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Technical University of Denmark

The effect of pH on the bioconcentration and toxicity of weak organic electrolytes

CH2O+O2 ⇆ CO2+H2O

Cecilie Rendal

DTU Environment
Department of Environmental Engineering

PhD Thesis
February 2013

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The synopsis part of this thesis is available as a pdf-file for download from the DTU research database ORBIT: http://www.orbit.dtu.dk

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Preface

The aim of this PhD thesis is to characterize the pH dependent bioconcentration and toxicity of organic electrolytes, to establish which pH levels are optimal for ecotoxicological tests, and to develop sound methods for maintaining stable pH levels in laboratory tests.

The thesis is a contextualized summary of five scientific papers covering most of the findings of the PhD project. The thesis also includes work which has not previously been published, namely the EC50 regressions in chapter 3.1, the work with rhodamine 6G in chapter 4.1, and the exploration of the ionized fraction in chapter 4.2.

The five scientific papers are included as appendices to this thesis.

I. Rendal C, Kusk KO, Trapp S. 2011. The effect of pH on the uptake and toxicity of the divalent weak base chloroquine tested on *Salix viminalis* and *Daphnia magna*. *Environmental Toxicology and Chemistry.* 30:354-359.

II. Rendal C, Kusk KO, Trapp S. 2011. Optimal choice of pH for toxicity and bioaccumulation studies of ionizing organic chemicals. *Environmental Toxicology and Chemistry.* 30:2395–2406.

III. Rendal C, Trapp S, Kusk KO. 2012. Critical evaluation and further development of methods for testing ecotoxicity at multiple pH using *Daphnia magna* and *Pseudokirchneriella subcapitata*. *Environmental Toxicology and Chemistry*. 31:1843-1852.

IV. Rendal C, Wang H, Christensen IMA, Kusk KO. 2012. A method for pH specific toxicity tests with *Pseudokirchneriella subcapitata* tested on three ionizing compounds. *Environmental Toxicology and Chemistry.* **Submitted.**

V. Anskjær GG, Rendal C, Kusk KO. 2012. Effect of pH on the toxicity and bioconcentration of sulfadiazine on *Daphnia magna*. *Chemosphere*. **Submitted.**

In this online version of the thesis, the articles are not included but can be obtained from electronic article databases e.g. via www.orbit.dtu.dk or on request from DTU Environment, Technical University of Denmark, Miljøvej, Building 113, 2800 Kgs. Lyngby, Denmark, reception@env.dtu.dk.

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Cecilie Rendal

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February 2013

Summary

Many of the compounds in use today have ionizing properties. Investigations have shown that around half of the compounds preregistered for REACH and over 70% of all pharmaceuticals are ionizing organic compounds. These compounds may pose a risk when they are released into the environment. Ionization, however, complicates the environmental risk assessment of these compounds because the uptake processes of the neutral fraction differ from the processes of the ionized fraction.

Acids are increasingly neutral at pH levels below the pK_a while bases are increasingly neutral at pH levels above the pK_a. Because the neutral fraction is more lipophilic than the ionized fraction, ionizing organic compounds are often taken up more efficiently when they are present in the neutral form. Several studies have thus shown that acids are more toxic and more bioconcentrating at lower pH levels while bases are more toxic and bioconcentrating at higher pH levels.

In this study the bioconcentration and toxicity of the bivalent weak base chloroquine was tested on *Salix viminalis* at pH levels of 6, 7, 8 and 9. It was found that both bioconcentration and toxicity are higher at high pH values where the compound is increasingly neutral. A simulation with the cell model showed similar results. However, to draw any conclusions concerning the behavior of acids and bases in general, it is necessary to move away from the one-compound approach, and start looking at a larger dataset.

Therefore a dataset was compiled from an extensive literature search, and based on this dataset, the toxicity and bioconcentration of electrolytes was found to be systematically higher at pH levels that favor the neutral species. In surface waters with pH levels between 6 and 9 the bioconcentration and toxicity of acids and bases can thus be expected to fluctuate with pH if pK_a values fall within the range of 3-10 for acids and 5-12 for bases. Toxicity tests with *Pseudokirchneriella subcapitata* for the acid salicylanilide and the bases trimethoprim and ethoxyquin were in accordance with these ranges. Zwitterions and amphoters show pH dependent toxicity and bioconcentration if they contain a pK_a value within the ranges given for acids and bases. Three theoretical exceptions were found to the above.

The first exception is when the recipient water has pH levels outside the normal range of pH 6-9. In such cases the given pK_a range is too narrow and must be adjusted to accommodate the extreme pH level.

The second exception is when the ionized fraction is toxic through a specific mode of action - an example was found in the literature for sulfonamides where it is the ionized species that exert the antibiotic effect. This, however, only presents an exception if the bacteria are unable to maintain homeostasis. Bioconcentration and toxicity experiments with *Daphnia magna* showed that the sulfonamide sulfadiazine behaves as a simple acid with higher toxicity and bioconcentration at low pH levels.

A final theoretical exception was identified for cations with delocalized charges: a group of compounds for which ionized and neutral fractions have equal or similar lipophilicity. The neutral and ionized fractions of these compounds are taken up at equal rates, but the added effect of electrical attraction makes it theoretically possible for the cation to be more toxic and more bioaccumulative. The existence of such an exception, however, could not be verified experimentally with the model compound rhodamine 6G.

The role of the ionized fraction for uptake and toxicity is often assumed to be negligible. An examination of the contribution of the ionized fraction to the bioconcentration of ionizing organic compounds showed that this fraction cannot safely be overlooked.

The work presented in this thesis suggests that the standard test procedures used to test toxicity and bioconcentration are not sufficient to fully illuminate the ecotoxicity of ionizing organic compounds unless the effect of pH is somehow considered.

The final work presented in this thesis is a series of schematic selection criteria designed to facilitate rapid and correct decisions about which pH level to choose for bioconcentration and toxicity experiments. These guidelines are based on the findings disclosed in this thesis, and on the discussion thereof. The regular use of these guidelines will help eliminate situations where bioconcentration or toxicity is underestimated. The guidelines are supported by simple and robust test methods for conducting pH specific toxicity tests with the common test organisms *Daphnia magna* and *Pseudokirchneriella subcapitata* in the pH range of 6-9.

Dansk sammenfatning

Mange kemiske stoffer, der produceres og anvendes i dag, har ioniserende egenskaber. En undersøgelse har vist, at omkring halvdelen af de stoffer, som blev forudregistreret til REACH, var ioniserende stoffer. Derudover er mere end 70 procent af alle lægemidler også ioniserende. Disse stoffer kan udgøre en risiko for miljøet, når de udledes. De kemiske forbindelsers ioniserende egenskaber er imidlertid med til at komplicere miljørisikovurderingen, idet optagsprocesserne for neutrale og ladede stoffer er forskellige.

Syrer er overvejende neutrale, når pH er under $pK_{a,}$ mens det omvendte gør sig gældende for baser. Optagelsen af de ioniserende stoffer er ofte mere effektiv, når de er i neutral form. Dette skyldes, at den neutrale andel er mere fedtopløselig end den ioniserede andel. Flere undersøgelser har vist, at syrer er mest toksiske og mest biokoncentrerende ved lavere pH, mens baser er mest toksiske og biokoncentrerende ved højere pH-niveauer.

I denne afhandling undersøges det, hvorledes pH påvirker den bivalente svage base chloroquin's biokoncentration og toksicitet. Piletræsstiklinger (*Salix viminalis*) blev brugt som forsøgsorganisme, og der blev udført forsøg ved pH: 6, 7, 8 og 9. Det viser sig, at både biokoncentration og toksicitet stiger med pH, når chloroquin bliver mere neutral. Simulering med en dynamisk Fick-Nernst-Planck model viser lignende resultater. Selvom forsøget påviser en effekt af pH, er det ikke muligt at drage generelle konklusioner om sammenhængen mellem pH og miljøpåvirkningerne af syrer og baser. Til at udpege generelle tendenser, har det derfor været nødvendigt at indsamle flere data.

Et større datamateriale er sammensat på baggrund af omfattende litteratursøgning. Datamaterialet har dannet grundlag for en undersøgelse, som viste, at elektrolytter er systematisk mere giftige og biokoncentrerende ved pH-niveauer, hvor den neutrale form fremkommer. I overfladevand med pH-værdier mellem 6 og 9 kan syrer og basers biokoncentration og toksicit forventes at variere med pH, hvis pK_a-værdier falder inden for området 3-10 for syrer og 5-12 for baser. Toksicitetstests med *Pseudokirchneriella subcapitata* for syren salicylanilide og for baserne trimethoprim og ethoxyquin var i overenstemmelse med ovenstående intervaller. Zwitterioner og amphoterer udviser pH-afhængig toksicitet og biokoncentration, hvis de indeholder en pK_a-værdi inden for de anførte intervaller for syrer og baser. Der blev dog udpeget tre teoretiske undtagelser til ovenstående konklusioner.

Første undtagelse er, når søvandet har pH uden for intervallet 6-9. I sådanne tilfælde vil det givne pK_a-interval være for snævert og bør justeres.

Anden undtagelse er, når den ioniserede del optages eller er toksisk gennem en specifik virkningsmåde. Et eksempel på dette blev fundet i litteraturen for sulfonamider. Her er det den ioniserede form, der udløser den antibiotiske effekt. Dog udgør sulfonamider kun en undtagelse, hvis bakterierne ikke kan opretholde homøostase. Biokoncentrerings- og toksicitetsforsøg blev udført med *Daphnia magna* og viste, at sulfadiazine opfører sig som en almindelig syre med højere toksicitet og biokoncentrering ved lave pH-niveauer.

Tredje undtagelse er teoretisk og blev udpeget for kationer med delokaliserede ladninger - en gruppe af stoffer, hvor de ioniserede og neutrale dele har omtrent samme fedtopløselighed. De neutrale og ioniserede arter optages i disse tilfælde lige effektivt, men indvirkningen af elektrisk tiltrækning gør det teoretisk muligt, at kationen er mere toksisk og mere bioakkumulerende. En sådan undtagelse kunne dog ikke verificeres eksperimentelt med stoffet rhodamin 6G.

I flere modeller og regressioner i litteraturen antages den ioniserede fraktions bidrag til det samlede optag antages at være ubetydeligt. Det påvises, at denne fraktion bidrager til det samlede optag, og derfor bør modeller tage højde for den ioniserede andel af stoffet.

Resultaterne af denne afhandling viser, at de almindelige testprocedurer, der anvendes til at teste toksicitet og biokoncentration, ikke er tilstrækkelige til fuldstændigt at belyse økotoksiciteten af ioniserende stoffer, med mindre der tages højde for pH's inflydelse. Derfor foreslår afhandlingen at teste, der hvor forbindelsen er mest neutral. På denne baggrund opstiller afhandlingen en række skematiske retningslinjer, der muliggør et hurtigt og korrekt valg af pH til økotoksikologiske tests med ioniserende stoffer. Ved brug af disse retningslinjer undgår man situationer, hvor bioakkumulering eller toksicitet undervurderes. Retningslinjerne understøttes af enkle og robuste metoder udviklet til pH specifikke tests med *Daphnia magna* og *Pseudokirchneriella subcapitata* indenfor pH området 6-9.

Reader's guide

Chapter 1 is an introduction to ionizing organic compounds in the environment. The problem definition is presented along with the objectives of the study.

Chapter 2 gives a brief overview of the basic theory that describes the behavior of electrolytes in aquatic systems and the processes that govern passive uptake by biota.

Chapter 3 deals with different approaches to predicting the bioconcentration and toxicity of ionizing organic compounds. This chapter includes predictive regressions with EC50 and a case study with the weak divalent base chloroquine where bioconcentration and toxicity are measured on *Salix viminalis*, and where the cell model is applied for comparison.

Chapter 4 focuses on characterizing the pH dependent behavior of acids, bases, zwitterions and amphoters. Here a number of statements are presented that describe the ecotoxicological behavior of these compounds when pH fluctuates in the range of pH 6 to 9. A number of exceptions are discussed, and two case studies are presented -one concerning the amphoter sulfadiazine, and one concerning the fluorescent dye rhodamine 6G. Finally the contribution of the ionized fraction to the overall bioconcentration and toxicity is discussed.

Chapter 5 presents selection criteria for appropriate pH levels for toxicity and bioconcentration tests with ionizing organic compounds. This chapter also gives recommendations for pH stabilization methods in toxicity tests with *Daphnia magna* and *Pseudokirchneriella subcapitata*.

1. Introduction

1.1 Environmental risk assessment of chemicals

Modern environmental risk assessment of chemicals typically employs results of standard ecotoxicological tests. In such tests, standard media generally ensures uniform conditions for the test species, and factors such as light and temperature are also controlled in the laboratory. However, conditions in the environment are not uniform. Nutrient composition, light conditions, temperature and the pH of natural waters fluctuate in the environment, and test conditions therefore rarely reflect the true conditions in the environment (Laskowski et al., 2010). Furthermore, organisms in the environment are susceptible to other stress factors such as predators, diseases and competition (Gurevitch et al., 2002). In recent years there has been a growing awareness of the discrepancy between natural conditions and laboratory test conditions, and a resulting focus on the difference between the measured effects of a compound compared to the "true" effect of a compound in the environment (Mayer et al., 2009; Kim et al., 2010).

The pH of water is a central parameter in water chemistry, as well as in aquatic ecosystems (Stumm & Morgan, 1981). In ecotoxicology and risk assessment pH is therefore important to consider, particularly for electrolytes, a class of compounds known to change dissociative state with changing pH. This study, which is firmly rooted in traditional ecotoxicology and environmental risk assessment of chemicals, focuses on pH and on the effect of pH on the bioconcentration and toxicity of ionizing organic compounds.

1.2 The use and frequency of ionizing organic compounds

The European Union has launched legislation for the Registration, Evaluation, Authorization and restriction of CHemicals (REACH). About 143000 compounds were registered by 2009 and a survey conducted by Franco et al. (2010) showed that 49% of these compounds were ionizing organic compounds. Pharmaceuticals are not included in REACH, and represent another large group of compounds which often have ionizing characteristics. A survey by Manallack (2007) showed that over 70% of all pharmaceuticals are ionizing organic compounds. These surveys make the clear point that ionizing organic compounds represent a significant portion of the compounds that are in production and use today, and thus a clear procedure for risk assessment of them is paramount.

1.3 Ionizing organic compounds in the environment

The pH in the environment is far from stable, and in rivers and lakes it easily fluctuates in the range of pH 6-9 over a single day (Stumm & Morgan, 1981), and extremes can occur, as for instance seen with acid mine drainage sites (Niento et al., 2007). The speciation of an ionizing compound is determined by the pK_a of the compound relative to the pH of the solution (Hasselbalch, 1916). In pharmacology it is well known that the speciation state of a drug influences lipophilicity, solubility and permeability and that these factors in turn affect the absorption, distribution and excretion of the drug (Manallack, 2007). Just as the pH in blood, organs, and cell compartments affects the uptake and distribution of medicines into the human body (Manallak, 2007), so does the pH in water affect the uptake of, not just pharmaceuticals, but all ionizing organic compounds into organisms in the aquatic environment. Because the pH levels in ecotoxicological tests are often either undefined (Arnot & Gobas, 2006) or prone to drift (Mayer et al., 2009), there is a risk that the tests do not accurately reflect the true toxicity and bioconcentration of the compound, and that the environmental impact of ionizing organic compounds is underestimated.

1.4 Problem definition

A large percentage of the anthropogenic compounds in production and use today are ionizing organic compounds. These compounds dissociate in water with changing pH conditions, and this pH dependence influences toxicity and bioconcentration. An understanding of the behavior of these compounds is necessary in order to develop methods to accurately test and predict the toxicity and bioconcentration and eventually also to provide sound risk assessment.

The aim of this PhD thesis is thus to characterize the pH dependent bioconcentration and toxicity of organic electrolytes, to establish the optimal pH levels for ecotoxicological tests with organic electrolytes, and to develop sound methods for maintaining specific pH levels in laboratory tests without causing negative effects on the test organisms. The following formulates the specific objectives of the study.

1.5 Objectives

I. To investigate whether there is an effect of pH on the toxicity and bioconcentration of ionizing organic compounds, and to determine how pH affects:

chloroquine: a divalent weak base (**Paper I**).
sulfadiazine: an amphoter (**Paper V**).
rhodamine 6G: a cation with delocalized charge.

II. To determine whether predictive regressions can be made for the EC50 of ionizing organic compounds.

III. To determine whether acids and bases can always be considered more toxic and more bioconcentrating when they are neutral and to identify possible exceptions (**Paper II**).

IV. To determine whether the pK_a of an electrolyte can be used to identify which compounds are likely to show pH dependent bioconcentration and toxicity (**Paper II**).

V. To investigate and quantify the contribution of the ionized fraction of an electrolyte to bioconcentration and toxicity, and to establish whether or not this fraction can be safely neglected.

VI. To develop pH selection criteria and methods for testing the toxicity of ionizing organic compounds at multiple pH using *Daphnia magna* and *Selenastrum capricornutum* (**Paper III** and **IV**).

2. Theory

The following is a brief introduction to acid-base theory in aqueous systems, followed by a description of the processes that govern the passive uptake and bioconcentration of organic electrolytes.

2.1 The dissociation of acids and bases

The term "ion" was first used by Michael Faraday in 1834. The word means "wanderer" in Greek, and refers to the movement of charged molecules through aqueous solution towards an anode or a cathode. It was also Faraday who introduced the terms "anion" and "cation" (Faraday, 1834).

The behavior of acids and bases however, was first described 1887 by the Swedish scientist Svante August Arrhenius in his theory of electrolytic dissociation (Arrhenius, 1887). Arrhenius defined acids as compounds (containing hydrogen) that upon dissociation can produce hydrogen ions, and bases as compounds (containing hydroxide) that can produce hydroxide ions:

Acids: $HA \rightleftharpoons H^+ + A^-$ Bases: $BOH \rightleftharpoons B^+ + OH^-$ (1)

The main limitation of the Arrhenius theory is that there are a number of compounds which can release H^+ or OH^- without containing a hydrogen atom or a hydroxide group, such as SO_3[1], N_2O_5[2], Na_2O[3], and K_2O[4] (Upadhya, 2012).

The limitations of the Arrhenius theory were addressed in 1923 with the Brønsted-Lowry classification. The theory was developed by Johannes Nicolaus Brønsted and Thomas Martin Lowry who both, independently, and within a few months of each other came to the same conclusions and published papers (Lesney, 2003). The classification makes a distinction between a hydrogen ion and a proton, and thus a Brønsted-Lowry acid is a compound that can donate a proton, and a base is a compound that can accept a proton (Brønsted, 1923; Lowry, 1923). With this theory each acid has a conjugate base, and each base has a conjugate acid.

---

[1] $(SO_3)_2 + H_2O \rightarrow SO_4^{2-} + 2H^+$

[2] $N_2O_5 + H_2O \rightarrow 2NO_3^- + 2H^+$

[3] $Na_2O + H_2O \rightarrow 2Na^+ + 2OH^-$

[4] $K_2O + H_2O \rightarrow 2K^+ + 2OH^-$

All Arrhenius acids and bases are included in the Brønsted-Lowry classification, but the Brønsted-Lowry classification is more general and includes a number of substances not covered by the Arrhenius theory.

The Lewis classification of acids and bases, also first published in 1923, was developed by Gilbert N. Lewis and defines an acid as a substance that can accept an electron lone pair from another molecule and thereby complete a stable group of one of its own atoms. A Lewis base is thus a compound which can donate a pair of electrons to a Lewis acid (Lewis, 1923).

Building on the mass action law of the Norwegian scientists Guldberg and Waage (1879), the American scientist Lawrence Joseph Henderson was the first to describe the relation between an acid, its salt, and the H^+ concentration. The equation, commonly referred to as the Henderson approximation takes the form (Henderson, 1908a,b):

$$[H^+] = K_a \frac{[acid]}{[salt]} \tag{2}$$

where K_a is the equilibrium constant for the dissociation process (also known as the acid dissociation constant) written as:

$$K_a = \frac{[H^+][A^-]}{[HA]} \tag{3}$$

The concept of using a standardized scale for the concentration of H^+ in solution was introduced in 1909 by the Danish scientist Søren Sørensen with the p_H scale (Sørensen, 1909), which was revised in 1924 to pH - a denomination that we still use today.

$$pH = -log_{10}[H^+] \tag{4}$$

In 1916, several years after the publication of the Henderson approximation (Henderson, 1908a,b), Karl Albert Hasselbalch coupled the Henderson approximation with the pH scale introduced by Sørensen (1909), giving the equation the format that we know today (Hasselbalch, 1916):

$$pH = pK_a + log_{10}\left(\frac{[A^-]}{[HA]}\right) \tag{5}$$

where pK_a is the negative decadic logarithm of acid dissociation constant:

$$pK_a = -log_{10}K_a \tag{6}$$

Equation 5 is today referred to as the Henderson-Hasselbalch equation, giving credit to both scientists (Po & Senozan, 2001).

It is possible to calculate the fraction of neutral compound, $f_{n,}$ of a monovalent acid or base at a given pH based on the Henderson-Hasselbalch equation (Franco, 2010):

$$f_n = \frac{1}{1+10^{\alpha(pH-pK_a)}} \tag{7}$$

where α is -1 for acids and +1 for bases. It follows that the fraction of ionized compound, $f_{i,}$ is $1-f_n$.

Multivalent ionizing organic compounds contain more than one ionizing group: multivalent acids have more than one acidic group, and multivalent bases have more than one basic group. Zwitterions, such as amino acids, contain both an acid and a base, and can thus contain both a negative and a positive electrical charge within the same molecule. An amphoter also has both acidic and basic groups; however for amphoters the pK_a of the acid is always higher than the pK_a of the base. For both amphoters and zwitterions, the iso-electric point is the midpoint between the pK_a of the acid and the pK_a of the base. The dissociation of various classes of electrolytes is exemplified in Figure 1, based on calculations with *equation 5*.

2.2 Ionic strength, activity and Debye-Hückel

The scientific contributions of Gilbert N. Lewis were not limited to acid-base theory. In fact, many years prior to the definition of Lewis acids and bases, he introduced the concept of fugacity (1901) and the companion concept of activity (1907) (Mackay & Arnot, 2011).

Activity is a measure of the “effective concentration” of a species in a mixture, and is defined as:

$$a_i = exp\left(\frac{u_i-u_i^o}{RT}\right) \tag{8}$$

a_i = the activity of the species i
u_i = the chemical potential of the compound under conditions of interest
u_i^o = the chemical potential of the compound under reference conditions
R = the universal gas constant (8.314 J mol^{-1} K^{-1})
T = the absolute temperature in Kelvin

In collaboration with Merle Randall it was also Lewis who introduced the concept of ionic strength, I.

The ionic strength (mol/L) as defined by Lewis and Randall in 1921, is a descriptor for the overall concentration of charges in a solution, and is calculated as (Solomon, 2001):

$$I = 0.5 \sum_{i=1}^{n} c_i z_i^2 \tag{9}$$

C_i = the formal concentration of the ion i (mol/L)
z_i = the charge number of the ion i

Today, almost a century after the introduction of the concepts of ionic strength and activity, these parameters are still regarded as major variables in water chemistry (Sastre de Vincente, 2004).

The activity can be calculated using and an activity coefficient (γ) based on the formal concentration, C (mol/L) (Trapp et al., 2010):

$$a = \gamma C \tag{10}$$

In very dilute solutions, γ approaches 1 for all ions.

Activity coefficients of ions can be calculated using the Debye-Hückel equation (Debye & Hückel, 1923). The Davies approximation is one of the many approximations for the Debye-Hückel equation. It is valid for I ≤ 0.5 M (Davies, 1962):

$$log\, \gamma_i = -A_i^z \left(\frac{\sqrt{I}}{1+\sqrt{I}} - 0.3I\right) \tag{11}$$

A is a constant that depends on the pressure and temperature.

The activity of neutral compounds increases with increasing ionic strength. The activity coefficient of a neutral species depends linearly on the ionic strength of the solution (Setschenow, 1889):

$$log\, \gamma = k_m I \tag{12}$$

k_m = the Setschenow coefficient.

The relation between ionic strength and activity is due to electrostatic interactions between ions as they are attracted to and repulsed by one another (Debye & Hückel, 1923). When there are many ions in solution, these interactions lower the activity of ions. The effective concentration thus refers to the activity of a given solute, while the formal concentration refers to the true concentration in solution (Debye & Hückel, 1923).

The pK_a of a compound is also sensitive to changes in ionic strength. The corrected pK_a can be calculated with the Debye-Hückel limiting law (Debye & Hückel, 1923):

$$pK_a^{'} = pK_a - \frac{1.824 \cdot 10^6}{(\varepsilon T)^{3/2}} \cdot |z|\sqrt{I} \tag{13}$$

$pK_a^{'}$ = the corrected pK_a value
ε = the dielectric constant of the solution (78.54 F m^{-1} for water at 298K)

Based on the review of the acid-base theory given above, we now focus on the known processes which govern the passive uptake and bioconcentration of both neutral and ionized molecules.

As shortly outlined in **Paper II**, the uptake and bioconcentration of neutral compounds is mostly a partitioning process between aqueous phases and lipids (Mackay & Fraser, 2000; Trapp et al., 2010). The following briefly introduces the concepts of lipophilicity, partitioning, permeability and diffusion across cellular membranes for both neutral and ionized species.

2.3 Lipophilicity and log D

As early as 1899, the botanist Charles Ernest Overton studied the affinity of compounds for lipids by measuring the distribution between olive oil and water. He related the distributions to the narcotic effects observed on tadpoles (Al-Awquati,1999). Today scientists no longer use olive oil to measure partition coefficients, but octanol. The partition coefficient, also known as the octanol-water partition coefficient K_{ow}, is measured experimentally for a single chemical species (usually the neutral species).

Investigations by Briggs (1987) and Kleier (1988) show that the log K_{ow} of a neutral compound is approximately 3.5 units higher than the ionized counterpart (Trapp & Horobin, 2005):

$$\log K_{ow(ion)} = \log K_{ow\,(neutral)} - 3.5 \tag{14}$$

It is also possible to measure the distribution of a mixture of ionic forms which gives the pH dependent distribution coefficient, D (Testa et al., 1996).

Intramolecular forces are the forces that keep the atoms of a molecule together, while intermolecular forces are forces of attraction or repulsion which act between neighboring particles (atoms, molecules or ions) (Bruice, 2003).

Lipophilicity is described by Testa et al. (1996) as a balance of intramolecular forces and intermolecular interactions involving a solute and the two phases between which it partitions.

The most important intermolecular electrostatic forces that affect the lipophilicity are ionic bonds, H-bonds, and dipole-dipole interactions (Pliška et al., 2008). Intramolecular forces include structural factors such as isomerism, tautomerism, steroisomerism, ionization, and molecular size (Testa et al., 1996).

For simple acids and bases D is greater at pH levels where the compound is in the neutral form (Figure 1: salicylanilide and trimethoprim). For multivalent compounds (Figure 1: chloroquine), the same pattern holds, although here the change in D with pH is greater, due to the loss or gain of several charges. For zwitterions and amphoters the highest lipophilicity is seen at the isoelectric point where there is a neutral or a net neutral charge on the compound (Figure 1: sulfadiazine and ciprofloxacin).

An interesting observation regarding the partitioning of ionizing organic compounds was made by O'Connor et al. (2012) while measuring log D for a series of electrolytes. They found a shift in the pK_a of the compounds when measuring the log D using the immobilized artificial membrane technique. They gave the explanation that the ionic nature of membranes causes electrostatic forces which act on the ionic fraction of the compound. These electrostatic forces are not seen when measuring log D in simple octanol-water systems (O´Connor, 2012).

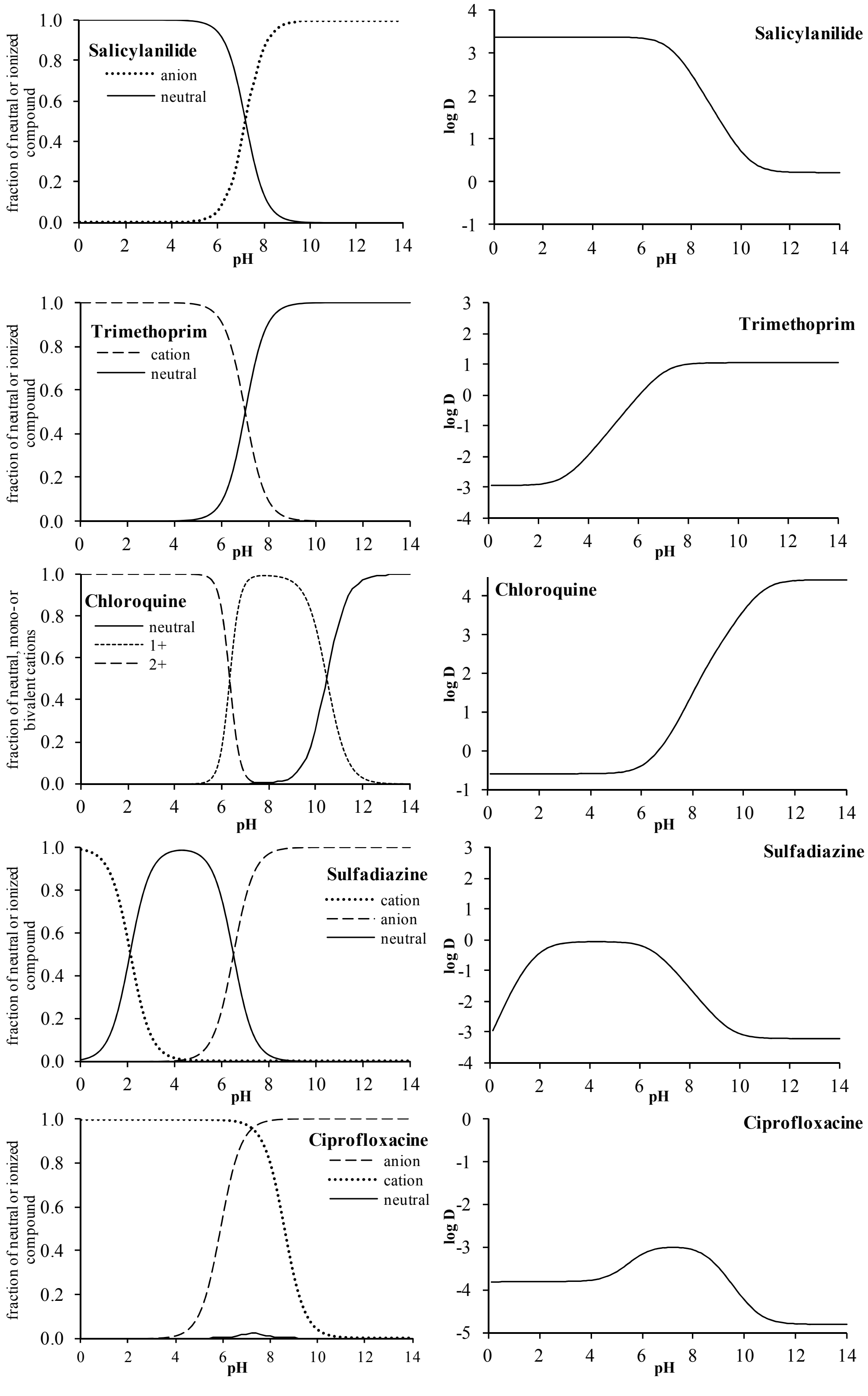


Figure 1. The dissociation behavior (ACD, 2008) and calculated log D (ACD, 2008) of a monovalent acid (salicylanilide), a monovalent base (trimethoprim), a divalent base (chloroquine), an amphoter (sulfadiazine), and a zwitterion (ciprofloxacin) with changing pH.

2.4 Uptake into cells: diffusion, permeability, sorption and the ion trap

The previously mentioned partition and tadpole experiments of Charles Ernest Overton lead him to believe that cells were surrounded by thin membranes (which he called lipoids) with the properties of oil (Heimburg, 2007). He formulated what is now known as the Overton rule, which states that the membrane permeability of a molecule is higher when the lipophilicity is higher. This is a conclusion that still holds true today (Al-Awqati, 1999).

The permeability, P (m/s) of a membrane can be calculated as:

$$P = \frac{DK_{ow}}{\Delta x} \qquad (15)$$

D = the diffusion coefficient

K_{ow} = the partition coefficient

Δx = the membrane thickness

The permeability of the ionized fraction is calculated with the same equation, but using log $K_{ow(ion)}$, *equation 14*. For neutral compounds, the diffusive flux across membranes follows Fick's 1st Law of Diffusion (Trapp, 2004):

$$J = -P(a_i - a_o) \qquad (16)$$

J = the unit flux (kg/m^2 /s)

P = the permeability of the membrane (m/s)

a_i = activity inside the membrane (kg/m^3)

a_o = activity outside the membrane (kg/m^3).

The ionic strength inside a cell is approximately 0.3 mol/L (Trapp, 2004), and the ionic strength for natural freshwater is typically much lower in the range of 0.02-0.06 mmol/L (Kim et al., 2005). The activity of a neutral compound can be calculated based on the ionic strength using the activity coefficient calculated using the Setschenow equation, *equation 12*.

Membranes in living cells have an electrical field caused by the ionic gradient created by the many imbedded pumps. Both the ionic gradient and the electrical field influence the uptake of ions (Berg et al., 2001). Thus Fick's 1st Law of Diffusion does not adequately describe the diffusion of ions on its own. Developed by the German physical chemist and physicist Walther Nernst, the Nernst equation describes the ratio of an ion inside (i) and outside (o) the cellular membrane due to electrochemical equilibrium (Nernst, 1889; Trapp et al, 2010):

$$\frac{a_i}{a_o} = \exp\left(\frac{-zFE}{RT}\right) \qquad (17)$$

z = the electric charge

F = the Faraday constant (96484.56 C/mol)

E = the membrane potential (volts)

R = universal gas constant (8.314 J/mol/K)

T = the absolute temperature in Kelvin

The flux of ions across a membrane is driven by both an electrical gradient and a chemical gradient. This is described by the Nernst-Planck equation, which gives the flux of ions (Briggs et al., 1961, cited in Trapp, 2004):

$$J = P\left(\frac{N}{e^{N}-1}\right)\left(a_o - a_i\, e^{N}\right) \qquad (18)$$

N = zEF/RT

Notice the inclusion of the charge number on the ion and the membrane potential. This inclusion ensures that the degree of electrical attraction and repulsion between ion and membrane is included in the uptake calculations.

The sorption of the neutral compound ($K_{neutral}$) to the lipid phase inside the cell can be calculated using the formula (Trapp et al., 2010):

$$K = Lipid\ content\ (g/g) \cdot 1.22 \cdot K_{ow} \qquad (19)$$

The sorption of the ionized fraction is calculated with the same equation, but using log $K_{ow(ion)}$, *equation 14*.

As outlined in **Paper II**, the ion trap is an uptake process which can only occur for ionizing organic compounds. When pH levels outside the cell allow the presence of a neutral fraction, this fraction is taken up rapidly because the membrane permeability of the neutral fraction is much higher than the membrane permeability of the ionized fraction. If the pH inside the cell is different from the pH outside cell, the compound may dissociate inside the cell. If this happens the compound will not be able to leave the cell at the same rapid rate that it entered.

The ion trap thus depends on different states of ionization outside and inside the cell. The neutral molecules establish the equilibrium across the cell membrane, while ions accumulate inside. Thus, the ion trap is strongest for acids when the pH outside the cell is several log units below the pH inside the cell, and for bases, the pH must be above the pH inside the cell. For both acids and bases, the ion trap is also stronger when the pK_a of the compound is close to the pH outside the cell. Examples of the ion trap have been observed and described by (Raven, 1975; Briggs et al., 1987; Trapp et al., 2008; Neuwoehner & Escher, 2010).

3. Predicting the bioconcentration and toxicity of ionizing organic compounds

The following considers techniques used to predict the bioconcentration and toxicity of electrolytes, starting with common regression techniques and then moving on to modeling techniques based on theoretical uptake processes. The chapter includes EC50 regressions based on the data from **Paper II**, and a case study of the divalent weak base chloroquine based on the results of **Paper I**.

3.1 BCF and EC50 regressions based on log K_{ow} and log D.

Bioconcentration is often quantified using the bioconcentration factor, BCF defined as the concentration in the organism divided by the concentration in the environment (Arnot & Gobas, 2006):

$$BCF = \frac{concentration\ in\ organism}{concentration\ in\ environment} \qquad (20)$$

BCF regressions with K_{ow} are commonly used to estimate BCF, such as the BCF regression by Veith et al. (1979) for fish:

$$\log BCF = 0.85 \cdot \log K_{ow} - 0.70 \qquad (21)$$

This regression is recommended in the Technical Guidance Document from the European Commission for neutral compounds (European Commission, 2003). This type of regression is often not applicable to electrolytes due to the difference in lipophilicity of neutral and ionized species, and BCF regressions specific to ionizing organic compounds are rare (Fu et al., 2009). One of the few investigations was made by Meylan et al. (1999), who investigated the relation between BCF and log K_{ow} of ionizing organic compounds. They suggested a series of guidelines instead of a mathematical solution due to limited data. Fu et al. (2009) worked with the same challenge and produced BCF regressions for acids and bases based on the pK_a and log K_{ow} of the compound.

The Technical Guidance Documents from European Commission suggest using a regression made for neutral compounds and multiplying with the fraction of neutral compound, f_n, *equation 7* (European Commission, 2003). Using f_n as a correction factor is builds on the assumption that the contribution of the ionized fraction to the total BCF is negligible. The validity of this approach is discussed further in chapter 4.2.

Fu et al. (2009) tested the use of a correction factor as suggested in the Technical Guidance Documents. They also tested the efficacy of exchanging K_{ow} with log D in normal BCF regressions. Both approaches gave good results, but it was found that log D was the superior predictor (Fu et al., 2009).

Similar regressions for predicting the toxicity of electrolytes are rare, and the few examples that could be found are specific to either class of compound (Saarikoski & Viluksela, 1982), (Zhao et al., 2010) or pH (Cronin et al., 2000).

In response to the quite limited regression work done with EC50 values, the following case study looks into the relation between measured EC50 and both log K_{ow} and log D.

Case study: Exploring the relation between EC50 and log D

To investigate how measured EC50 values for acids and bases correlate with log K_{ow} and log D the dataset from **Paper II** is analyzed.

Based on the results of Fu et al. (2009), we know that BCF correlates well with log D. The relation between EC50 and log D of acids and bases however, has not been fully investigated. In response to objective II, we formulate a hypothesis.

Hypothesis 1. *The EC50 of acids and bases correlates with log D and log* K_{ow}.

The dataset of **Paper II** is based on 37 studies from the literature where the effect of pH on either the toxicity or the bioconcentration of ionizing organic compounds was documented. Data could be extracted from 14 of these, yielding a total of 233 measured EC50 values and 139 measured BCF values. The dataset is thus heterogeneous in nature, and contains data from experiments using different test species, test durations and pH stabilization methods.

The 233 EC50 values from **Paper II** are plotted against both log K_{ow} and log D in Figures 2 (acids) and 3 (bases). Regressions are given for the entire dataset in subfigures **a** and **c**, and as a collection of regressions for each individual compound in subfigures **b** and **d**.

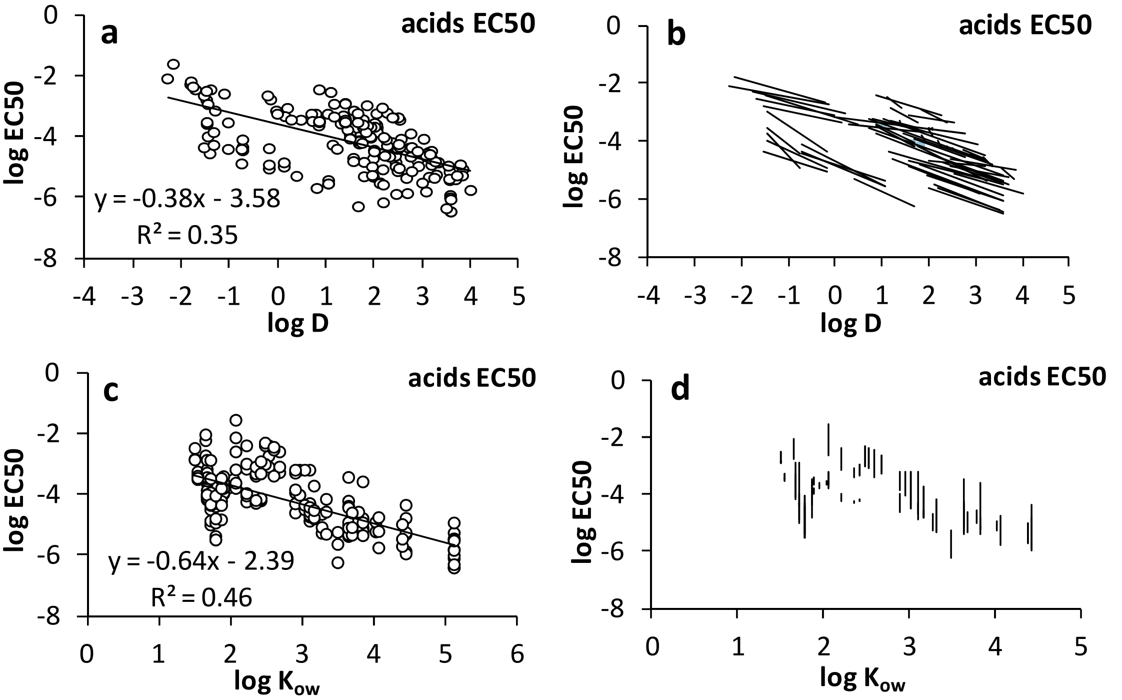


Figure 2. log D and log K_{ow} and correlations with EC50 (mol/L) for acids. **a** and **b** show regressions for the entire dataset, while **c** and **d** show regressions for each individual compound (at various pH levels). Log D and log K_{ow} are from ACD (2008). Data is from **Paper II.**

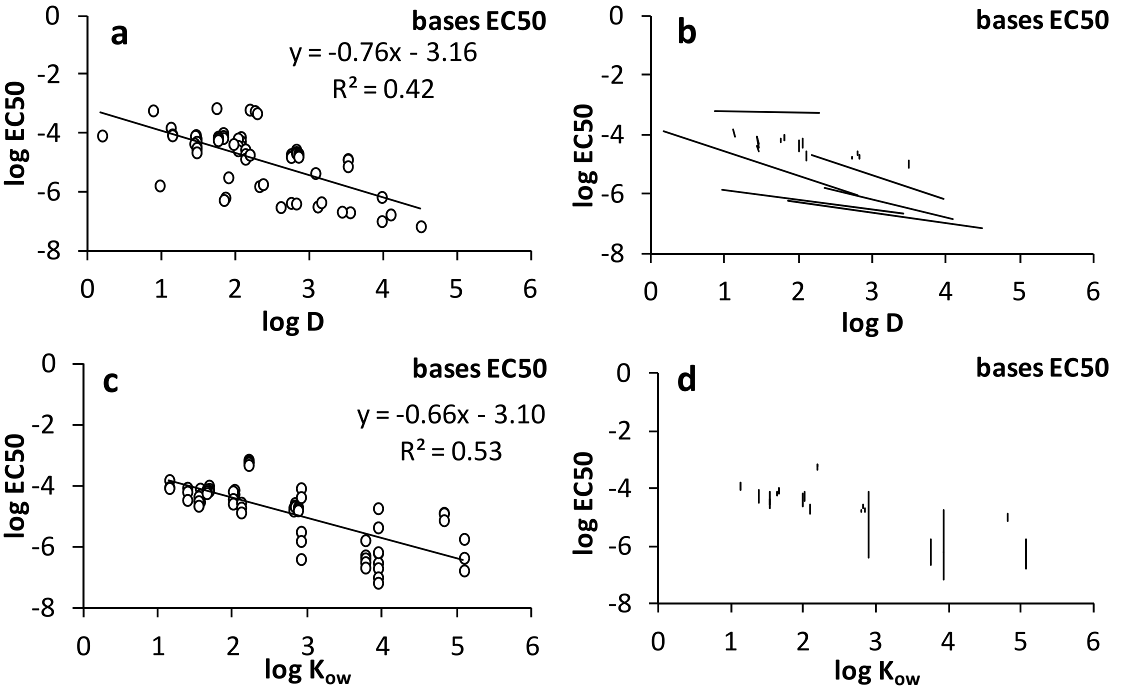


Figure 3. log D and log K_{ow} correlations with EC50 (mol/L) for bases. **a** and **b** show regressions for the entire dataset, while **c** and **d** show regressions for each individual compound (at various pH levels). Log D and log K_{ow} are from ACD (2008). Data is from **Paper II.**

For both acids and bases log K_{ow} functions as the best predictor for EC50, with R^2 values of 0.46 and 0.53 respectively, ($\alpha < 0.05$). Hypothesis 1 is thus confirmed. In comparison Fu et al. (2009) found BCF correlations with log D with R^2 values of 0.62 for both acids and bases. It is likely that some of the scatter in Figures 2 and 3 is caused by the heterogeneous nature of the data. If this is the case it is likely that the predictive ability of the correlations for EC50 can be improved by sorting data by test species or compound class.

Because log D includes the partitioning of the ionized fraction it is a parameter that contains more information than log K_{ow}, but despite this higher level of information, log D was found to be an inferior predictor. In Figure 2 and 3 subfigures **c** and **d** show the EC50 correlations for each individual compound. When the slopes of these many individual regressions are plotted against the average pH of the tests for each compound relative to the pK_a of the compound a pattern emerges (Figure 4). At pH levels where the compounds are predominantly ionized ($pH - pK_a \geq 0$ for acids, and $pH - pK_a \leq$ for bases) the slopes are close to zero. However, at pH levels where the compounds are predominantly neutral the slopes are increasingly negative until they eventually reach infinite (indicating a vertical line). This indicates that the greatest change in toxicity occurs at pH levels below the pK_a for acids, and above the pK_a for bases. In other words, pH affects toxicity most when over half of the compound is present in the neutral form.

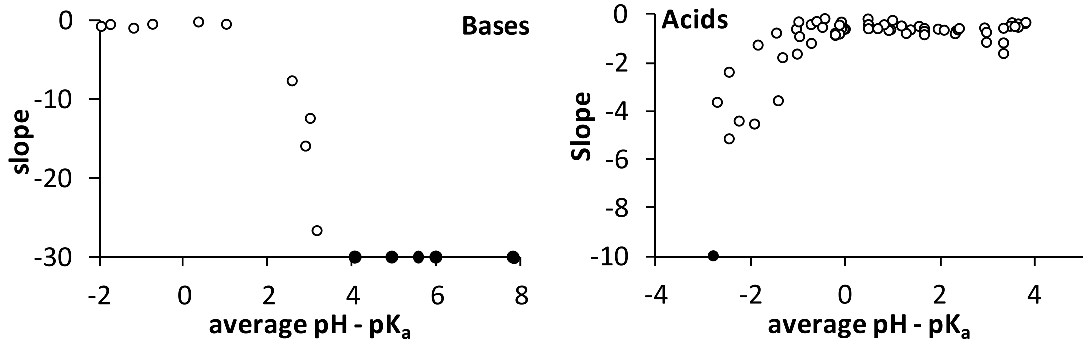


Figure 4. The slopes of EC50 regressions for individual compounds at various pH levels plotted against the average pH of tests relative to the pK_a of the compound (ACD, 2008). Data is from **Paper II**. The dots that are filled indicate that there was no change in log D and that a slope could not be calculated.

Based on the results of this case study we can conclude that there is a significant correlation between log EC50 and both log K_{ow} and log D. Furthermore, the information in Figure 4 suggests that it may be possible to predict the slope of regressions between EC50 and log D. This may be a useful supplement to normal regressions (Figure 2 and 3).

While regressions are a common and useful approach to predicting both bioconcentration and toxicity, more process-oriented models have been developed which are based on much of the theory presented in chapter two. The following is a description of the cell model - a process oriented model which simulates the uptake of electrolytes into a cell. The model is used to explore the uptake of ionizing organic compounds at various pH, and an example simulation is performed for the weak base chloroquine in a case study based on **Paper I**.

3.2 The cell model: uptake and distribution of electrolytes into cells

With regards to bioconcentration, a number of steady-state models have been created based on the Nernst-Planck equations, taking the ion trap into account (Raven, 1975; Rigitano et al., 1987; Inoue, 1998). Other ion trap models include Kleier (1988) and Neuwoehner & Escher (2010). The cell model (Trapp 2004; Trapp et al., 2005) is a dynamic model based on the approach of Raven (1975), but with the inclusion of lipophilic adsorption.

The overall equation for the cell model is (Trapp et al., 2004):

$$J = P_n\left(a_{n,o} - a_{n,i}\right) + P_i\left(\frac{N}{e^N - 1}\right)\left(a_o - a_i\, e^N\right) \tag{22}$$

P_n = permeability of the neutral fraction (m/s)
P_i = the permeability of the ionized fraction (m/s)

The model is described in detail in (Trapp et al., 2004), and takes all the effects described in chapter two into account including dissociation, adsorption, membrane permeability, electrical attraction, the Nernst equilibrium and the ion trap.

The cell model has the advantage that it calculates the uptake processes of both the ionic and neutral molecules, and can identify which uptake processes dominate. It has the added advantage that it can predict in which intracellular compartments the compound will accumulate.

Based on the theoretical considerations presented in chapter 2, we expect the factors of dissociation, lipophilicity and diffusion of electrolytes to cause a lower accumulation when the compound is electrically charged.

As described in **Paper II**, the cell model was used to investigate the effect of pH on the bioconcentration of monovalent acids and bases. The main chemical input parameters of pK_a, valency and log K_{ow} were varied over the plausible chemical space while pH was varied between 4 and 10.

These simulations confirmed a higher uptake of acids at low pH levels, with no combination of pK_a and K_{ow} resulting in an exception to this finding. For bases the accumulation was found to be higher at high pH levels with the exception of cations with delocalized charges - a class of compounds for which the change in lipophilicity of the ionized fraction and the neutral fraction is small (Duvvuri et al., 2004). This exception will be discussed in more detail in chapter 4.

For both acids and bases the ion trap effect was found to be strongest when the pK_a of the compound is close to the pH outside the cell. For bases the pH must be higher outside the cell than inside, and for acids the pH must be lower inside than outside.

The following case study is an example simulation with the cell model using the divalent weak base chloroquine.

Case study: the divalent weak base chloroquine

Chloroquine is a drug commonly used to prevent and to treat malaria, and has been widely used on a global scale because it is one of the cheapest alternatives (Zurita et al., 2005). It is a divalent weak base, with pK_a values of 10.47 and 6.33 and a log K_{ow} of 4.37 (ACD, 2008). The dissociation behavior and the log D is illustrated in Figure 1: chloroquine. In the pH range of 6 to 9, which can be considered the relevant range in rivers and lakes (Stumm & Morgan, 1981), the drug will be present in three dissociative states: neutral (CQ), protonated (CQ^+), and twice protonated (CQ^{++}).

The compound has been ecotoxicologically tested on *Daphnia magna*, *Chlorella vulgaris*, *Poeciliopsis lucida* (cells), and *Vibrio fischeri* by Zurita et al. (2005), who concluded that the compound should be classified as harmful to the environment. The ecotoxicological tests performed by Zurita et al. (2005) were conducted at standard test conditions, and do not take a possible change in toxicity with changing pH into account. In response to this, and to objective I for chloroquine, we can formulate the following hypothesis.

Hypothesis 2. *The toxicity and bioconcentration of chloroquine is higher at high pH levels where the compound is increasingly neutral.*

To test this hypothesis we set up an experiment with willow tree cuttings (*Salix viminalis*), using the willow tree test as described by Trapp et al. (2000). An example simulation with the cell model was also performed for an unicellular algae.

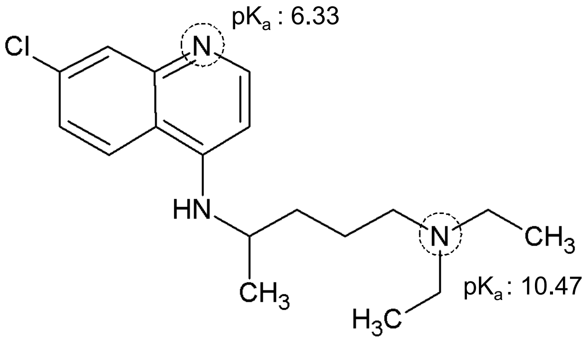


Figure 5. The structure of chloroquine (ACD, 2008) modified from **Paper I**.

Using transpiration inhibition as an endpoint, EC10 values of 16, 13, 3 and 1 mg/L were calculated for pH levels of 6, 7, 8 and 9, respectively. The uptake of chloroquine was measured in the same experiment, and also showed increasing accumulation at higher pH levels with 67-hour bioconcentration values of 71 ±41, 261 ±54, 326 ±48 and 696 ±113 for pH levels of 6, 7, 8 and 9 respectively (Figure 6). The methods are described in detail in **Paper I**. These measurements confirm hypothesis 2.

The bioconcentration of chloroquine in an algae cell was modeled using the cell model (*equation 22*). In this example the cell model includes the following compartments/organelles: vacuole, mitochondria, nucleus, chloroplast, and cytosol. Furthermore, the pK$_a$ shift described by O´Connor et al. (2012) is included. The parameters of the organelles are given in Table 1.

Table 1. Cellular characteristics used in the cell model.

	volume (µm^3)	area (µm^2)	lipid %	Electrical Potential mV (measured)		pH (measured)	
Cytosol	13	480	0.2	-140	(Blumwald et al., 2000)	7.3	(Bethman et al., 1995)
Chloroplast	33	676	13	+100	(Vredenburg & Tonk, 1975)	5.5	(Heldt et al., 1973)
Vacuole	19	101	2	+113	(Bethman et al., 1995)	5.0	(Bethman et al., 1995)
Mitochondria	2	58	15	-150	(Colombini, 2004)	8.0	(Llopis et al., 1998)
Nucleus	5	20	3	-5	(Mazzanti et al., 2001)	7.6	(Seksek & Bolard, 1996)

Volumes are calculated based on volume percentage measurements by Garret (2004) on the green algae *Pseudokirchneriella subcapitata*, in combination with the average cell volume of a *Pseudokirchneriella subcapitata* (72 μm^3) given in (ISO, 2008). For those organelles where no percentage measurements were available, volumes were estimated from micrographs. Apart from the parameters used to describe the cell (Table 1), the cell model requires charge number (z), pK_a and K_{ow} values for the test compound.

The cell model yielded bioconcentration factors of 35, 50, and 740 for pH levels of 6, 7, 8, and 9, indicating an increasing bioconcentration with increasing pH (Figure 6).

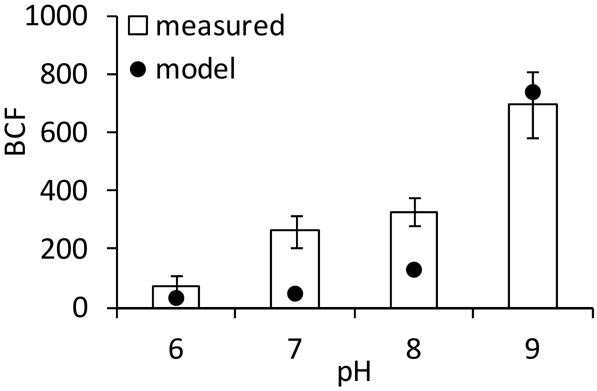


Figure 6 The measured BCF of chloroquine by *Salix viminalis* at pH levels of 6, 7, 8 and 9 at 67 hours of exposure (**Paper I**). Error bars give the standard error. The points are calculated bioconcentration factors from the cell model.

A schematic overview of the uptake of chloroquine is given in Figure 7, and each of the processes is briefly described in the following.

The membrane permeability of chloroquine (*equation 15*) follows the order $PCQ>>P\ CQ^+ >>PCQ^{++}$, indicating that the highest membrane permeability of chloroquine is when the compound is present in the neutral form – namely at pH levels above pK_{a2} (10.47). The values of P given in Figure 7 are calculated using membrane data from Riederer (1995).

Inside the cell chloroquine will dissociate according to the intracellular pH, which is around 7.3 (Bethman et al., 1995).

For a base, such as chloroquine, the ion trap occurs when pH inside the cell is lower than pH outside the cell. For chloroquine the ion trap is strongest at pH levels of 8 and 9 and contributes to the higher toxicity and bioconcentration found at these pH levels compared to pH levels of 6 and 7. For bases, the ion trap can be enhanced by the presence of acidic compartments, such as vacuole with a pH of 5 (Bethman et al., 1995).

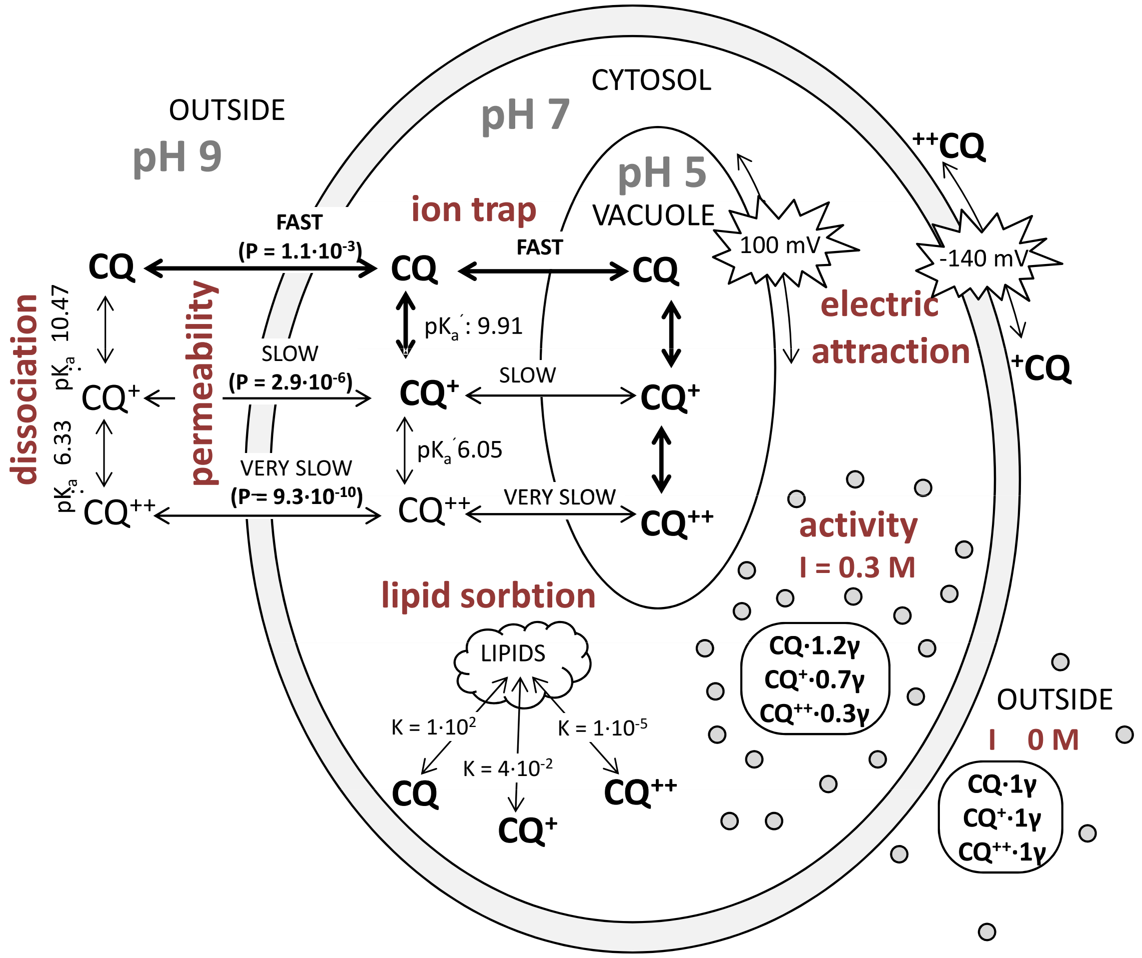

OUTSIDE
pH 9
CYTOSOL
pH 7
pH 5
VACUOLE
ion trap
FAST
(P = 1.1·10-3)
FAST
CQ
CQ
CQ
dissociation
pKa 10.47
pKa 6.33
permeability
pKa´: 9.91
SLOW
(P = 2.9·10-6)
SLOW
CQ+
CQ+
CQ+
pKa´6.05
VERY SLOW
(P = 9.3·10-10)
VERY SLOW
CQ++
CQ++
CQ++
100 mV
-140 mV
++CQ
+CQ
electric
attraction
activity
I = 0.3 M
lipid sorbtion
LIPIDS
K = 1·10²
K = 4·10-2
K = 1·10-5
CQ
CQ+
CQ++
CQ·1.2γ
CQ+·0.7γ
CQ++·0.3γ
OUTSIDE
I 0 M
CQ·1γ
CQ+·1γ
CQ++·1γ

Figure 7. Uptake of chloroquine (CQ). P is the permeability, K is the distribution coefficient between lipids and water, I is the activity, γ is the activity coefficient, pK`$_a$ is the corrected pK$_a$ (for ionic strength). The membrane thickness and diffusion coefficient data for calculation of P is taken from Riederer et al. (1995).

The ionic strength (I) inside plant cells is usually in the range of 0.3 to 0.5 mol/L (Trapp, 2004). This high I level affects the activity, and with an intracellular ionic strength of 0.3 M the activity of neutral compounds is increased ($\gamma = 1.23$), while the activity of CQ^+ is decreased ($\gamma = 0.74$) and the activity of CQ^{++} is decreased even further ($\gamma = 0.30$). The high ionic strength inside the cell also has an effect on the pK$_a$ of the compound, and the corrected pK$_a$ can be calculated using the Debye-Hückel limiting law (*equation 13*). With an intracellular ionic strength of 0.3 M the pK$_a$ of chloroquine changes from 6.33 and 10.47 to 6.05 and 9.91 respectively.

Inside the cell, sorption to lipids also occurs. The effect of this can be calculated using *equation 19*. The sorption coefficients (K) for the various chloroquine species are given in Figure 6, and follow the order: $K_{CQ} >> K_{CQ+} >> K_{CQ++}$, based on the assumption that the log K_{ow} loses 3.5 units for every charge CQ gains, *equation 14*. Thus we expect a much higher sorption of CQ when it is neutral. With a cytosolic pH level of 7.3 less than 1% of CQ is fully neutral, this fraction however, will accumulate in the lipids of the cell.

Due to the ionic gradients established by cellular pumps, there is an electrical charge on cellular membranes. The charge on the outer cell membrane is typically in the range of -140 mV (Blumwald et al., 2000). The positively charged CQ^+ and CQ^{++} will be attracted by this electrical membrane charge, and this interaction can increase the uptake. The Nernst ratio (*equation 17*) gives a measure of the theoretical accumulation due to the electrical potential. Assuming an outer membrane potential of -140 mV, the Nernst ratio at the outer membrane is approximately 250 for CQ^+ and 6500 for CQ^{++}. This illustrates that the electrical attraction of both the single and especially the double charge is a factor that increases the uptake. For chloroquine electrical attraction is greater at pH levels where the charged forms CQ^+ and CQ^{++} dominate.

Based on the experimental results and the modeling presented here, we can conclude that there is an effect of pH on chloroquine, and that this effect is higher when the compound is more neutral. Thus hypothesis 2 is verified: chloroquine is more toxic at higher pH levels.

However, to draw any broader conclusions about ionizing organic compounds it is not sufficient to study individual compounds. Therefore the next chapter considers acids and bases in general using a dataset compiled of many different substances.

4. The ecotoxicological effects of acids and bases at variable pH

This chapter addresses objectives III, IV and V by analyzing the dataset from literature compiled in **Paper II**. Additionally the chapter presents two case studies, namely the experimental work with antimicrobial agent sulfadiazine from **Paper V**, and some previously unpublished work with the cationic dye rhodamine 6G.

4.1 Mapping the pH-dependent behavior of acids and bases.

The predictions of the cell model showed that the uptake and toxicity of electrolytes is highest when the compound is in the neutral form. This was confirmed experimentally for the single compound chloroquine. However, in accordance with objective III it relevant to ask whether this is true for all compounds. We formulate the following hypothesis:

Hypothesis 3. *Acids and bases are always more toxic and bioconcentrating when they are in the neutral form.*

As outlined in **Paper II** the magnitude of the pH induced change of BCF and EC50 varied from compound to compound. We know that the pH dependent uptake behavior of electrolytes depends on both the pH of the test and on the pK_a of the compound (as dictated by the Henderson-Hasselbalch equation). This issue is addressed by objective IV, and leads to the following hypothesis:

Hypothesis 4. *The pK_a of the compound determines the magnitude of change in toxicity and bioconcentration with pH.*

The heterogeneous nature of the data makes direct comparison of toxicity and bioconcentration difficult due to the different pH ranges tested, and the difference in pK_a values of the individual compounds. It is therefore necessary to normalize data for both pH and pK_a. The data collected in the review was filtered to include only compounds for which BCF or EC50 was measured on both sides of the pK_a. Data was then normalized for each compound such that the BCF or EC50 value where the compound was fully neutral (or almost fully neutral) was set to 1. The remaining points were then calculated relative to this. The normalized data is plotted against pH- pK_a in Figure 8.

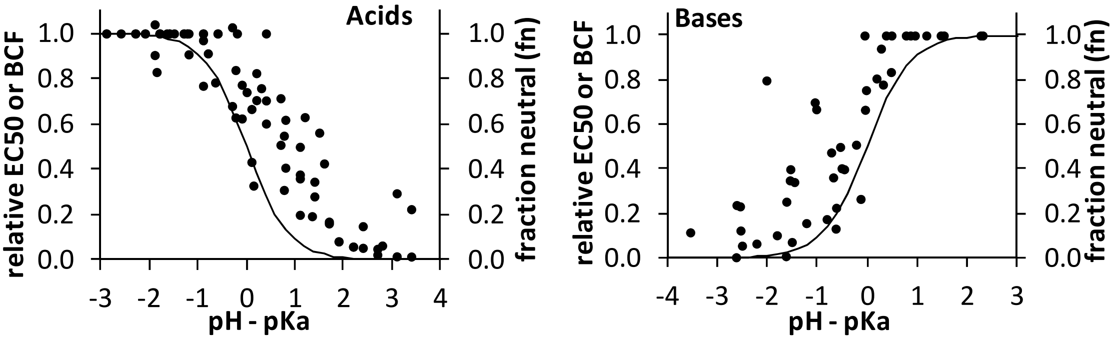


Figure 8. The normalized toxicity or bioconcentration of acids. Modified from **Paper II**. The curve illustrates the fraction of neutral compound (f_n) as given by the Henderson-Hasselbalch equation.

The relative toxicity and bioconcentration appears to follow the degree of speciation as described by the Henderson-Hasselbalch equation (Figure 8). Some of the scatter may be explained by the very heterogeneous nature of the data which is based on work from different laboratories, with different test organisms, exposure times, endpoints, and different pH stabilization methods. The discrepancy between the neutral fraction (solid line in Figure 8) and relative EC50 or BCF may also be due to the contribution of the ionized fraction. Indeed, most data points are above the fraction of neutral compound f_n (*equation 7*) supporting this assumption. The contribution of the ionized fraction of acids and bases to toxicity and bioconcentration is discussed in more detail in chapter 4.1.

An alternative explanation follows from the findings of O'Connor et al. (2012) who found a shift of pK_a when measuring the log D using the immobilized artificial membrane technique. The ionic nature of membranes causes electrostatic forces which act on the ionic fraction of the compound increasing the uptake. These electrostatic forces are not seen when measuring log D in simple octanol-water systems.

For acids it can be observed from Figure 8 that the greatest relative change with pH occurs when pH – pK_a is in the range of -1 to 3. This is the range for which dissociation changes most (f_n, solid line), and thus also the range with the maximal change in log D. In natural aquatic environments with pH in the range of 6-9, we can therefore expect a change in uptake and toxicity for acids with pK_a values in the range of 3 to 10.

Simulations with the cell model (as described in chapter 3) confirmed that there is no change in bioconcentration in the range of pH 6-9 for acids with pK_a outside in this range. The toxicity tests in **Paper IV** with the weak acid salicylanilide (tested on *Pseudokirchneriella subcapitata*) also confirm this finding. With a pK_a of 7.2 (ACD, 2008) salicylanilide is within the given range and shows higher toxicity at low pH levels with measured EC50 values (95% confidence intervals) of 1.1 mg/L (0.8-1.4), 1.7 mg/L (1.6-1.8) and 4.6 mg/L (4.0-5.4) for pH levels of 7, 8 and 9 respectively. The dissociation curve and log D for salicylanilide can be seen in figure 1: salicylanilide. Experimental procedures are described in detail in **Paper IV**.

For bases it can be observed from Figure 8 that the greatest relative change with pH occurs when pH - pK_a is in the range of -3 to 1. This is the range for which dissociation changes most (f_n, solid line), and thus also the range with the maximal change in log D. In natural aquatic environments with pH in the range of 6-9, we can thus expect a change in uptake and toxicity for bases with pK_a values in the range of 5 to 12.

Simulations with the cell model (as described in chapter 3) confirmed that there is no change in bioconcentration in the range of pH 6-9 for bases with pK_a outside in this range. The toxicity tests in **Paper IV** with the weak bases ethoxyquin and trimethoprim (tested on *Pseudokirchneriella subcapitata*) also confirmed this finding. With a pK_a of 5 (ACD, 2008) ethoxyquin is at the very lower limit for where a change in toxicity can be expected, and indeed toxicity tests at pH levels of 6, 7 and 8 showed no significant change in toxicity with pH. With a pK_a of 7.2 trimethoprim is within the given pK_a range and toxicity tests confirmed increasing toxicity with increasing pH with EC50 (95% confidence interval) of 105 mg/L (94-117), 95 mg/L (82-110) and 78 mg/L (71-84) for pH levels of 7, 8 and 9 respectively. The dissociation curve and log D for trimethoprim can be seen in figure 1: trimethoprim. Experimental procedures are described in detail in **Paper IV**.

Based on the apparent patterns in Figure 8 and on model simulations, it is possible to conclude the following:

Statement I. Acids are more toxic and bioconcentrating at low pH levels where the neutral form predominates. In waters with pH levels in range of 6 - 9, acids will show pH dependent toxicity and bioconcentration if pK_a values are in the range of 3 - 10.

Statement II. Bases are more toxic and bioconcentrating at high pH levels where the neutral form predominates. In waters with pH levels in range of 6 - 9, bases will show pH dependent toxicity and bioconcentration if pK_a values are in the range of 5 - 12.

The behavior of multivalent electrolytes is more complicated, in particular if anionic and cationic groups are both present, and there is a lack of pH variable test measurements for these compounds. However, based on the log D behavior of these compounds, and based on the observed behavior of monovalent acids and bases we expect a higher toxicity when the neutral form of the compound occurs.

If both the acidic and basic groups are within the given pK_a ranges of statements I and II, we speculate that the compound will have the greatest ecotoxicological effects at the isoelectric point (the midpoint between the pK_a value of the acids and the pK_a value of the base).

If the acidic pK_a is within the given pK_a range, but the basic pK_a is not, the we expect the compound to show pH dependent behavior as an acid and should be treated as such. If the situation is reversed, and only the basic pK_a is in the given pK_a range, then the compound is expected to show pH dependent behavior as a base.

Based on these considerations we can add a tentative statement to the list above:

Statement III. The ecotoxicological effects of zwitterions and amphoters may be sensitive to pH fluctuations in the naturally occurring pH range of 6-9 if either one or both of the below is true:

- At least one acidic pK_a is in the range of 3-10.
- At least one basic pK_a is in the range of 5-12.

The stipulation that zwitterions and amphoters are more toxic at the isoelectric point if both the acidic and basic pK_a are within the given ranges has not been experimentally confirmed. However, there are examples of cases where amphoters behave as simple acids due to very low basic pK_a values. Such an example is illustrated with the sulfonamide sulfadiazine in **Paper III**, and will be discussed later in this chapter in a brief case study.

Returning to hypothesis 3 stating that "A*cids and bases are always more toxic and bioconcentrating when they are in the neutral form*" we can conclude that this is generally the case. However, as the following will clarify, there are a number of exceptions. Concerning the hypothesis 4 stating that "*The* pK_a *of the compound determines the magnitude of change in toxicity and bioconcentration with pH*" we can conclude that this also appears to be true, and pK_a ranges were defined for acids and bases within which a change in toxicity and bioconcentration can be expected. However the following will also identify cases where these ranges are not applicable.

The statements outlined above are, as mentioned, based on both the 233 EC50 values and the 139 BCF values of the collected dataset. However, the three following exceptions to the statements were identified:

Exception I. Unexpected toxicity changes for compounds with pK_a values outside the ranges given in statements I-III above.

Exception II. Cases where the ionized fraction is more toxic through a specific mode of action.

Exception III. Compounds for which log D does not change much with ionization: cations with delocalized charge.

Each of these exceptions will be discussed briefly in the following. The discussion includes two small experimental case studies illuminating the behavior and ecotoxicological effects of the amphoteric sulfonamide antibiotic sulfadiazine and the cationic fluorescent dye rhodamine 6G.

Exception 1: Unexpected changes in toxicity with pH

The dataset revealed three examples of compounds that showed an effect of pH with pK_a values outside ranges given in statements I-III.

The first example consists of a series of methyl phenols with pK_a values between 10.07 and 10.32. These acids have pK_a values just outside the pK_a range of statement I, yet they show an average factor of change in toxicity with pH of 1.5 (Cronin et al., 2000). Considering the uncertainties generally involved in bioconcentration and toxicity tests, an average factor of change of 1.5 is not alarming, and may even be considered negligible, however other examples show a larger effect.

The most striking example was found for a series of 13 anilines tested for toxicity towards *Daphnia magna* (Cronin et al., 2000). The toxicity of these bases was seen to systematically increase with increasing pH levels despite the fact that all the anilines have pK_a values well below the pK_a range in statement II

(-4.3 to 4.61). These compounds are fully ionized at all the tested pH levels. The factor of change was admittedly low, in the range of 1.2 - 2.7, but because it was documented for each of the thirteen compounds it cannot be neglected. A possible explanation for this increased toxicity could be an intolerance of the test organisms for the pH levels.

In the last example, a series of phenoxyacetic acids with pK_a values between 2.98 and 3.13 showed an average factor of change in bioconcentration of 42 (Briggs et al., 1987; Rigitano et al., 1987). This is a large factor of change considering the pK_a values at the very limits of where an effect is expected. The explanation is probably that in each case the authors tested the bioconcentration in the range of pH 4 - 8. Statements I-III were made for the pH range of 6-9, which is the common pH range found in natural surface waters (Stumm & Morgan, 1981). This example underlines a need for extra vigilance in cases where the recipient waters have pH levels outside the norm, such as acid mine drainage sites.

Exception 2: The toxic ionized fraction - the example with sulfonamides.

Compounds with a specific mode of action involving the ionized fraction or compounds which are taken up not by simple diffusion but by active uptake of the ionized fraction can be exceptions to statements I-III. An example was found with sulfonamides. The antibiotic effects of sulfonamides are induced by the ionic form which interrupts the folic acid cycle in bacteria (Henry, 1943).

Strictly speaking, sulfonamides are amphoters, but because the pK_a of the basic group is low (1-3) this group is permanently neutral at most biologically relevant pH levels. The basic group can therefore rarely affect the pH induced change in toxicity. With acid pK_a levels in the range of 6.8 - 8.5 sulfonamides act as acids in the pH range of 6 - 9, and become increasingly ionized as pH increases.

The uptake and toxicity of these compounds was simulated by Zarfl et al. (2008), who by the help of the cell model showed that the uptake and effect of sulfonamides depends both on the intracellular and extracellular pH. The uptake of the sulfonamides is greatest at low pH where the compounds are more neutral. Once inside the cell it is the anionic species which interrupts the folic acid cycle. Thus it is the extracellular pH which determines the total uptake into the cell, but the intracellular pH which determines how much of the compound is present in the toxic anionic form (Zarfl et al., 2008). So despite the toxicity of the anionic form, these compounds are still expected to be most toxic at low external pH levels.

A series of toxicity tests on two bacterial strains by Trappe et al. (2008) showed that for one strain (*Pantoea agglomerans*) the effect of the sulfonamides was greatest at low external pH levels where the sulfonamides are present in the neutral state. Another strain (*Pseudomonas aeruginosa*) showed the opposite trend, with higher toxicity at higher pH values where the sulfonamides are present in the ionized state. The authors suspected this difference to be caused by an inability of *Pseudomonas aeruginosa* to maintain stable internal pH levels (homeostasis) which causes high concentrations of the toxic anionic form inside the cell at high pH levels, explaining the results. The majority of microorganisms (such as *Panteoa agglomerans*), however, are able to maintain homeostasis.

A true exception would be found for compounds where the ionized fraction has a mode of action which targets the outer membrane. Such compounds will be more toxic at high pH levels for acids, and low pH levels for bases. For sulfonamides, however the exception from statements I-III is limited to microorganisms which are unable to maintain homeostasis. Sulfonamides are therefore expected to behave as acids with regards to other organisms. The following case study is a brief ecotoxicological investigation of the sulfonamide antibiotic sulfadiazine based on experiments with *Daphnia magna*. The case study is based on the results presented in **Paper III**, and answers the question posed in objective I for sulfadiazine.

Case study: the amphoteric antibiotic sulfadiazine

Sulfadiazine is a common antibiotic used in animal feed and livestock production (Thiele-Bruhn, 2008). With an acid pK_a of 6.5 and a basic pK_a of 1.57 (ACD, 2008) sulfadiazine is an amphoter, but the basic group is outside the range where it affects the pH dependent behavior of the compound at usual pH ranges (6 to 9) (Figure 9). The dissociation behavior and log D of sulfadiazine can be seen in Figure 1: sulfadiazine. The pH dependent toxicity and bioconcentration of sulfadiazine has only been sparingly investigated (Trappe, 2008), and we formulate the following hypothesis:

Hypothesis 5. *The toxicity and bioconcentration of the amphoter sulfadiazine increases with decreasing pH when tested on* Daphnia magna.

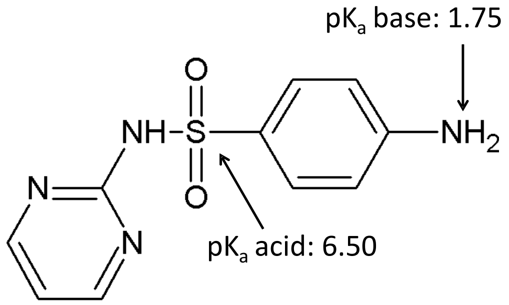


Figure 9. The structure of sulfadiazine modified from (ACD, 2008)

In **Paper V** we tested the toxicity of sulfadiazine at the three pH levels 6.0, 7.5 and 8.5 using the buffering methods described in **Paper IV**. We found EC50 values of 27, 188, and 310 mg/L, respectively in the 48h *Daphnia magna* test. This is in line with the expected with a clearly higher toxicity at pH 6 where the compound is predominantly neutral (f_n = 0.76), than at pH 8.5 where it is predominantly ionized (f_n = 0.01).

We also measured the bioconcentration at two pH levels (Figure 10), and found a higher bioconcentration at pH 6.5 (50 ±7.1 ml/g) than at pH 8.5 (36 ±4.5 ml/g). The method is described in detail in **Paper IV**.

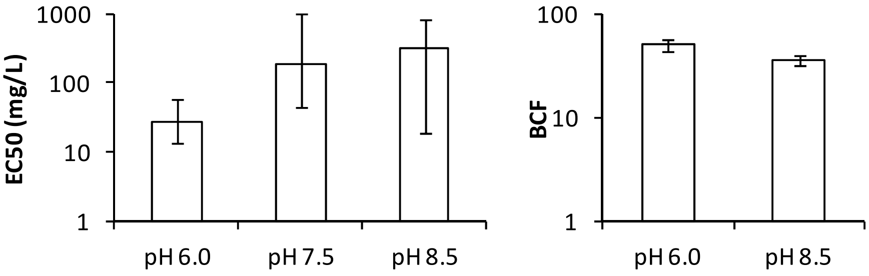


Figure 10. The measured 48 hour EC50 at pH levels of 6.0, 7.0 and 8.0 to *Daphnia magna* and the measured BCF on *Daphnia magna* at pH 8.5 and 6.0.

The experiments confirm hypothesis 5: when tested on *Daphnia magna* sulfadiazine is more toxic and more bioconcentrating at low pH levels where the neutral form predominates.

Exception 3: Cations with delocalized charges

As briefly outlined in **Paper II**, if there is no change in the lipophilicity of a compound with pH, then we expect no significant difference in the partitioning behavior of ionized and neutral fractions. Ionized and neutral fractions will cross membranes at similar speeds, and therefore the ion trap will not have any effect. This makes electrical attraction and repulsion a relevant process. The attraction of cations to the negative charges generally found on membranes will cause the ionized fraction of these bases to accumulate more than the neutral fraction. This leads to a possible exception (as previously mentioned) to statement II - that bases are taken up more efficiently in the neutral state which occurs at high pH levels.

The lipophilicities of the neutral and ionized fractions are equal or similar for compounds with delocalized charges (Berneth, 2012). A delocalized charge is a charge that is spread over a conjugated series of molecules (alternating double and single bonds) (Bertheth, 2012). Because the charge is spread over a large area of the molecule (rather than fixed to a single atom) the effect of the charge is weaker and thus the difference in polarity of the charged and neutral species is much reduced, as confirmed by (Reymond et al., 1999, cited by Duvvuri et al., 2004). Thus, cations with delocalized charges may be an exception to statement II. This is further investigated with rhodamine 6G in the following.

Case study: the cationic dye rhodamine 6G

Rhodamine 6G is an aminoxanthene dye with a pK_a of 7.5 (Duvvuri, 2004). The structure of rhodamine 6G is given in Figure 11, where the broken line indicates the conjugated system across which the charge can travel. Rhodamine 6G was chosen as a test compound due to the large size of the chromophore (22 atoms), and due to its frequent use in histology and its use as a fluorescent probe.

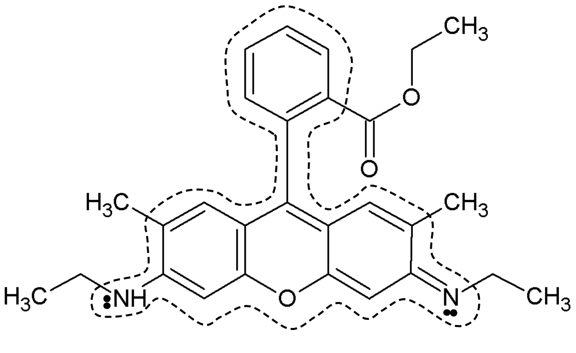


Figure 11. The structure of rhodamine 6G. The broken line indicates the area of the compound over which the charge is spread. The figure is modified from (ACD, 2008).

Rhodamine 6G is known to accumulate in cellular organelles with a high membrane charge - which is common for energy producing units such as mitochondria and chloroplasts. This distribution pattern seems to confirm the fact that electrical attraction plays a major role in the uptake and accumulation of rhodamine 6G. In line with the questions raised by objective I, this case study tests the following hypothesis:

Hypothesis 6. *Rhodamine 6G, a cation with delocalized charge, has a log D which does not change significantly with pH. Rhodamine 6G is therefore more toxic in the ionized form.*

If the hypothesis is confirmed, it will present a clear exception to statement II.

The log D of rhodamine 6G was investigated at pH levels of 4, 5, 6, 7, 8, and 9 using the shake flask method (OECD, 1995) with spectrophotometric measurements in both the aquatic and the octanol phase. For each pH level the experiment was performed using three ratios of water:ocanol (always with a total volume of 6 ml). The volume ratios were 0.5:5.5, 1.0:5.0, and 1.5:4.5. Each volume ratio was tested in triplicate, as were individual concentrations.

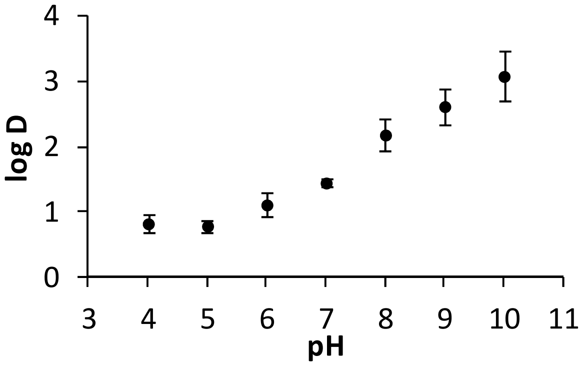


Figure 12. Measured log D of rhodamine 6G. Error bars indicate standard deviation.

The log D of rhodamine 6G changes significantly with pH (Figure 12). This result conflicts with the findings of Dvuuri et al., (2004) who determined that the partition coefficients of rhodamine 6G and rhodamine 123 do not significantly change upon ionization. The change in log D from the fully ionized state at low pH to the fully neutral state at high pH is approximately 2 units. This proves that the lipophilicity of the neutral fraction is higher than that of the ionized fraction. The difference, however, is smaller than the average 3.5 units predicted by *equation 14*. Based on these results we can thus refute the first half of hypothesis 5: rhodamine 6G is significantly more liphilic in the neutral form than in the ionized form.

The toxicity of rhodamine 6G towards *Daphnia magna* was investigated. The standard procedure of OECD was used (OECD, 1996), with the addition of buffers as recommended in **Paper III**. A total of 12 concentrations were tested at each pH level, with three replicates (each with 5 animals) at each concentration. The dose-response curve was calculated using the software ToxCalc (ToxCalc, 2001).

The dose-response curves (Figure 13) clearly show that the toxicity of rhodamine is higher at pH 8 (where the neutral form predominates), than at pH 6 (where the ionized from predominates).

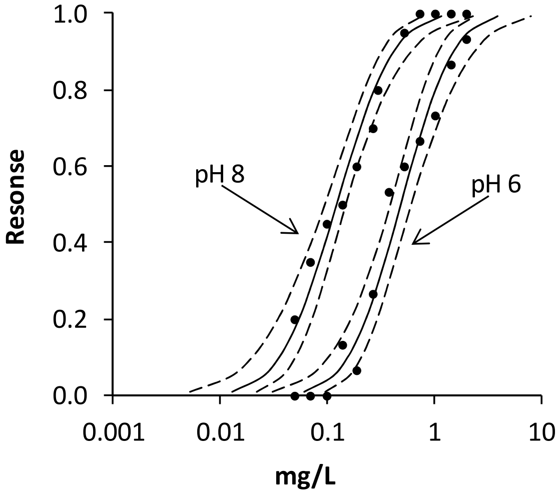


Figure 13. 48-hour dose-response curves for rhodamine 6G tested on *Daphnia magna* at pH levels of 6 and 8.The broken lines indicate the 95% confidence intervals, and the points indicate the mobility response of the daphnids.

EC50 values of 0.50 and 0.12 mg/L were obtained for pH levels of 6 and 8 respectively, indicating a change in toxicity of approximately a factor four. This factor of change is not unusual for a base with pK_a at around 7.5 (based on the dataset from the review), and thus the hypothesis that cationic dyes with delocalized charges constitute and exception to statement II could not be experimentally confirmed. The results indicate that the change in lipophilicity (albeit lower than average) still outweighs the electrical attraction of the ionized fraction. It is remains unknown whether an ion with the same lipophilicity (log D) as the neutral species has equal, higher or lower toxicity - if such a case even occurs.

4.2 Exploring the role of the ionized fraction

Having now established that it appears to be a general rule (with a number of exceptions) that acids are more toxic at low pH levels and bases are more toxic at high pH levels we come to the assumption on which much of the current risk assessment bases on: that the contribution of the ionized fraction to the bioconcentration and toxicity is negligible. The permeability of a neutral compound is typically between 1,000 and 10,000 times faster than the permeability of its ionized counterpart (Raven, 1975), and it is often assumed that the neutral species alone is able to cross the membrane (Neuwoehner &

Escher, 2010). As previously discussed many of the current approaches to dealing with the risk assessment of ionizing organic compounds rely on this assumption (European Commission, 2003; Saarikoski & Viluksela, 1982). Based on the questions raised by objective V we first examine the hypothesis:

Hypothesis 7. *The ionized fraction of an electrolyte does not significantly contribute to toxicity and bioconcentration.*

Figure 14 shows EC50 and BCF values for acids and bases plotted against pH-pK_a. The area where the compound is fully ionized is highlighted in the figure.

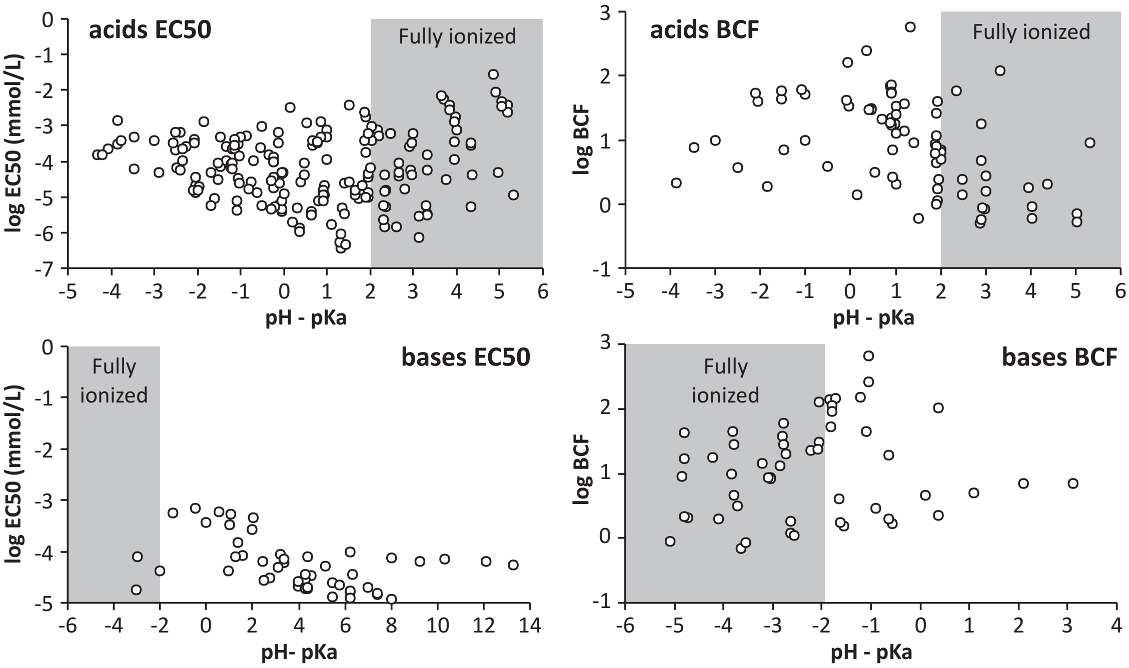


Figure 14. Bioconcentration and toxicity values for acids and bases plotted against pH- pK_a. The grey areas indicate where the compounds are fully ionized.

EC50 and BCF values have clearly been measured for both acids and bases at pH levels where the compound is completely ionized. This indicates that the ionized fraction can in itself indeed be both toxic and bioconcentrating. However, as previously described in **Paper II**, few cases are found in the data where the ionized fraction is more toxic than the neutral fraction. So despite the fact that the ionizing fraction appears to be toxic in some cases, it remains the least toxic and least bioconcentrating form of the compound in all cases in the present dataset.

Although we have now established that the ionized fraction can be bioconcentrating and can cause toxicity in itself, the quantitative contribution of the ionized fraction in a mixture situation still remains unknown.

We calculate the bioconcentration of compounds based on the assumption that only the neutral fraction contributes to the accumulation. Using the measured BCF of the fully neutral compound we predict the BCF by multiplying by the fraction of neutral compound (f_n) at various pH levels. These predicted values are compared to measured values, and we assume that whatever fraction of the uptake cannot be explained by the neutral fraction is explained by the ionized fraction.

Figure 15 shows the results of this calculation for the bioconcentration of both acids and bases from the dataset in **Paper II**.

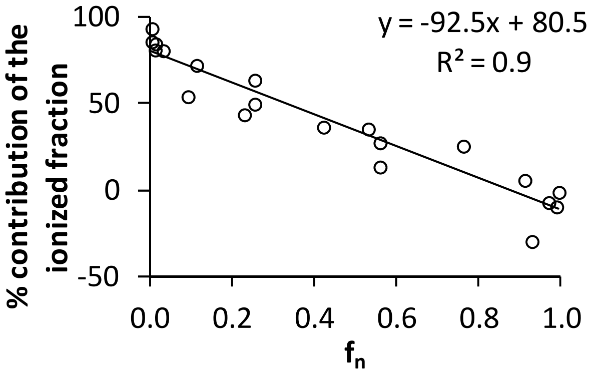


Figure 15. Contribution of the ionized fraction of the electrolyte to the total BCF as a function of f_n.

Figure 15 shows that the contribution of the ionized fraction to the bioconcentration is close to zero when the compound is fully neutral and close to 100% when the compound is almost fully ionized.

The contribution of the ionized fraction cannot be safely dismissed (Figure 15).We accept that the ionized fraction can play an important role in the bioconcentration of ionizing organic compounds. The weakness of this analysis is the small size of the dataset, and the fact that it is composed mainly of phenols and only a few basic compounds.

In much the same way as log D considers both neutral and ionized fractions of the electrolyte, we have now shown that both ionized and neutral fractions of compound contribute to the bioconcentration of ionizing organic compounds. It could therefore be reasonable to expect that the change in bioconcentration is proportional to the change in D.

We therefore examine the change in EC50 and BCF with pH in relation to the change in D with pH. The following hypothesis is formulated:

Hypothesis 8. *The factor of change in toxicity and bioconcentration with pH is equal to the factor of change in lipophilicity (D) with pH.*

Figure 16 shows the ratio between the pH induced factor of change in toxicity and BCF divided by the pH induced factor of change in D. To confirm the hypothesis we expect to see a constant ratio of 1, which would indicate an equal factor of change in toxicity and D.

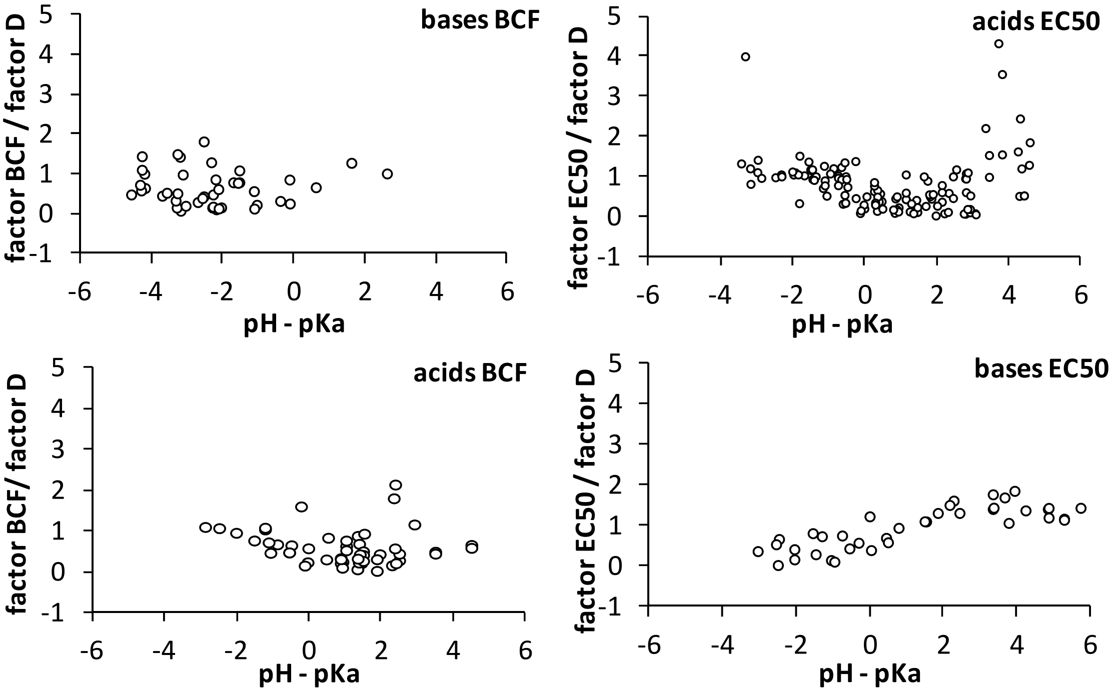


Figure 16. The ratio of factor of change in BCF or EC50 and factor D for both acids and bases.

We see that the vast majority of points are in the range of 0 - 2 indicating that the change in toxicity and BCF is indeed closely related to the change in D. We further notice that for both acids and bases the factor of change in D is greater than the change in both EC50 and BCF when the neutral form predominates. This could indicate that the ionized fraction contributes to both the toxicity and the bioconcentration, but does not contribute significantly to the apparent lipophilicity of the compound (D).

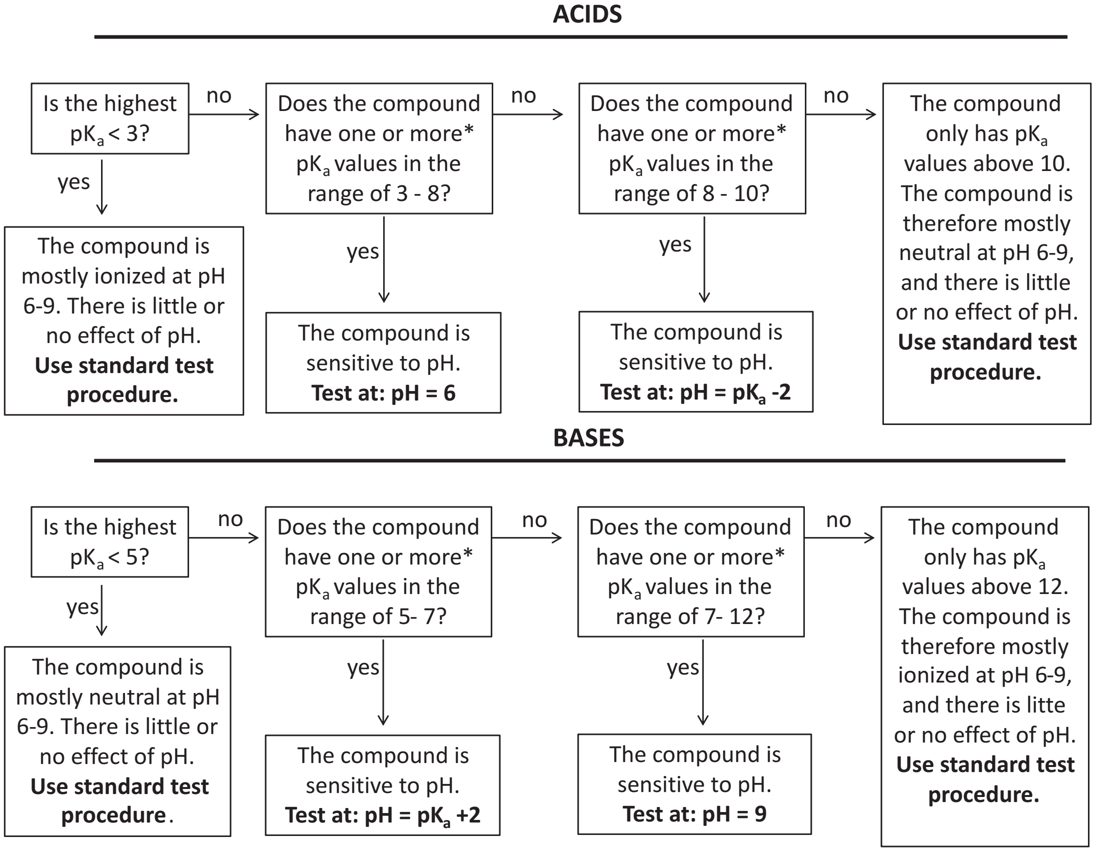
Based on the results and discussions presented in this chapter, it can thus be concluded that acids, bases, zwitterions and amphoters are most toxic at pH levels that induce the neutral form, and statements I-III describe which compounds (based on pK_a) can be considered susceptible to pH changes in BCF and toxicity. The investigations of the ionized fraction and the relation to log D point to the conclusion that the ionized fraction plays an important role for both BCF and EC50 - especially when it dominates.

5. Recommendations and practical methods

In response to objective VI, the following deals with the practical aspects of the ecotoxicological testing of ionizing organic compounds – namely the selection of appropriate pH levels for tests, and the maintenance of stable pH levels throughout the tests.

5.1 Selecting pH levels for ecotoxicological tests

Figure 17 gives criteria for selecting appropriate pH levels for ecotoxicological testing of acids and bases based on statements I and II (chapter four). The guide is thus based on both experimental results from the literature, and on the simulations of the cell model. The guide is meant as supplement to current guidelines to avoid the underestimation of the toxicity and bioconcentration of ionizing organic compounds due to the pH of the test medium. Note that if the compound has more than one pK_a in the ranges defined in the figure, it is the highest pK_a that rules for bases, and the lowest pK_a that rules for acids.



ACIDS
Is the highest pKa < 3?
no
yes
The compound is mostly ionized at pH 6-9. There is little or no effect of pH. Use standard test procedure.
Does the compound have one or more* pKa values in the range of 3 - 8?
yes
The compound is sensitive to pH. Test at: pH = 6
no
Does the compound have one or more* pKa values in the range of 8 - 10?
yes
The compound is sensitive to pH. Test at: pH = pKa -2
no
The compound only has pKa values above 10. The compound is therefore mostly neutral at pH 6-9, and there is little or no effect of pH. Use standard test procedure.
BASES
Is the highest pKa < 5?
no
yes
The compound is mostly neutral at pH 6-9. There is little or no effect of pH. Use standard test procedure.
Does the compound have one or more* pKa values in the range of 5- 7?
yes
The compound is sensitive to pH. Test at: pH = pKa +2
no
Does the compound have one or more* pKa values in the range of 7- 12?
yes
The compound is sensitive to pH. Test at: pH = 9
no
The compound only has pKa values above 12. The compound is therefore mostly ionized at pH 6-9, and there is litte or no effect of pH. Use standard test procedure.

Figure 17. Schematic guides for selecting a test pH level for acids and bases based on the pK_a of the compound. The guide can be used for both mono- and multivalent acids and bases.
* If the compound has more than one pK_a in this range the highest pK_a rules for bases, and the lowest pK_a rules for acids.

For zwitterions and amphoters with two pK_a values (one acidic and one basic) the schematic guidelines in Figure 18 can be used. It should be noted that the suggestion in Figure 18 are based on statement III which is not based on data, modeling or experimental results – but on theory. This guide should therefore be used with caution.

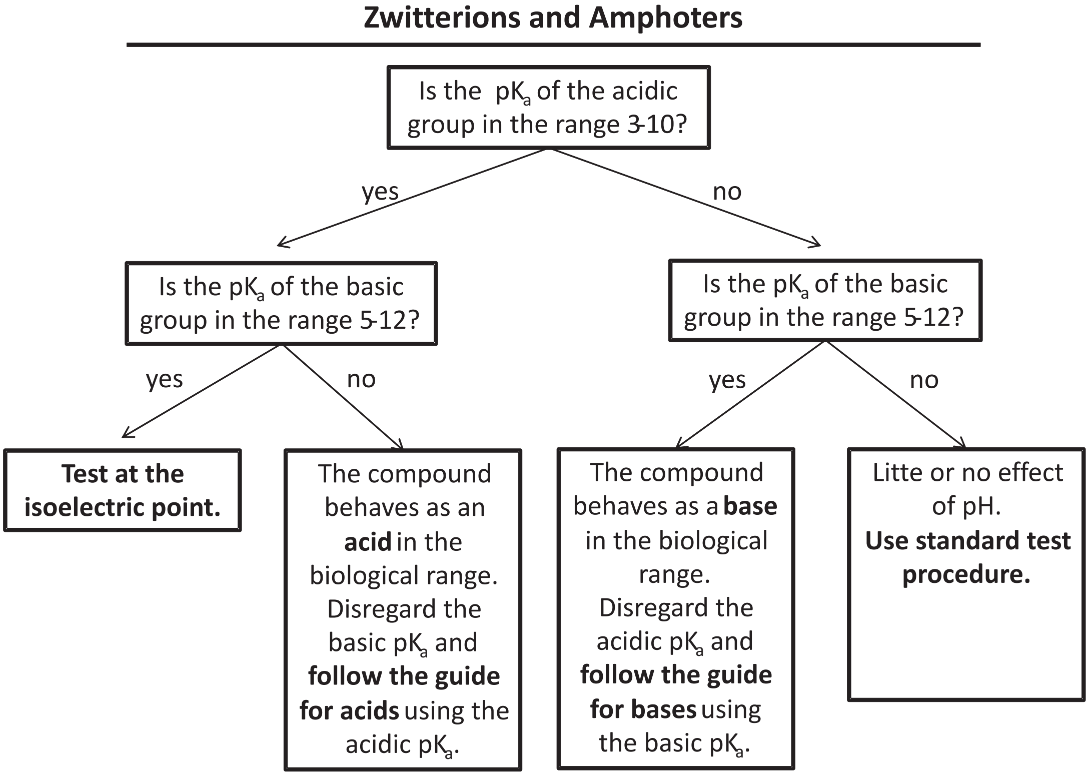

Zwitterions and Amphoters
Is the pKa of the acidic group in the range 3-10?
yes
no
Is the pKa of the basic group in the range 5-12?
Is the pKa of the basic group in the range 5-12?
yes
no
yes
no
Test at the isoelectric point.
The compound behaves as an acid in the biological range. Disregard the basic pKa and follow the guide for acids using the acidic pKa.
The compound behaves as a base in the biological range. Disregard the acidic pKa and follow the guide for bases using the basic pKa.
Litte or no effect of pH. Use standard test procedure.

Figure 18. A schematic guide to choosing an optimal pH level for ecotoxicological tests on simple zwitterions and amphoters (one acidic and one basic group) based on compound pK_a.

For zwitterions and amphoters with three or more pK_a values individual assessment may be necessary based on both speciation behavior and log D. Here the recommendation is to test at the pH level (in the range of 6-9) where log D is highest.

The systematic use of Figures 17 and 18 in relation to the ecotoxicological testing of electrolytes will help reduce some of the uncertainty in the testing of these compounds, and avoid unnecessary underestimation of the ecotoxicological effects.

The suggestions in Figures 17 and 18 are only valid when recipient waters have pH fluctuations in the within the range of 6-9. Further, the suggestions assume that test organisms can tolerate the recommended pH levels and the buffers needed to retain stable pH. Finally the suggestions may not be valid for cations with delocalized charges, nor for compounds where the ionized fraction is taken up through a specific pathway, or exerts a specific mode of action on the outer membranes.

As a further aid, the following section discusses methods for stabilizing pH in the test media and gives solutions for pH specific tests with *Daphnia magna* and *Pseudokirchneriella subcapitata*.

5.2 Meeting the methodological challenges

Maintaining a stable pH level in standard tests can be a challenge, particularly because there are currently no guidelines or standards for testing at specific pH levels (Meyer, 1998; Altenburger, 2010). This is the central challenge that **Papers III-IV** take up.

In **Paper III**, a review of the methods employed in the literature leads to a series of experiments to develop easy robust methods for testing pH at specific levels using *Daphnia magna* and *Pseudokirchneriella subcapitata*.

Taking inspiration from the work of Good et al. (1966), we define an applicable buffer as a compound that has negligible toxic or inhibitory effects on the test organism, while maintaining a stable pH. We formulate two criteria for the use of buffers in toxicity tests:

Criterion I. The applied concentration must be at least a factor of three below the measured EC10 of the compound.

Criterion II. The pH drift of the buffered media must not exceed ±0.2 pH units from the start to the end of the experiment with no pH adjustment underway.

Seven common buffering agents were tested for toxicity and pH drift using *Daphnia magna* and *Pseudokirchneriella subcapitata*. The pH of each of the tested concentrations within each toxicity test was monitored to document the pH drift. Based on these experiments it was possible to determine which compounds could live up to our criteria for an applicable buffer, and to determine applicable concentrations for toxicity tests with the two test organisms. The experimental procedure is described in **Paper III.**

With the methods developed in **Paper III**, it is possible to conduct acute immobilization tests with *Daphnia magna* in the pH range of 6.0 to 9.5 for 24 hours, and in the pH range of 6.0 to 8.0 for 48 hours. With *Pseudokirchneriella subcapitata* buffers can be used to maintain pH at levels of 7.5 and 8.0 (Table 2).

Table 2. Recommendations for buffer concentrations for toxicity tests with *Daphnia magna* and *Pseudokirchneriella subcapitata* from **Paper III**.

		D. magna **24 h**	***D. magna*** **48 h**	***P. subcapitata*** **48 h**	***P. subcapitata*** **72 h**
	pH 7.5	5.3	3.3	5.3	4.5
TRIS	pH 8.0	5.1	3.5	1.9	2.0
	pH 8.5	4.3	-	-	-
Phosphate	pH 6.0	2.1	-	-	-
MES	pH 6.0	19	9.2	-	-
MOPS	pH 7.0	21	8.9	-	-
HEPES	pH 7.8	30	12	-	-
CHES	pH 9.5	22	-	-	-

CHES: N-cyclohexyl-2-aminoethanesulfonic acid, TRIS: 2-amino-2-hydroxymethyl-propane-1,3-diol, HEPES: (N-morpholino)ethanesulfonic acid, MOPS: 3-(N-morpholino)propanesulfon acid

As discussed in **Paper III**, *Pseudokirchneriella subcapitata* cultures are sensitive to most organic buffering agents. Driven by the desire for a wider range of possible pH levels in algae tests, **Paper IV** explores the possibility of using the carbonate system as the main buffer in closed vials, based on the method of Christensen et al. (2009). Target pH levels were achieved by adjusting the concentration of $NaHCO_3$ in solution and injecting various volumes of CO_2 into the headspace of the closed system. By this method it was possible to conduct tests in the range of pH 6 to 9 with a drift within ±0.1 of the target pH (Table 3).

Table 3. Recommendations for pH stabilization of toxicity tests with *Pseudokirchneriella subcapitata* by adjusting the carbonate system in closed 20 mL vials with 4 mL test solution. Results are from **Paper IV**.

	$NaCO_2$	**CO_2**
pH 6	50 mg/L	900 μL
pH 7	150 mg/L	200 μL
pH 8	500 mg/L	100 μL
pH 9	500 mg/L	0

Similar methods as those presented here can be developed for other test organisms. The advantage of having a standardized method for testing toxicity is that effect concentrations become more comparable. As discussed in **Paper III**, a pH drift of 0.2 units may cause a percent wise change in dissociation of 11% if the pK_a of the compound is close to the pH of the water. This can have a significant effect on the toxicity or bioconcentration. Therefore minimizing the pH drift in pH specific toxicity tests with ionizing organic compounds is extremely important for accurate risk assessment of these compounds.

6. Conclusion

This study has confirmed that there is an effect of pH on the toxicity and bioconcentration of ionizing organic compounds. A significant effect of pH on the toxicity of the following compounds was documented: chloroquine (a divalent base), rhodamine 6G (a base with delocalized charge) and sulfadiazine (an amphoter). In all cases the toxicity of the compounds increased with the fraction of neutral compound: sulfadiazine was more toxic at lower pH levels, while the bases chloroquine and rhodamine 6G were more toxic at higher pH levels. Bioconcentration measurements showed the same trends for chloroquine and sulfadiazine.

The observed pH dependence is caused by the higher lipophilicity of the neutral fraction and the lower lipophilicity of the ionized fraction. BCF regressions based on both log K_{ow} and log D exist in the literature for ionizing organic compounds, and this study presents new regressions for EC50 using both log K_{ow} and log D. Although log K_{ow} was a superior predictor, it was found that the slope of individual compounds for regressions with log D and EC50 is related to pH of the exposure solution and the pK_a of the compound.

Analysis of a dataset compiled from the literature confirmed that the greatest toxicity and bioconcentration of acids and bases occurs at pH levels that favor the neutral fraction. This occurs at pH levels below the pK_a for acids and above the pK_a for bases. It was shown that acids with pK_a values in the range of 3-10, and bases with pK_a values in the range of 5-12 show pH dependent toxicity and bioconcentration, while compounds with pK_a values outside this range are not expected to be sensitive to pH. Amphoters and zwitterions are also expected to be sensitive to fluctuating pH values if one or more pK_a fall within the ranges given for acids or bases.

Three exceptions to the above were identified, including situations where the recipient waters have pH levels outside the normal range of 6-9, situations where a compound has a specific mode of action or uptake pathway that involves the ionized fraction, and situations bases have delocalized cationic charges that reduce or eliminate the difference of lipophilicity between the neutral and ionized fractions.

Because the neutral fraction is typically taken up more effectively it is often assumed that the contribution of the ionized fraction is negligible. An investigation of the ionized fraction revealed that ionized molecules can be both toxic and bioconcentrating. Further, it was illustrated that the contribution of the ionized fraction to the overall toxicity and bioconcentration is significant - particularly when the ionized fraction dominates.

Schematic guidelines for selecting optimal pH levels for bioconcentration and toxicity tests were presented. Systematic use of these will help eliminate situations where toxicity and bioconcentration of organic electrolytes is underestimated due to pH fluctuations. These guidelines call for ecotoxicological tests at specific pH levels in the range of pH 6 – 9.

To overcome the challenge of maintaining specific pH level throughout ecotoxicological tests, test methods were developed for toxicity tests with *Daphnia magna* and *Pseudokirchneriella subcapitata*. These test methods enable testing at pH level in the range of 6 to 9.5 for 24 hour tests with *Daphnia magna* and 6 to 9 for tests with *Pseudokirchneriella subcapitata*. In combination with the schematic pH selection guidelines these methods create a robust toolbox that will ensure an uncomplicated and precise test procedure to measure the toxicity of ionizing organic compounds.

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8. Papers

- **I.** Rendal C, Kusk KO, Trapp S. 2011. The effect of pH on the uptake and toxicity of the divalent weak base chloroquine tested on *Salix viminalis* and *Daphnia magna*. *Environmental Toxicology and Chemistry.* 30:354-359.

- **II.** Rendal C, Kusk KO, Trapp S. 2011. Optimal choice of pH for toxicity and bioaccumulation studies of ionizing organic chemicals. *Environmental Toxicology and Chemistry.* 30:2395–2406.

- **III.** Rendal C, Trapp S, Kusk KO. 2012. Critical evaluation and further development of methods for testing ecotoxicity at multiple pH using *Daphnia magna* and *Pseudokirchneriella subcapitata*. *Environmental Toxicology and Chemistry*. 31:1843-1852.

- **IV.** Rendal C, Wang H, Christensen IMA, Kusk KO. 2012. A method for pH specific toxicity tests with *Pseudokirchneriella subcapitata* tested on three ionizing compounds. *Environmental Toxicology and Chemistry.* **Submitted.**

- **V.** Anskjær GG, Rendal C, Kusk KO. 2012. Effect of pH on the toxicity and bioconcentration of sulfadiazine on *Daphnia magna*. *Chemosphere*. **Submitted.**

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