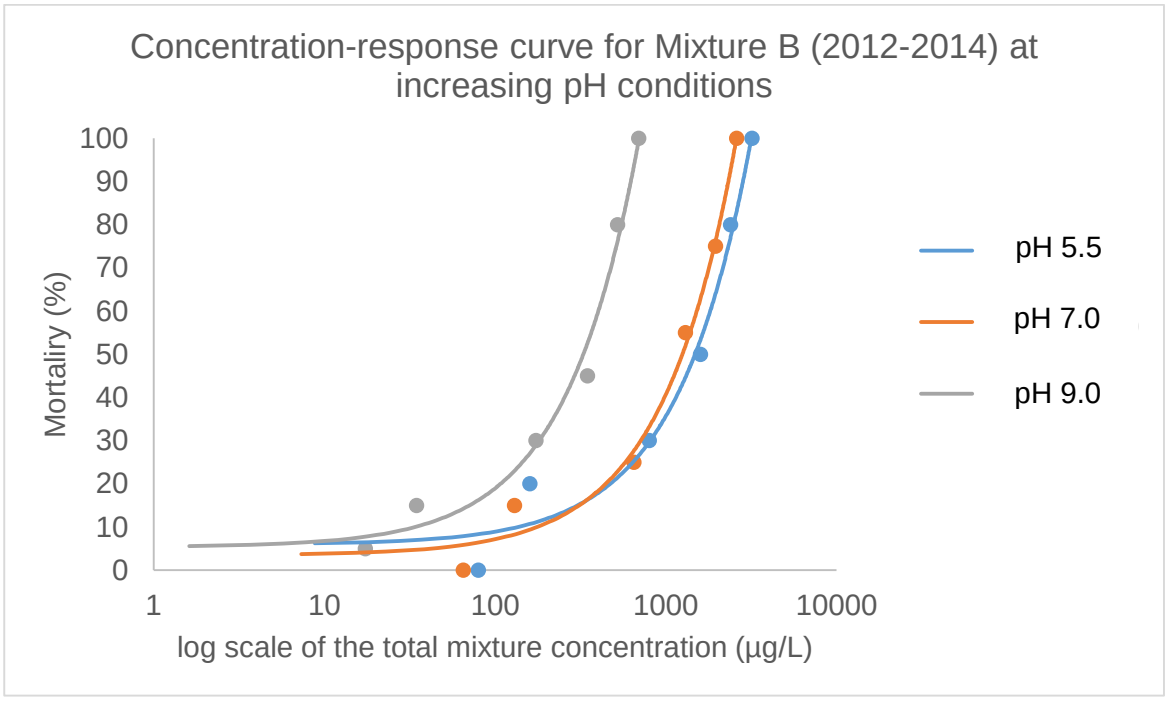
Figure 5. The change of the studied antidepressants ratio (%) which dispensed by doctor, hospital, pharmacy or under personal administration over a decade from 2009 to 2018 in England (NHS). The 3 different average groups, including group A (2009-2011), B (2012-2014), and group C (2015 to 2018), were compared to determine the change of the studied antidepressants consumption ratio (%).

The toxicity of mixture of antidepressants were increased compared to single antidepressant toxicity tests at increasing pH levels. The results suggest that the toxicity of antidepressant mixtures become more toxic, and become even more toxic at alkali conditions. In terms of 21d EC50 reproduction at pH 7.5, there were two different endpoints, reproduction enhancement (SSRIs) or inhibition (SNRI or TCA). EC50 of SSRIs decreased twice between CTP, FLX and SRT, respectively. In contrast, EC50 of DUL and DOS had a 9-folds difference to each antidepressant. The single toxicity data from Chapter 2 was needed to run the model to compare the mixture effects to single compound effects. Also, the order of single antidepressant toxicity could be used to determine each effectiveness in mixture.

***Daphnia magna* 48h mixture acute tests**

In the 48h mixture acute tests, no mortality was observed in the control treatments. The target pHs were also well maintained as the Chapter 2 experiments. The measured concentration of all tested samples were above limit of detection and limit of quantification except the controls (Appendix 2). The concentration-response curves for each mixture was demonstrated in Figure 6.





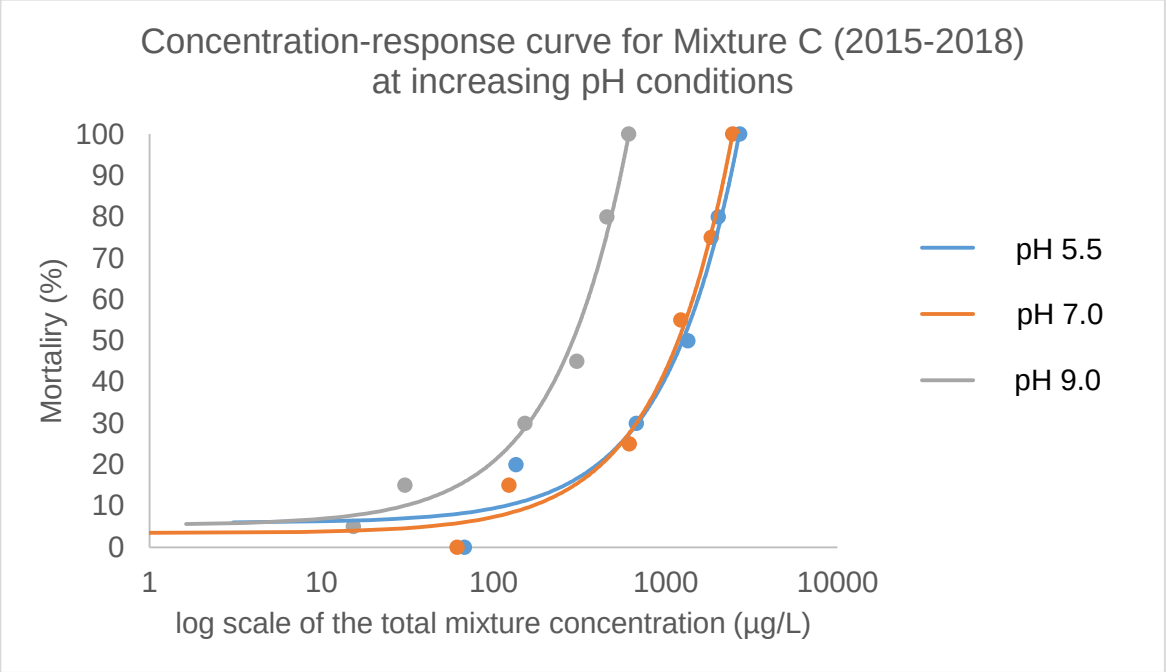


Figure 6. Concentration-response graphs for mixture acute toxicity at pH 5.5, 7.0 and 9.0.

Table 5. A summary table of mixture LC50 for each pH based on total concentrations.

Mixture	pH	LC50 (µg/L)	CI 95% (µg/L)
A	5.5	1535.7	1467.5-1608.2
	7.0	1340.3	1267.9-1403.7
	9.0	383.0	334.8-427.8
B	5.5	1597.9	1527.7-1653.4
	7.0	1300.7	1246.1-1362.6
	9.0	347.5	301.3-387.7
C	5.5	1353.3	1293.3-1402.8
	7.0	1231.7	1163.4-1298.2
	9.0	305.1	268.5-331.9

The acute toxicity of mixtures were extremely similar to each other over the last decade based on our graphs (Fig.6). The LC50 of mixture A were 1535.7, 1340.3, and 383.0 µg/L at pH 5.5, 7.0 and 9.0, respectively. Mixture B had similar toxicity of 1597.9, 1300.7, and 347.5 µg/L at same pH conditions. Lastly, the LC50 of mixture C were 1353.3, 1231.7, and 305.1 µg/L at increasing pH (Table 5). The acute toxicity of mixtures did not seems to be increasing over the last decade. However, lower concentration of each component was exposed at same pH over the last decade. Therefore, the acute toxicity of mixtures were increased at increasing pH over the last decade.

***Daphnia magna* 21d mixture chronic tests**

In the 21d chronic mixture tests, no mortality was observed in the controls (Appendix 1). The target pH was also well maintained as the Chapter 2 experiments. The measured concentration of all tested samples were at least above 80% on limit of detection and limit of quantification except the control and solvent control samples (Appendix 2). The concentration-response curves for each mixture was demonstrated in figure 7. *D. magna* was exposed with the 2 different mixtures of antidepressants (SSRIs or SNRI+TCA) which had reproduction enhancement and inhibition effects, respectively. Interestingly, the toxicity of both mixtures were slightly increased from A to B. Moreover, the toxicity between B to C increased almost double compared to toxicity change between A to B.

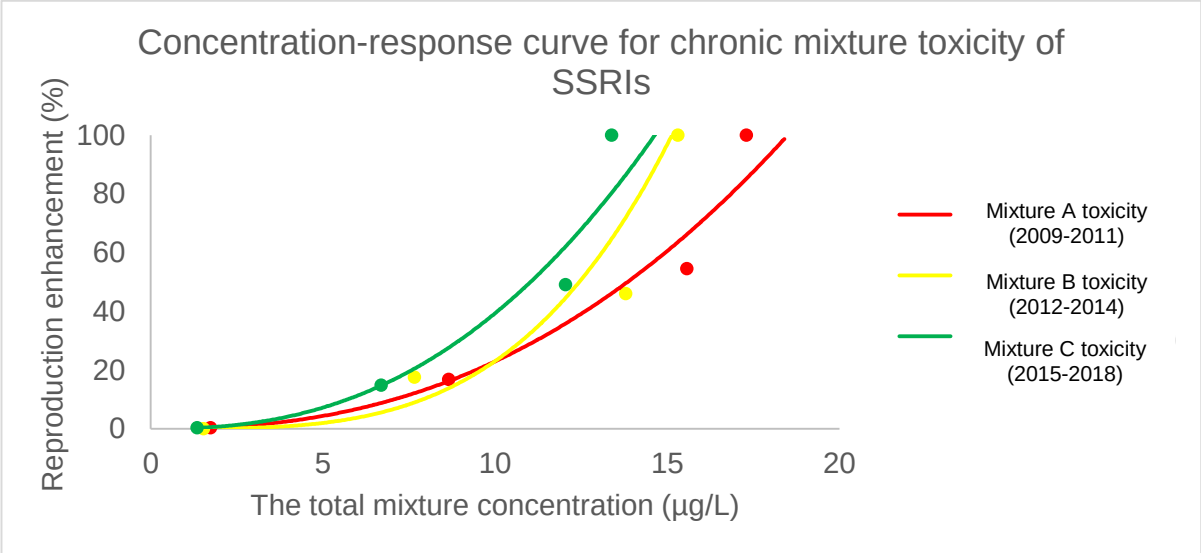


Figure 7a. Concentration-response graphs for SSRIs mixture chronic toxicity. Each line demonstrates the changes in mixture toxicity between 2009 to 2018.

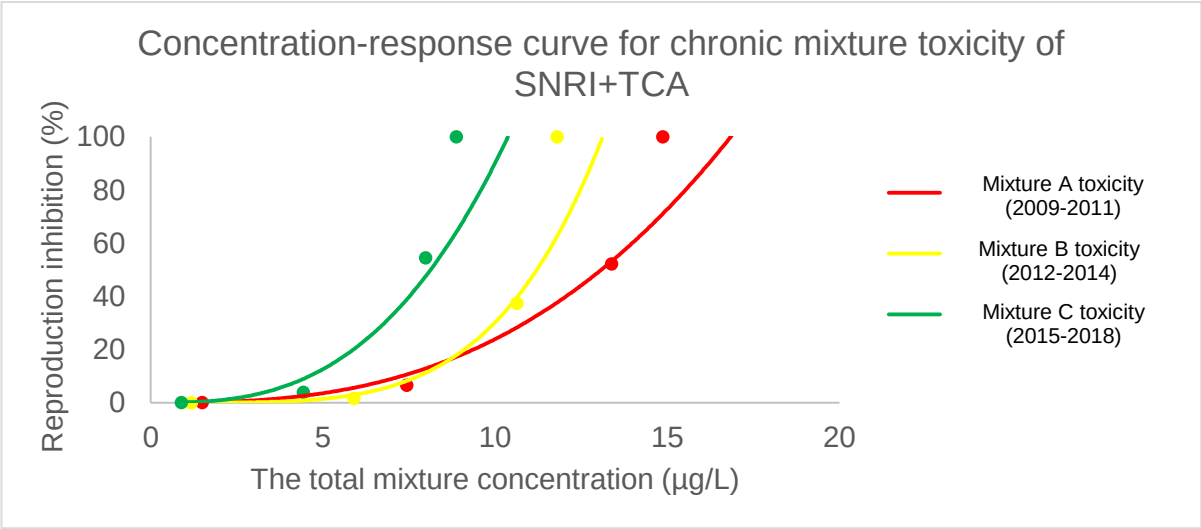


Figure 7b. Concentration-response graphs for SNRI+TCA mixture chronic toxicity. Each line demonstrates the changes in mixture toxicity between 2009 to 2018.

Table 6. A summary table of mixture EC50 (reproduction enhancement or inhibition) based on total concentrations.

Mixture		EC50 (µg/L)
SSRIs (reproduction enhancement)	A	14.8
	B	12.5
	C	11.0
SNRI+TCA (reproduction inhibition)	A	13.1
	B	11.2
	C	8.13

For the SSRIs mixtures, generally reproduction enhancement was observed in *D. magna* (Fig 7a). EC50 of reproduction enhancement for each mixture (A, B, and C) was estimated at 14.8, 12.5, and 11.0 µg/L after exposed for 21 days, respectively (Table 6). The SSRIs mixture EC50 were significantly ($p < 0.05$) different over the last decade. It is fact that the usage of SRT was increased more than 50%, while the CPT and FLX usage was decreased more than 20% each over the decade.

For the SNRI and TCA mixtures, *D. magna* reproduction was generally inhibited (Fig 7b). EC50 of reproduction inhibition for each mixture (A, B, and C) was calculated at 13.1, 11.2, and 8.13 µg/L, respectively (Table 6). Also, the EC50 of SNRI+TCA mixture were significantly ($p < 0.05$) different over the decade. The usage of DOS was increased approximately 50%, while the DUL usage was decreased approximately 50% over the decade.

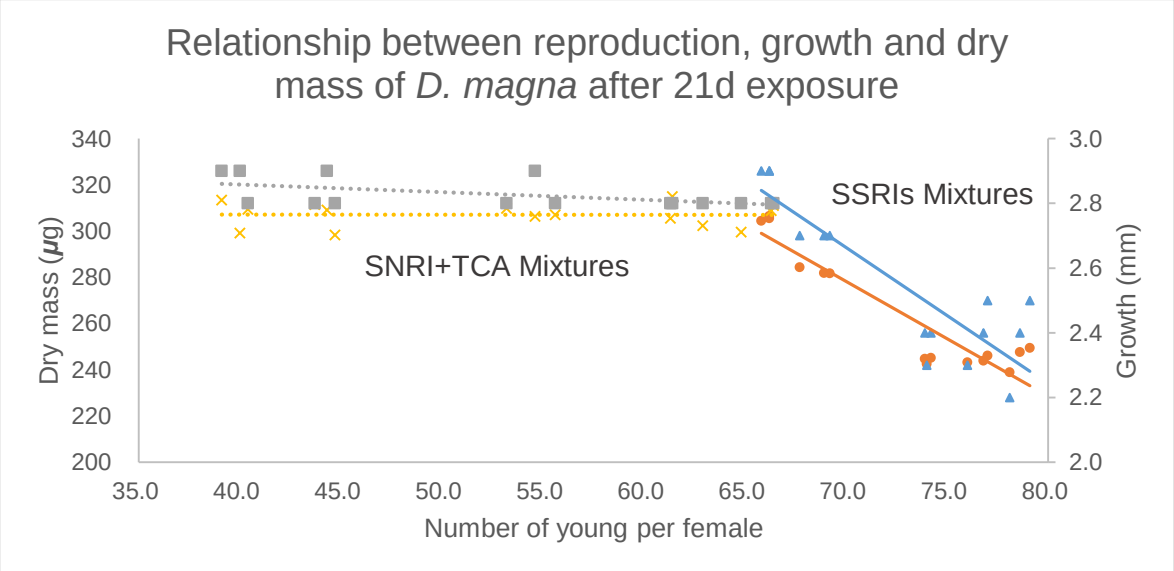


Figure 8. Relationship between the number of young per female, growth (mm) and dry mass (µg) after exposing the SSRIs mixtures or SNRI+TCA mixtures on of *D. magna* for 21 days. Solid line and symbols (blue) = reproduction vs. growth (SSRIs); Solid line and symbols (orange) = reproduction vs. dry mass (SSRIs); Solid symbols and dotted line (grey) =

reproduction vs. growth (SNRI+TCA); Solid symbols and dotted line (yellow) = reproduction vs. dry mass (SNRI+TCA)

The growth of *Daphnia magna* demonstrated a marked difference between the different mixtures exposure, SSRIs or SNRI+TCA, after 21 days (Fig. 8). The steepness of relationship between reproduction and growth was decreased from -0.0023 (SNRI+TCA) to -0.042 (SSRIs), which become nearly 18 times steeper when exposed with SSRIs with increasing reproduction. Moreover, the relationship between number of young per female and dry mass was also decreased from -0.0056 (SNRI+TCA) to -4.963 (SSRIs), which become almost 900 times steeper with SSRIs mixture exposure.

Mixture modelling using concentration addition (CA) model

The chronic effects of each antidepressant class were not changed in mixture study compare to Chapter 2. Based on the CA model, we could assume that the mixtures that consist with similar effects at single exposure would be more accurately predicted by CA model. Therefore, the EC50 (TU1.0) obtained from CA model from acute and chronic tests were compared to the measured mixture acute and chronic toxicity to determine the accuracy of CA predictability. Moreover, the relationship between growth, mass and reproduction of *D. magna* after exposed for 21 days to different mixtures was determined by comparing the steepness of each dataset.

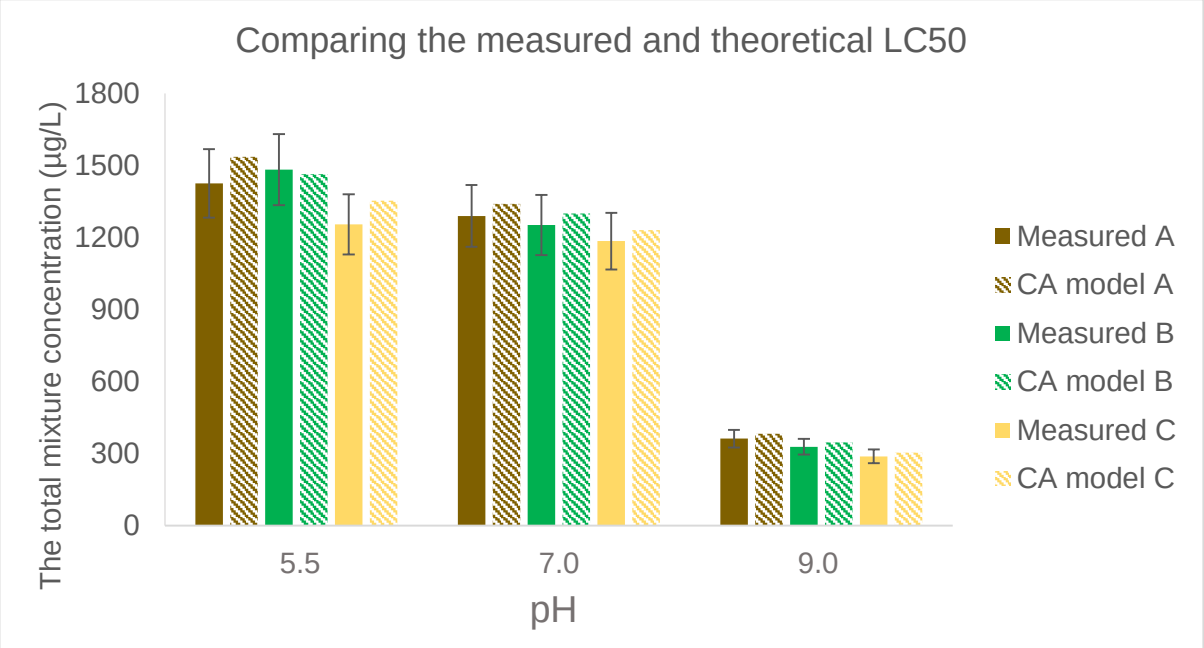


Figure 9. Acute toxicity (mortality) of mixture A, B and C on *D. magna* at pH 5.5, 7.0 and 9.0. Each filled bar represents the measures LC50 from each mixture (±95% Confidence Interval, CI). Dashed bars represent the estimated LC50 based on CA model.

Figure 9 illustrates the estimated and measured toxicity of mixture A, B and C at pH 5.5, 7.0 and 9.0. Our single acute toxicity data was used to calculate the different

concentration for each component at increasing pH conditions. As we expected, the concentration of antidepressants were relatively lower compared to antidepressants concentrations at higher pH levels because all of the single acute toxicity of antidepressants were increased at increasing pH conditions. As a results, CA model was very accurately matched to the antidepressants mixture acute toxicity (Fig. 8). Measured mixture acute toxicities based on the total mixture concentrations (95% CI; theoretical toxicity) were, i.e. 1425.4 (1352.8-1586.2; 1535.7), 1290.0 (1121.5-1398.2; 1340.3) and 362.7 (344.7-390.8; 383.0) µg/L for mixture A (pH 5.5-9.0), 1483.0 (1384.2-1554.3; 1463.9), 1251.9 (1174.2-1338.5; 1300.7) and 329.0 (308.4-354.2; 347.5) µg/L for mixture B (pH 5.5-9.0), 1254.9 (1193.3-1374.2; 1353.2), 1185.0 (1091.3-1297.4; 1231.5), and 288.8 (95% 254.8-331.2; 305.1) µg/L for mixture C (pH 5.5-9.0), respectively.

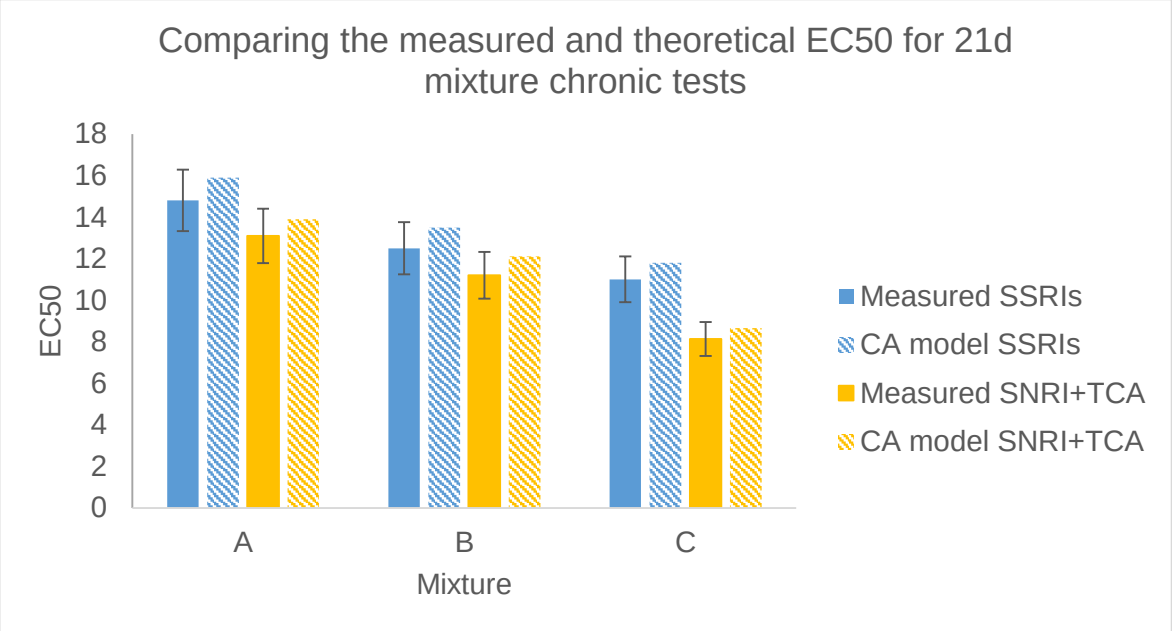


Figure 10. Chronic toxicity (reproduction) of mixture A, B and C on *D. magna* at pH 7.5. Each filled bar represents the measures EC50 (blue for reproduction enhancement; yellow for reproduction inhibition) from each mixture (±95% Confidence Interval, CI). Dashed bars represent the estimated EC50 (blue for reproduction enhancement; yellow for reproduction inhibition) based on CA model.

The theoretical EC50 obtained by the CA model was compared to the measured EC50 determined from 21d chronic mixture tests (Fig. 10). As we expected, the CA model was well fitted to the measured mixture toxicity. Moreover, the toxicity of SSRIs and SNRI+TCA mixtures were increased over last decade. Measured SSRIs mixture chronic toxicities based on the total mixture concentrations (95% CI; theoretical toxicity) were, i.e. 14.8 (13.1-16.2; 15.9) µg/L for mixture A, 12.5 (11.0-14.1; 13.5) µg/L for mixture B, and 11.0 (9.8-12.3; 11.8) µg/L for mixture C. Measured SNRI+TCA mixture chronic toxicities based on the total mixture concentrations (95% CI; theoretical toxicity) were, i.e. 13.1 (11.2-14.3; 13.9) µg/L for mixture A, 11.2 (10.3-12.4; 12.1) µg/L for mixture B, and 8.13 (7.42-8.84; 8.65) µg/L for mixture C.

3.3. Discussion

Antidepressants are normally prescribed only with a single medication to human because there are potential to have serotonin syndrome by overdose or mechanical disruption. However, the aquatic organisms are mostly likely to be exposed to more than a single antidepressant. Measuring the actual toxicity of the mixture compounds in different aquatic environmental conditions such as pH levels is considered to be difficult in real life. We have determined the PEC of antidepressants over last 10 years using the NHS data to calculate the ratio of the pharmaceuticals for the different mixtures between the time periods (Appendix 2). Using the data from NHS, the antidepressant mixture toxicity over last ten decade in the UK was determined by testing CA model

Nevertheless, common major drawbacks of the CA and IA models can be highlighted by the following background assumptions. Firstly, in the reality of risk assessment, living organisms and the environment may be exposed to both similarly and dissimilarly acting chemicals simultaneously. However, both CA and IA models do not consider mixed similarly and dissimilarly acting chemical groups to simplify model development (Loewe and Muischnek, 1926; Bliss, 1939; Plackett and Hewlett, 1952; Mwense et al., 2004). Secondly, the use of CA and IA models can be strictly limited unless accurate MoAs of all mixture constituents are readily available (Borgert et al., 2004; Lambert and Lipscomb, 2007). Knowledge of such MoAs remains lacking (European Commission, 2009). Lastly, both models assume that no interactions (e.g., synergism, antagonism, and potentiation) occur among mixture components (Plackett and Hewlett, 1952; Altenburger et al., 2003).

Since the MoA of mixture components are the main factor that decide the predictions for both models, the endpoints could be different. For example, Moser et al. (2005 and 2006) tested the mixture with 5 components which had 2 different endpoints using the CA model. Interaction between the mixture components, such as synergism, was not assumed over the study, but the different effects were found at each different part of organs of organisms, blood and brain. The adverse effects of mixtures with no interactions (e.g., synergism, antagonism, and potentiation) would solely be observed if the effects were found at each different part of the exposed organism. Our study had 2 different endpoints at reproduction only, which the interactions are expected to be observed with various mixture combinations. However, both models cannot be used to estimate the toxicity because it is against the assumption which no interactions occur among mixture components. Therefore, CA was selected for our mixture study to determine the mixture toxicity of antidepressants.

Effect of pH on mixtures of antidepressant by exposing acutely to *D. magna*

We could assume that our different class of antidepressants share the similar MoA because acute tests were very well fitted to CA model. In terms of mechanisms of different classes of antidepressants, SSRIs would increase the secretion of peptide hormones to increase the rate of synthesis or secretion of ecdysteroids and terpenoids, which led to the oxygen demand and the frequency of ecdysis increased significantly (Campos et al., 2012). However, some of SSRIs could bind to other receptors and different SSRIs have different affinities for different receptors. It will induce the *D. magna* to be more vulnerable to antidepressants. Such as, SNRI would down regulate the IL-2 to increase the ROS or slows the T-lymphocytes proliferation to increase mortality. Also, TCA would adversely affect CYP2C19, isoform of CYP450, to decrease the rate of pumping out the excess of toxicants in their body or antidepressants. This combination of adverse effects of the antidepressants would cause the mixture toxicity to be increased at increasing pH conditions.

The concentration of each component were calculated by using the obtained single acute toxicity (LC50) to explain the mixture toxicity at increasing pH conditions. In addition, the deduced concentrations were also decreased in terms of time periods from A to C. Therefore, we could clearly say that the lethal toxicity of antidepressant mixtures have been increasing from the past to the most recent years. Most importantly, some of the concentration of each component in acute mixture was already lower than the PEC in some part of the world (FLX and SRT). Also, it is fact that there are much diverse antidepressants existing in the aquatic environment than our studied antidepressants. As CA model was fitted very well, the toxicity could be increased if greater number of mixture components present. In the real world, this means that the lethal toxicity would be decreased (stronger) by the mixtures with a greater number of pharmaceutical components at increasing pH in the UK.

Effect of different antidepressant mixtures on *D. magna* reproduction

Our study have studied 2 different mixtures to determine the effect on reproduction, growth or dry mass of *D. magna* for 21 days. The first mixture, SSRIs, were aimed to determine the reproduction enhancement because we have observed the reproduction enhancement and growth inhibition effects from single exposure tests in Chapter 2. The second mixture, SNRI+TCA, each component had reproduction inhibition and no effects on growth from single exposure tests in Chapter 2, therefore we were expected to observe the reproduction inhibition and no effects on growth on *D. magna*.

All of our measured mixture toxicities were fitted in a good shape to the toxicity prediction with CA models. SSRIs mixtures were obviously expected to be fitted to CA model because the mixture was composed with same class of antidepressants. In a point of MoA, SSRIs increased serotonin postsynaptic activity to stimulate ecdystroids and

juvenile hormone which are responsible for controlling oogenesis and vitellogenesis (Campos et al., 2012), resulting in an increased reproduction and decreased offspring size and maturation age in our study. Although the SNRI+TCA mixture was expected to be fitted to IA model because the mixture was composed with the different antidepressant classes, it was fitted well to CA model. We assumed that the DOS (TCA) adversely affect to the CYP2C19, isoform of CYP450, by reducing the rate of pumping out the toxicants, including antidepressants. It explains the change in reproduction inhibition effect by SNRI and TCA mixture that when the TCA concentration ratio was greater than SNRI (Dey et al., 2015).

The SSRIs exposure will result in the reproduction enhancement which could misunderstand beneficial effects on aquatic invertebrate population. However, Barbosa et al. (2017) studied that the next generation from the exposed aquatic invertebrates were reduced in size and much more vulnerable to antidepressant lethal toxicity. Moreover, the reproduction enhancement effect of SSRIs observed with fishes (Nakamura et al., 2008). The increased number of aquatic invertebrates and fishes could adversely affect the food-webs in the aquatic system. Moreover, much active ionic exchange could be observed thought gill if aquatic organism adapted to high alkali condition (pH 10; Wilkie and Wood, 1996). This will lead to have greater chance to be adversely affected with dissociated ions from antidepressants at basic condition. The SNRI+TCA exposure would obviously adverse to aquatic organisms because their population will be reduced by reproduction inhibition effects. For instance, increased food consumption and decreased sexual hormones are known for one of antidepressant side-effect. Two of the antidepressant side-effects are the reduction of sexual desire and increase of appetite. If we assume this effect also happen to aquatic organism, Jaeschke (2002) reported that the aquatic organism had fratricidal killing and euthanasia after exposed with TCA. It will accelerate the population reduction, not only with the reproduction inhibition effect solely from SNRI+TCA.

In the UK, the consumption of antidepressants with relatively weaker toxicity has been decreasing while the antidepressants with relatively stronger toxicity has been increasing from the last decade (Appendix 3). The concentration of antidepressants in aquatic environment need to be reduced regardless to their toxicity strength. We could design the future antidepressants concentration in aquatic environment by regulating the products or disposal to reduce the risk of antidepressant mixture toxicity. Lastly, we have clearly determined the effect of mixture toxicity on aquatic invertebrates in different concentration ratio of each component.

Effect on *D. magna* growth and dry mass with the antidepressant mixtures

In figure 7, the growth and dry mass have relatively similar trends to each other. For instance, SSRIs mixture demonstrated the decrease in growth and dry mass in same time while number of young per female was increased. Growth reduction was also observed at our

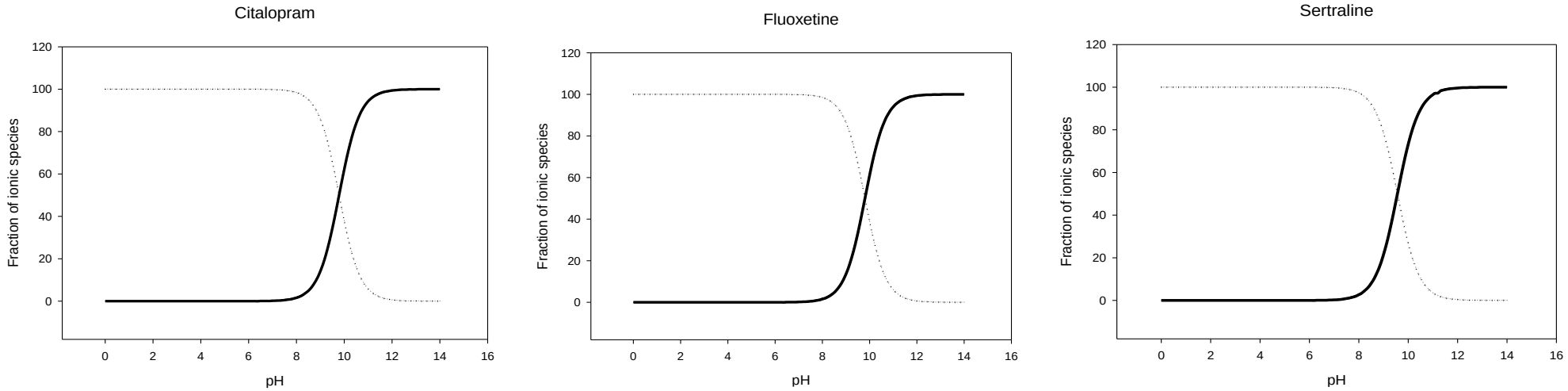
single SSRI exposure experiments, therefore it could be explained with the same context with the single exposure tests in Chapter 2. Moreover, we could assume that the greater amount of energy was allocated to the reproduction compared to the growth which led to reduction in growth (Gilbert, 2012). However, the growth or dry mass reduction was not observed with SNRI+TCA mixture exposure on *Daphnia magna* over all the different mixture ratio. This also could be explained by the effect of SNRI or TCA that reduced the reproduction under energy allocation theory to maintain the growth of *Daphnia magna* using the reproduction source energy. Lastly, we can also assume that the eggs are the major mass component of *D. magna* and the size of daphnids varies depend on the eggs.

3.4 Conclusion

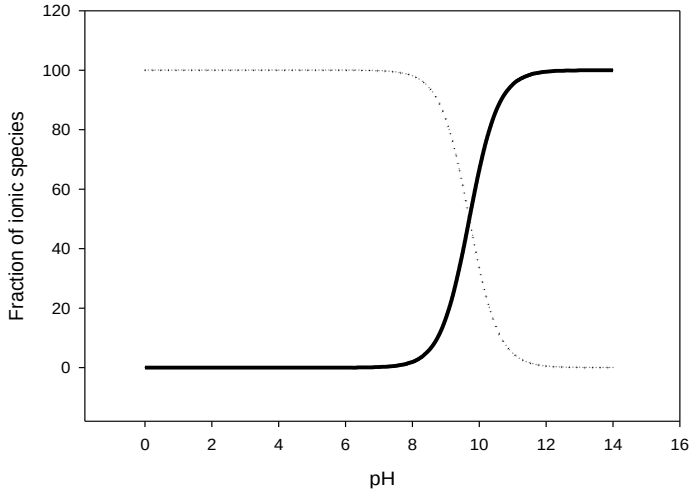
The consumption of CTP, FLX and DOS have decreased, while SRT and DUL have increased rapidly over the last decade in England. Based on this data, the 3 different PEC of each antidepressant were calculated to determine the different ratio of mixture between the 3 time periods (A = 2009-2011; B = 2012-2014; C = 2015-2018). Concentration addition (CA) model was selected to estimate mixture toxicity of antidepressant. Single LC50 and EC50 from Chapter 2 was used to predict the each antidepressant concentration in each mixture at increasing pH. The predicted mixtures with 3 different ratio (A, B and C) were exposed to *D. magna* for 48 hours at increasing pH. The chronic effects of tested antidepressants were same as the single chronic exposure. SSRIs or SNRI+TCA mixtures were exposed to *D. magna* for 21 days at pH 7.5. Measured mixture toxicity was compared to the predicted toxicity (TU1.0) at each increasing pH. CA model predicted accurately the all of the mixture tests by satisfying the CI 95% of measured mixture toxicity. Interestingly, our mixture results were explained by the order of antidepressant toxicity from Chapter 2 and change in concentration ratio over last decade explains. This study has been conducted novel approaches of antidepressant mixtures on *D. magna* with pH variation interactions between the different classes of antidepressants and different chemophysical factors in freshwater invertebrates warrant further studies.

Appendix and Supplementary data

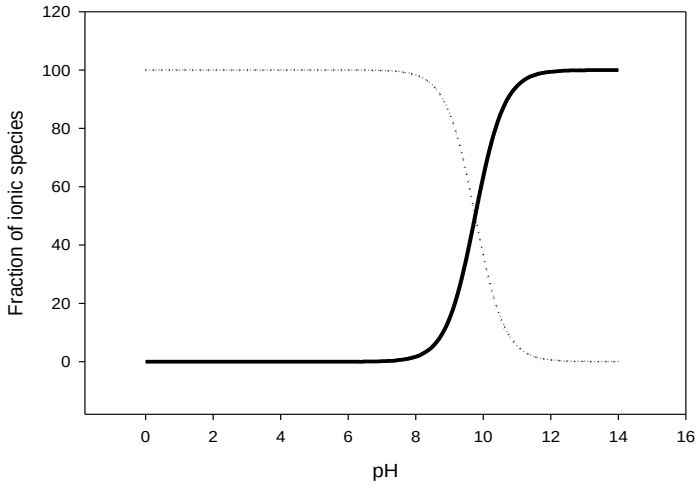
Appendix 1. Fraction of ionic species of tested antidepressants, including citalopram, fluoxetine, sertraline, duloxetine, and dosulepin as a function of pH. The curves were constructed or the basis of the ionic component distribution calculated from the Henderson-Hasselbalch equation using the acid and base dissociation constants (Chemaxon, 2019). Single-line and dotted line represent the fraction of uncharged ions and charged ions, respectively.



Duloxetine



Dosulepin



Appendix 2. The toxicity of 2 mM of Phosphate buffer (pH 5.5-6.0) and Tris-HCl buffer (pH 7.0-9.0) over 48 hours (acute) or 21days (chronic) to *D. magna*.

48hr acute test

Concentration (mM)	pH5.5		pH6.0		pH7.0		pH7.5		pH8.0		pH9.0	
	24hr	48hr	24hr	48hr	24hr	48hr	24hr	48hr	24hr	48hr	24hr	48hr
Control	0.000	-0.020	0.260	-0.020	0.006	0.019	0.020	0.040	-0.024	-0.005	-0.034	0.001
0.5	0.050	0.070	0.090	0.110	0.088	0.178	0.090	0.110	0.080	0.241	-0.078	-0.246
1	0.020	0.120	0.060	0.030	0.060	0.095	0.100	0.190	0.035	0.054	-0.101	-0.193
2	0.010	0.000	0.030	-0.010	0.045	0.020	-0.030	0.000	0.026	0.009	-0.010	-0.065

Highlighted pH concentration (2mM) has chosen for the follow acute and chronic experiments, because all concentration showed no mortality and 2mM showed the minimum pH variation

21d chronic test

Concentration (ug/L)	Adult survival (%)		No. of young per female			First day of reproduction (day)			No. of Young per brood			Growth (mm)			Population growth rate (r)
	mean	SD	mean	SD	SE	mean	SD	SE	mean	SD	SE	mean	SD	SE	
C	100	-	64.5	5.3	1.7	9.5	0.5	0.2	5.4	0.5	0.2	2.90	0.1	0.0	0.338
pH5.5 (2mM)	100	-	63.3	7.9	2.5	9.6	0.5	0.2	5.3	0.7	0.2	2.80	0.1	0.0	0.334
pH6.0 (2mM)	100	-	62.5	6.6	2.1	9.5	0.5	0.2	5.3	0.5	0.2	2.80	0.1	0.0	0.338
pH7.0 (2mM)	100	-	63.0	7.5	2.4	9.5	0.5	0.2	5.2	0.4	0.1	2.90	0.1	0.0	0.338
pH7.5 (2mM)	100	-	65.7	3.1	1.0	9.5	0.5	0.2	5.4	0.5	0.2	2.80	0.1	0.0	0.333
pH8.0 (2mM)	100	-	65.1	7.0	2.2	9.5	0.5	0.2	5.4	0.5	0.2	2.80	0.1	0.0	0.336
pH9.0 (2mM)	100	-	64.0	6.1	1.9	9.4	0.5	0.2	5.7	0.7	0.2	2.90	0.1	0.0	0.339

Appendix 3. Chemical analysis for each antidepressant in acute and chronic tests

Citalopram (CTP) 48hr acute test

	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (mg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**
Control	na	0	<LOD*	100	na	0	<LOD*	100
Solvent control	na	0	<LOD*	100	na	0	<LOD*	100
0.31	1.488	16.2	0.27	85.7	1.497	15.5	0.26	82.1
0.63	1.488	37.9	0.62	99.7	1.497	32.6	0.54	85.8
1.25	1.488	81.5	1.34	106.9	1.497	72.8	1.19	95.5
2.50	1.488	153.4	2.51	100.5	1.497	146.3	2.40	95.9
5.00	1.488	312.5	5.12	102.3	1.497	293.4	4.80	96.1
10.0	1.488	672.6	11.0	110.1	1.497	638.9	10.46	104.6
Mean	1.488	159.3	3.48	100.7	1.497	149.9	3.27	95.0
%RSD	0.000	146.1	117.4	7.1	0.000	147.5	118.8	7.9

*Limit of detection (LOD) = 0.052; Limit of quantification (LOQ) = 0.157

Fluoxetine (FLX) 48hr acute test

	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (mg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**
Control	na	0	<LOD*	100	na	0	<LOD*	100
Solvent control	na	0	<LOD*	100	na	0	<LOD*	100
0.08	2.998	5.34	0.06	83.2	2.876	5.82	0.07	92.8
0.16	2.998	12.8	0.18	116.1	2.876	11.2	0.16	100.1
0.31	2.998	24.1	0.36	114.5	2.876	22.3	0.33	105.5
0.63	2.998	41.7	0.63	101.2	2.876	45.8	0.70	111.4
1.25	2.998	80.8	1.24	99.4	2.876	76.6	1.18	94.1
2.50	2.998	152.2	2.36	94.2	2.876	144.2	2.23	89.3
Mean	2.998	39.6	0.81	101.1	2.876	38.2	0.78	99.1
%RSD	0.000	133.7	107.7	10.4	0.000	131.6	105.5	7.2

*Limit of detection (LOD) = 0.037; Limit of quantification (LOQ) = 0.114

Sertraline (SRT) 48hr acute test

	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (mg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**
Control	na	0	<LOD*	100	na	0	<LOD*	100
Solvent control	na	0	<LOD*	100	na	0	<LOD*	100
0.08	6.587	1.02	0.09	110.8	6.542	0.98	0.08	106.4
0.16	6.587	2.12	0.18	116.2	6.542	2.07	0.18	113.4
0.31	6.587	4.08	0.35	112.3	6.542	3.93	0.34	108.1
0.63	6.587	6.68	0.58	92.1	6.542	6.47	0.56	89.2
1.25	6.587	12.9	1.11	89.0	6.542	14.2	1.22	98.0
2.50	6.587	30.1	2.60	103.9	6.542	32.8	2.83	113.2
Mean	6.587	7.1	0.82	103.0	6.542	7.6	0.87	103.5
%RSD	0.000	144.0	115.7	9.4	0.000	148.7	120.2	8.0

*Limit of detection (LOD) = 0.038; Limit of quantification (LOQ) = 0.114

Duloxetine (DUL) 48hr acute test

	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (mg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**
Control	na	0	<LOD*	100	na	0	<LOD*	100
Solvent control	na	0	<LOD*	100	na	0	<LOD*	100
0.31	1.102	90.8	0.28	89.9	1.117	91.6	0.28	90.4
0.63	1.102	230.2	0.59	94.5	1.117	212.8	0.55	88.3
1.25	1.102	497.6	1.19	94.8	1.117	483.1	1.15	92.2
2.50	1.102	887.5	2.05	82.1	1.117	890.4	2.06	82.3
5.00	1.102	2027.9	4.59	91.8	1.117	2275.4	5.14	102.8
10.0	1.102	3976.8	8.92	89.2	1.117	4287.8	9.61	96.1
Mean	1.102	963.9	2.94	92.8	1.117	1030.1	3.13	94.0
%RSD	0.000	144.6	112.9	6.4	0.000	147.5	115.9	7.4

*Limit of detection (LOD) = 0.157; Limit of quantification (LOQ) = 0.474

Dosulepin (DOS) 48hr acute test

	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (mg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (mg/L)**	**Recovery (%)**
Control	na	0	<LOD	100	na	0	<LOD	100
Solvent control	na	0	<LOD	100	na	0	<LOD	100
0.08	7.026	17.7	0.07	86.8	7.134	18.8	0.07	91.8
0.16	7.026	38.2	0.14	89.7	7.134	38.2	0.14	89.7
0.31	7.026	96.4	0.35	110.6	7.134	90.6	0.33	104.1
0.63	7.026	201.4	0.72	114.6	7.134	178.1	0.63	101.5
1.25	7.026	413.7	1.47	117.3	7.134	334.9	1.19	95.0
2.50	7.026	780.1	2.76	110.4	7.134	745.8	2.64	105.6
Mean	7.026	193.4	0.92	103.7	7.134	175.8	0.83	98.5
%RSD	0.000	142.5	113.3	11.0	0.000	146.3	116.9	5.8

*Limit of detection (LOD) = 0.016; Limit of quantification (LOQ) = 0.048

Citalopram (CTP) 21d chronic test

	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (µg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**
Control	na	0	<LOD	100	na	0	1.25	100
Solvent control	na	0	<LOD	100	na	0	1.25	100
7.50	1.451	0.64	7.19	95.9	1.502	0.74	8.12	108.2
15.0	1.451	1.61	16.19	107.9	1.502	1.68	16.84	112.3
30.0	1.451	3.35	32.34	107.8	1.502	3.42	32.98	109.9
60.0	1.451	7.1	67.13	111.9	1.502	6.98	66.02	110.0
120	1.451	14.5	135.79	113.2	1.502	13.9	130.22	108.5
240	1.451	28.7	267.54	111.5	1.502	29.0	270.32	112.6
Mean	1.451	7.0	87.70	106.0	1.502	7.0	65.88	107.7
%RSD	0.000	143.9	113.7	6.1	0.000	144.6	141.8	4.6

*Limit of detection (LOD) = 2.70; Limit of quantification (LOQ) = 8.19

Fluoxetine (FLX) 21d chronic test

	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (µg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**
Control	na	0	<LOD	100	na	0	0.38	100
Solvent control	na	0	<LOD	100	na	0	0.38	100
2.50	2.848	0.14	2.19	87.7	2.921	0.13	2.06	82.5
5.00	2.848	0.3	4.26	85.3	2.921	0.29	4.13	82.7
10.0	2.848	0.76	10.22	102.2	2.921	0.72	9.70	97.0
20.0	2.848	1.62	21.35	106.8	2.921	1.58	20.84	104.2
40.0	2.848	2.94	38.44	96.1	2.921	2.88	37.67	94.2
80.0	2.848	5.81	75.60	94.5	2.921	5.7	74.69	93.4
Mean	2.848	1.4	25.35	96.6	2.921	1.4	18.73	94.2
%RSD	0.000	140.7	110.5	7.5	0.000	141.8	138.9	8.4

*Limit of detection (LOD) = 0.79; Limit of quantification (LOQ) = 2.39

Sertraline (SRT) 21d chronic test

	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (µg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**
Control	na	0	<LOD	100	na	0	0.25	100
Solvent control	na	0	<LOD	100	na	0	0.25	100
1.00	6.529	0.31	0.98	98.0	6.488	0.27	0.89	88.5
2.00	6.529	0.68	1.85	92.7	6.488	0.59	1.64	82.0
4.00	6.529	1.34	3.41	85.3	6.488	1.26	3.22	80.6
8.00	6.529	3.2	7.80	97.5	6.488	3.08	7.52	94.0
16.0	6.529	7.01	16.80	105.0	6.488	6.84	16.40	102.5
32.0	6.529	14.4	34.24	107.0	6.488	14.1	33.54	104.8
Mean	6.529	3.4	10.85	98.2	6.488	3.3	7.96	94.1
%RSD	0.000	149.9	118.5	7.0	0.000	151.6	146.8	10.0

*Limit of detection (LOD) = 0.57; Limit of quantification (LOQ) = 1.74

Duloxetine (DUL) 21d chronic test

	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (µg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**
Control	na	0	<LOD	100	na	0	0.29	100
Solvent control	na	0	<LOD	100	na	0	0.29	100
12.5	1.355	1.31	13.22	105.7	1.362	1.22	12.33	98.6
25.0	1.355	2.58	25.75	103.0	1.362	2.42	24.17	96.7
50.0	1.355	4.42	43.91	87.8	1.362	4.39	43.62	87.2
100	1.355	8.97	88.82	88.8	1.362	8.45	83.69	83.7
200	1.355	21.1	208.55	104.3	1.362	19.2	189.80	94.9
400	1.355	38.4	379.31	94.8	1.362	37.0	365.49	91.4
Mean	1.355	9.6	126.60	98.1	1.362	9.1	89.96	94.1
%RSD	0.000	141.5	112.8	7.0	0.000	142.6	142.2	6.5

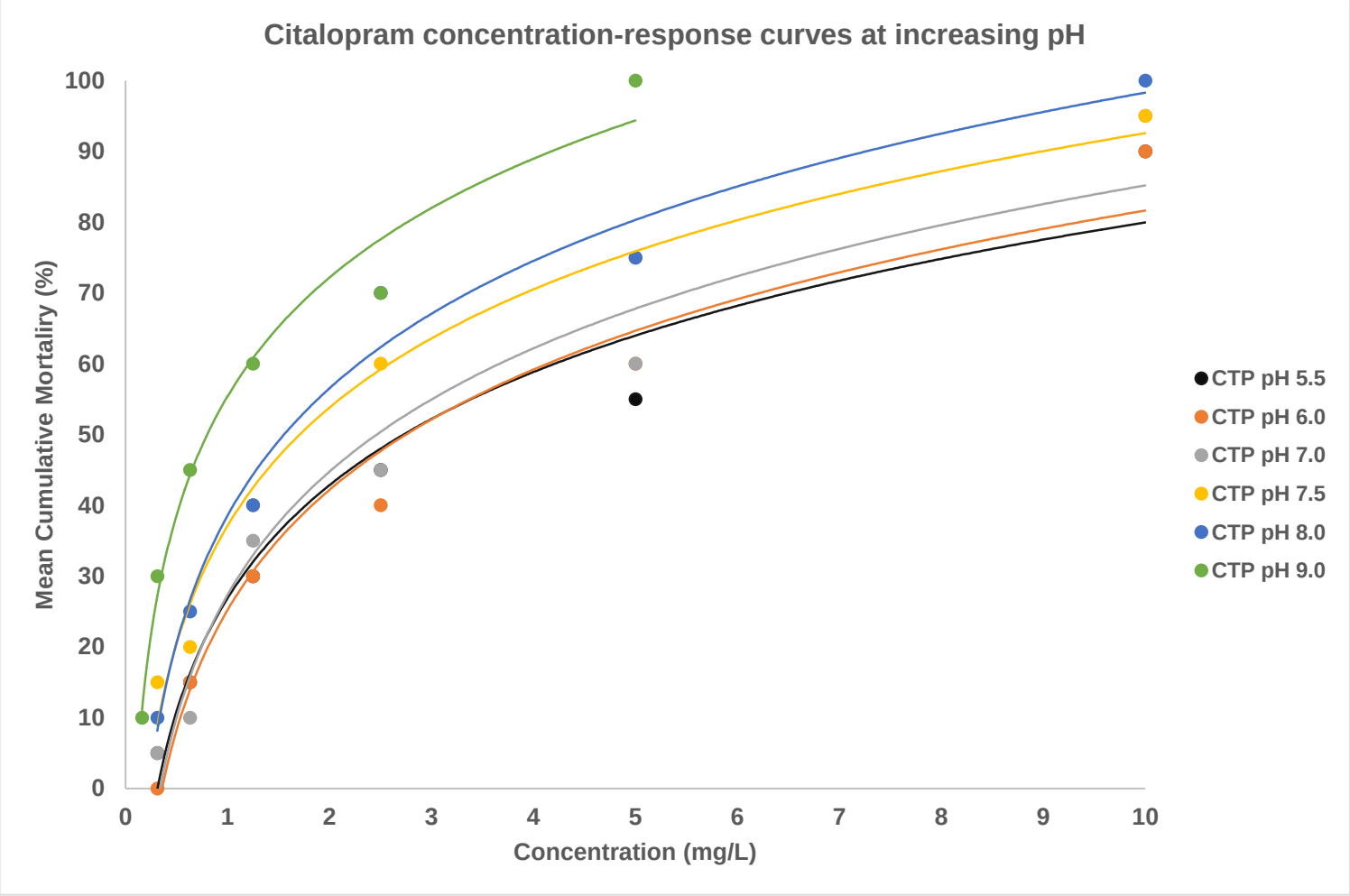
*Limit of detection (LOD) = 5.58; Limit of quantification (LOQ) = 16.9

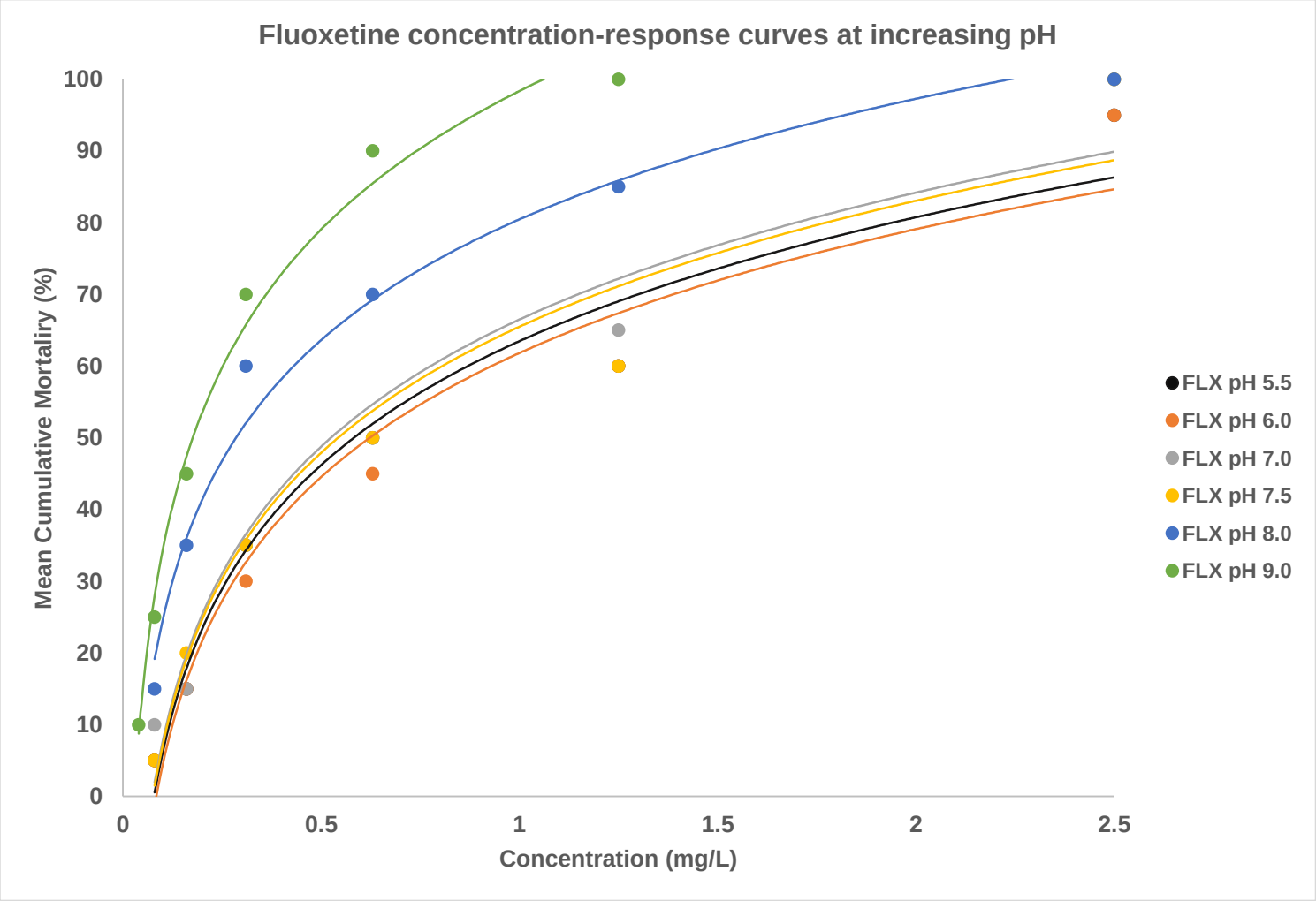
Dosulepin (DOS) 21d chronic test

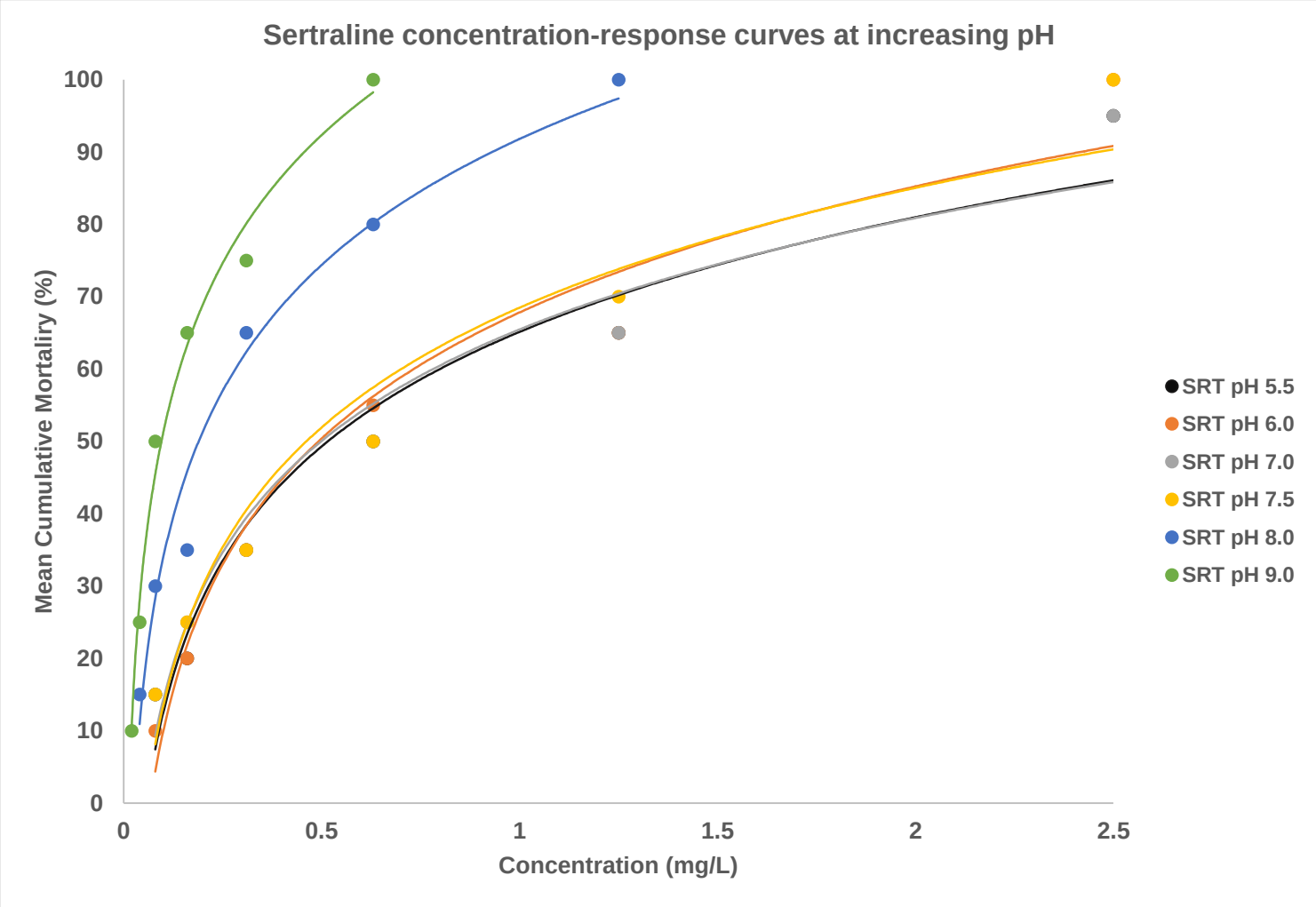
	Toxicity new samples (0h)				Toxicity old samples (48h)			
Injection (µg/L)	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (µg/L)**	**Recovery (%)**
Control	na	0	<LOD	100	na	0	-0.26	100
Solvent control	na	0	<LOD	100	na	0	-0.26	100
2.50	6.989	1.92	2.11	84.3	7.054	1.88	2.06	82.4
5.00	6.989	4.39	5.15	103.0	7.054	4.21	4.93	98.6
10.0	6.989	8.64	10.38	103.8	7.054	8.13	9.75	97.5
20.0	6.989	15.2	18.46	92.3	7.054	14.8	17.97	89.8
40.0	6.989	30.9	37.79	94.5	7.054	29.1	35.57	88.9
80.0	6.989	62.3	76.45	95.6	7.054	60.8	74.61	93.3
Mean	6.989	15.4	25.06	96.7	7.054	14.9	18.05	93.8
%RSD	0.000	140.0	112.7	6.7	0.000	141.2	143.2	6.8

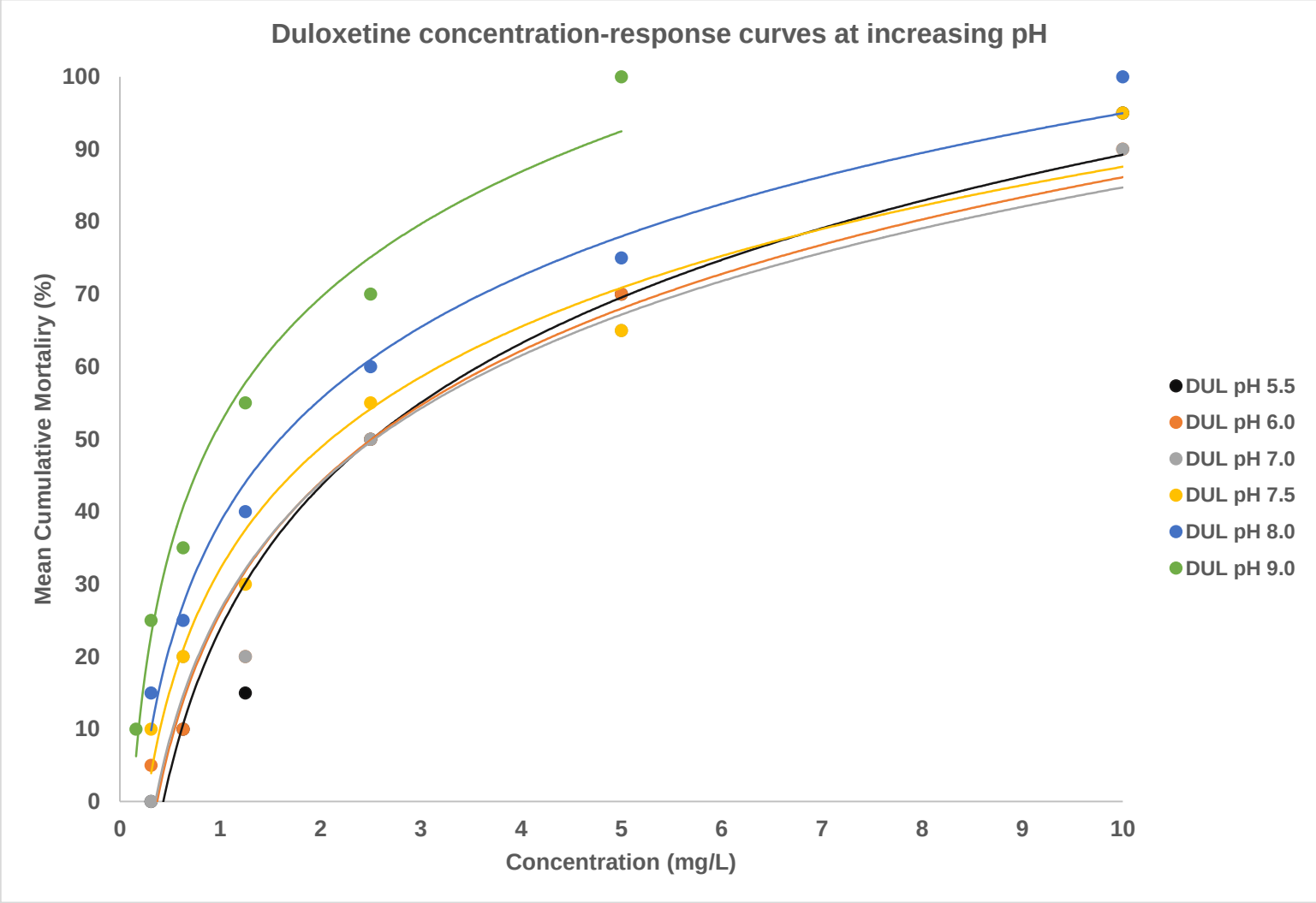
*Limit of detection (LOD) = 1.20; Limit of quantification (LOQ) = 3.63

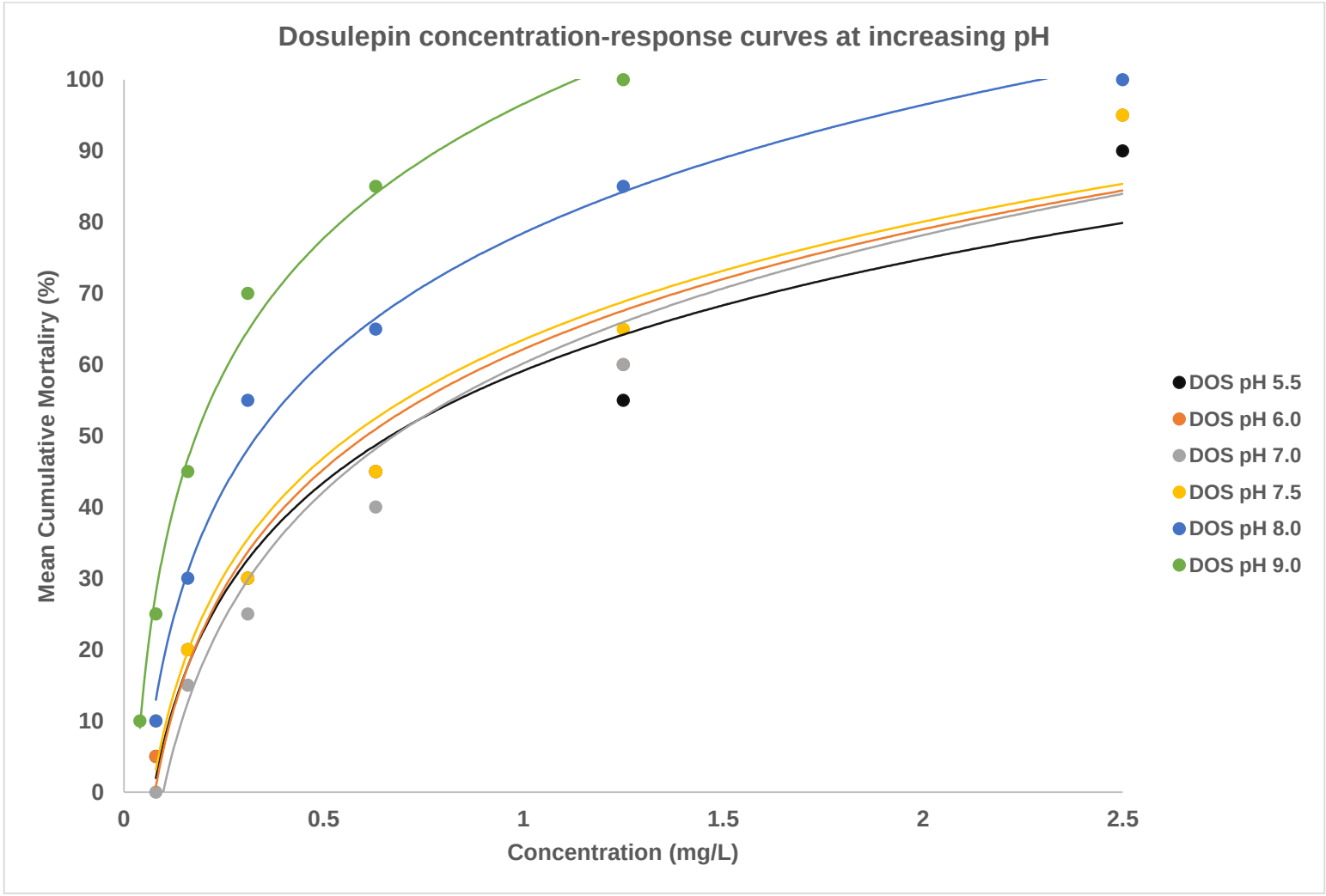
Appendix 4. Concentration-response curve for the acute tests




Fluoxetine concentration-response curves at increasing pH
Mean Cumulative Mortality (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
Concentration (mg/L)
FLX pH 5.5
FLX pH 6.0
FLX pH 7.0
FLX pH 7.5
FLX pH 8.0
FLX pH 9.0


Sertraline concentration-response curves at increasing pH
Mean Cumulative Mortality (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
Concentration (mg/L)
SRT pH 5.5
SRT pH 6.0
SRT pH 7.0
SRT pH 7.5
SRT pH 8.0
SRT pH 9.0


Duloxetine concentration-response curves at increasing pH
Mean Cumulative Mortality (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
Concentration (mg/L)
DUL pH 5.5
DUL pH 6.0
DUL pH 7.0
DUL pH 7.5
DUL pH 8.0
DUL pH 9.0


Dosulepin concentration-response curves at increasing pH
Mean Cumulative Mortaliry (%)
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
2.5
Concentration (mg/L)
DOS pH 5.5
DOS pH 6.0
DOS pH 7.0
DOS pH 7.5
DOS pH 8.0
DOS pH 9.0

Appendix 5. Chemical analysis for each antidepressant in chronic mixture tests

SSRIs Mixture A 21d chronic test

		Toxicity new samples (0h)				Toxicity old samples (48h)			
Antidepressant	**Injection (ug/L)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**
CTP	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.92	1.482	nd	na	na	1.498	nd	na	na
	4.59	1.482	0.39	4.87	106.1	1.498	0.28	3.85	83.9
	8.27	1.482	0.84	9.05	109.4	1.498	0.75	8.21	99.3
	9.19	1.482	0.92	9.79	106.5	1.498	0.81	8.77	95.4
FLX	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.57	2.911	nd	na	na	2.945	nd	na	na
	2.84	2.911	1.06	2.75	96.8	2.945	0.99	2.56	92.3
	5.11	2.911	2.11	5.23	102.3	2.945	2.02	5.04	98.2
	5.68	2.911	2.48	6.10	107.4	2.945	2.36	5.94	96.7
SRT	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.24	6.628	nd	na	na	6.696	nd	na	na
	1.22	6.628	nd	na	na	6.696	nd	na	na
	2.19	6.628	0.17	2.58	117.9	6.696	0.13	2.06	94.2
	2.43	6.628	0.19	2.84	116.9	6.696	0.17	2.58	117.9

*Limit of detection (LOD) = 2.70 (CTP), 0.79 (FLX), 0.57 (SRT); Limit of quantification (LOQ) = 8.19 (CTP), 2.39 (FLX), 1.74 (SRT)

SSRIs Mixture B 21d chronic test

		Toxicity new samples (0h)				Toxicity old samples (48h)			
Antidepressant	**Injection (ug/L)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**
CTP	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.73	1.482	nd	na	na	1.498	nd	na	na
	3.65	1.482	0.31	3.84	105.2	1.498	0.28	3.34	91.5
	6.57	1.482	0.76	6.77	103.0	1.498	0.75	6.41	97.6
	7.29	1.482	0.82	7.58	104.0	1.498	0.81	7.12	97.7
FLX	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.45	2.911	nd	na	na	2.945	nd	na	na
	2.25	2.911	0.88	2.38	105.8	2.945	0.99	2.08	92.4
	4.06	2.911	1.78	4.12	101.5	2.945	2.02	3.85	94.8
	4.51	2.911	1.98	4.64	102.9	2.945	2.36	4.39	97.3
SRT	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.35	6.628	nd	na	na	6.696	nd	na	na
	1.76	6.628	nd	na	na	6.696	nd	na	na
	3.16	6.628	0.23	3.31	104.7	6.696	0.13	3.01	95.3
	3.51	6.628	0.26	3.64	103.7	6.696	0.17	3.34	95.2

*Limit of detection (LOD) = 2.70 (CTP), 0.79 (FLX), 0.57 (SRT); Limit of quantification (LOQ) = 8.19 (CTP), 2.39 (FLX), 1.74 (SRT)

SSRIs Mixture C 21d chronic test

		Toxicity new samples (0h)				Toxicity old samples (48h)			
Antidepressant	**Injection (ug/L)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**
CTP	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.54	1.482	nd	na	na	1.498	nd	na	na
	2.68	1.482	nd	na	na	1.498	nd	na	na
	4.82	1.482	0.34	5.01	103.9	1.498	0.31	4.85	100.6
	5.36	1.482	0.42	5.58	104.1	1.498	0.38	5.42	101.1
FLX	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.35	2.911	nd	na	na	2.945	nd	na	na
	1.75	2.911	0.72	2.03	116.0	2.945	0.68	1.92	109.7
	3.16	2.911	1.52	3.48	110.1	2.945	1.42	3.27	103.5
	3.51	2.911	1.63	3.82	108.8	2.945	1.51	3.42	97.4
SRT	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.45	6.628	nd	na	na	6.696	nd	na	na
	2.26	6.628	0.17	2.58	114.2	6.696	0.16	2.41	106.6
	4.06	6.628	0.31	4.24	104.4	6.696	0.28	4.11	101.2
	4.51	6.628	0.35	4.68	103.8	6.696	0.29	4.59	101.8

*Limit of detection (LOD) = 2.70 (CTP), 0.79 (FLX), 0.57 (SRT); Limit of quantification (LOQ) = 8.19 (CTP), 2.39 (FLX), 1.74 (SRT)

SNRI+TCA Mixture A 21d chronic test

		Toxicity new samples (0h)				Toxicity old samples (48h)			
Antidepressant	**Injection (ug/L)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**
DUL	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.92	1.289	nd	na	na	1.274	nd	na	na
	4.59	1.289	nd	na	na	1.274	nd	na	na
	8.27	1.289	0.91	9.27	112.1	1.274	0.82	8.98	108.6
	9.19	1.289	1.01	10.26	111.6	1.274	0.93	9.42	102.5
DOS	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.57	7.193	nd	na	na	7.095	nd	na	na
	2.84	7.193	2.81	3.20	112.8	7.095	2.76	3.01	106.0
	5.11	7.193	4.76	5.60	109.7	7.095	4.57	5.03	98.4
	5.68	7.193	5.01	5.91	104.1	7.095	4.82	5.72	100.7

*Limit of detection (LOD) = 5.58 (DUL), 1.20 (DOS); Limit of quantification (LOQ) = 16.9 (DUL), 3.63 (DOS)

SNRI+TCA Mixture B 21d chronic test

		Toxicity new samples (0h)				Toxicity old samples (48h)			
Antidepressant	**Injection (ug/L)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**
DUL	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.73	1.289	nd	na	na	1.274	nd	na	na
	3.65	1.289	nd	na	na	1.274	nd	na	na
	6.57	1.289	0.73	6.68	101.7	1.274	0.70	6.46	98.3
	7.29	1.289	0.82	7.37	101.1	1.274	0.79	7.11	97.5
DOS	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.45	7.193	nd	na	na	7.095	nd	na	na
	2.25	7.193	2.18	2.35	104.4	7.095	2.11	2.06	91.6
	4.06	7.193	3.92	4.11	101.2	7.095	3.84	4.01	98.8
	4.51	7.193	4.28	4.63	102.7	7.095	4.11	4.45	98.7

*Limit of detection (LOD) = 5.58 (DUL), 1.20 (DOS); Limit of quantification (LOQ) = 16.9 (DUL), 3.63 (DOS)

SNRI+TCA Mixture C 21d chronic test

		Toxicity new samples (0h)				Toxicity old samples (48h)			
Antidepressant	**Injection (ug/L)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**	**Retention Time (min)**	**Peak Area**	**Measured concentration (ug/L)**	**Recovery (%)**
DUL	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.54	1.289	nd	na	na	1.274	nd	na	na
	2.68	1.289	nd	na	na	1.274	nd	na	na
	4.82	1.289	0.52	4.94	102.5	1.274	0.46	4.72	97.9
	5.36	1.289	0.61	5.42	101.1	1.274	0.58	5.21	97.2
DOS	Control	na	0	<LOD	100	na	0	<LOD	100
	Solvent control	na	0	<LOD	100	na	0	<LOD	100
	0.35	7.193	nd	na	na	7.095	nd	na	na
	1.75	7.193	1.92	1.83	104.6	7.095	1.76	1.71	97.7
	3.16	7.193	3.28	3.22	101.9	7.095	3.12	3.08	97.5
	3.51	7.193	3.45	3.62	103.1	7.095	3.34	3.42	97.4

*Limit of detection (LOD) = 5.58 (DUL), 1.20 (DOS); Limit of quantification (LOQ) = 16.9 (DUL), 3.63 (DOS)

Appendix 6. The PEC (µg/L) of each antidepressant in 3 different groups of year. The PEC (µg/L) of each pharmaceuticals were calculated using the amount of consumed antidepressants on the each time periods data and formula 6. The calculated PEC was used to calculate the concentration of each chemicals in different combinations of mixtures.

	PEC (µg/L)				
Year	**CTP**	**FLX**	**SRT**	**DUL**	**DOS**
09 to 11 (A)	2.65	1.63	0.70	0.16	0.61
12 to 14 (B)	2.24	1.38	1.08	0.22	0.34
15 to 18 (C)	1.81	1.18	1.52	0.27	0.18

Supplementary data

1. **Chemical prioritization**

Antidepressants Rank in RQ Order						
	Name	**RQ**	**Chemical Class**		**pK_a**	**K_{ow}**
1	Dosulepin	0.830	TCA	tricyclic antidepressant	9.76	4.68
2	Fluoxetine	0.476	SSRI	selective serotonin reuptake inhibitors	9.80	4.65
3	~~Lofepramine~~	~~0.224~~	~~TCA~~	~~tricyclic antidepressant~~	~~7.50~~	~~7.26~~
4	Sertraline	0.186	SSRI	selective serotonin reuptake inhibitors	9.85	5.29
5	Citalopram	0.174	SSRI	selective serotonin reuptake inhibitors	9.78	3.74
6	Duloxetine	0.143	SNRI	serotonin–norepinephrine reuptake inhibitors	9.34	4.29
7	Mirtazapine	0.070	NaSSA	α-2 adrenergic receptor antagonist		
8	Amitriptyline	0.054	TCA	tricyclic antidepressant		
9	Imipramine	0.009	TCA	tricyclic antidepressant		
10	Nortriptyline	0.008	TCA	tricyclic antidepressant		

2. ***D. magna* reference data**

UoY Environmental Toxicology Lab

***Daphnia magna* Acute Reference Toxicity Test Result**

Toxicant: Sodium Chloride (NaCl) / Media: ADaM
Manager: Jegak Seo/ Analyst: Jegak Seo
Period: February 2019 – July 2019

Test No.	Ending Date	EC50	cum. mean	cum. sd	cum. low CI	cum. high CI	analyst	pass/fail	note	95% C.I.		concentrations (g/L)
MRes_REF_01	8-Feb-19	3.82					SJG	P		3.53	4.04	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_02	22-Feb-19	3.97	3.89	0.105	3.68	4.11	SJG	P		3.68	4.21	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_03	8-Mar-19	3.95	3.91	0.081	3.75	4.08	SJG	P		3.59	4.21	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_04	22-Mar-19	3.79	3.88	0.091	3.70	4.06	SJG	P		3.32	4.10	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_05	5-Apr-19	3.82	3.87	0.083	3.70	4.04	SJG	P		3.35	4.13	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_06	19-Apr-19	3.78	3.85	0.083	3.68	4.02	SJG	P		3.27	4.09	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_07	3-May-19	3.87	3.85	0.076	3.70	4.01	SJG	P		3.42	4.17	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_08	17-May-19	3.96	3.87	0.079	3.71	4.03	SJG	P		3.54	4.24	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_09	31-May-19	4.00	3.88	0.086	3.71	4.06	SJG	P		3.62	4.28	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_10	14-Jun-19	3.76	3.87	0.090	3.69	4.05	SJG	P		3.22	4.08	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_11	28-Jun-19	3.76	3.86	0.092	3.68	4.05	SJG	P		3.22	4.08	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_12	12-Jul-19	3.76	3.85	0.092	3.67	4.04	SJG	P		3.22	4.08	0, 3.43, 3.95, 4.54, 5.22, 6.00
MRes_REF_13	26-Jul-19	3.78	3.84	0.091	3.67	4.03	SJG	P		3.27	4.09	0, 3.43, 3.95, 4.54, 5.22, 6.00

Abbreviations

%RSD	Percent relative standard deviation
5-HT	Serotonin
APIs	Active pharmaceuticals ingredients
BCF	Bioconcentration factors
CA	Concentration Addition
CI	Confidence interval
CTP	Citalopram
DDD	Defined daily doses
DOS	Dosulepin
DUL	Duloxetine
ECx	Effective Concentration at x%
EE2	Ethinyl estradiol
FLX	Fluoxetine
GP	General Practitioner
GSL	General sales list
HPLC	High-performance Liquid Chromatography
IA	Independent Action
IL-2	Interleukin 2
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MoA	mode of action
NE	Norepinephrine
NSAID	Non-steroidal anti-inflammatory drugs
P	Pharmacy medicines
PEC	Predicted environmental effect concentration
pK_a	Acid dissociation constant
POMs	Prescription-only medicines
ROS	Reactive oxygen species
SNRI	Serotonin-Norepinephrine Reuptake Inhibitors
SPE	Solid-Phase Extraction
SRT	Sertraline
SSRI	Selective Serotonin Reuptake Inhibitors
TCA	Tricyclic antidepressants

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