Development and application of new analytical methods based on chromatographic and electrophoretic separations to assess the environmental behavior of anthropogenic pollutants and their uptake in fungi and chironomids

Dissertation

der Mathematisch-Naturwissenschaftlichen Fakultät

der Eberhard Karls Universität Tübingen

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

vorgelegt von

Anna-Jorina Wicht

aus München

Tübingen

2018

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen.

Tag der mündlichen Qualifikation:	14.09.2018
Dekan:	Prof. Dr. Wolfgang Rosenstiel
1. Berichterstatter:	Prof. Dr. Carolin Huhn
2. Berichterstatter:	Prof. Dr. Heinz-R. Köhler

TABLE OF CONTENT

Abstract 6
Zusammenfassung 7
1 Introduction 9
1.1 Analysis of environmental contaminants in biota 9
1.2 Ecotoxicological effects 10
1.2.1 Correlation of internal concentration and exposure concentrations 10
1.2.2 Effects of nanoparticles on pesticide bioavailability and transformation 11
1.3 Application of electromigrative techniques for concentration of ionic and ionizable contaminants 12
2 QuEChERS Extraction in ecotoxicology: Analytical procedure and applications to biota - a review 14
2.1 Abstract 14
2.2 Introduction 14
2.2.1 Ecotoxicological interest 15
2.2.2 Classical methods 15
2.2.3 QuEChERS extraction procedure 15
2.3 Analytical considerations for sample preparation 16
2.3.1 Homogenization techniques and sample amounts 18
2.3.2 Comparison of extraction procedures 19
2.3.3 Extraction solvents 20
2.3.4 Removal of critical matrix components: lipids and waxes 20
2.3.5 Dispersive solid phase extraction materials 21
2.3.6 Acetate and citrate buffered extractions 22
2.3.7 Analytical methods compatible with QuEChERS extracts 23
2.4 Application 23
2.4.1 Environmental monitoring 23
2.4.2 Analysis of transformation products 25
2.4.3 Quantification strategies 26
2.4.4 Recoveries and limit of detection 28
2.5 Disadvantages and limitations 30
2.6 Conclusion and future perspectives 30
3 Determination of carbamazepine transfer from exposed midge larvae to adult midges by QuEChERS extraction and HPLC-MS/MS analysis 32
3.1 Abstract 32
3.2 Introduction 32
3.2.1 Chironomids and contaminant transfer 32

3.2.2 The pharmaceutical carbamazepine 33
3.2.3 Effects of carbamazepine in chironomids 33
3.2.4 Quantification of carbamazepine in biota 34
3.3 Experimental 34
3.3.1 Reagents, chemicals and consumables 34
3.3.2 Biological exposure tests 34
3.3.3 Analysis of exposure medium samples 35
3.3.4 Extraction procedure of midges and larvae 35
3.3.5 Instrumental methods 35
3.3.6 Data analysis 36
3.4 Results and Discussion 36
3.4.1 Method development 36
3.4.2 Optimization of QuEChERS extraction procedure 38
3.4.3 Method validation 39
3.5 Application 41
3.5.1 Analysis of exposure medium 41
3.5.2 Analysis of carbamazepine transfer from larvae to midges during exposure studies 41
3.5.3 Bioconcentration factor 44
3.6 Conclusion 44
4 Quantification of thiacloprid in chironomids exposed to the neonicotinoid in the presence of nanoparticles with a miniaturized QuEChERS procedure by HPLC-MS 45
4.1 Abstract 45
4.2 Introduction 45
4.2.1 The pesticide thiacloprid 45
4.2.2 Relevance of nanoparticles in the environment 46
4.2.3 Effects of nanoparticles 46
4.2.4 Ecotoxicological impact of micropollutants bound to nanoparticles 47
4.2.5 Ecotoxicological studies with chironomids 48
4.2.6 Analytical aspects of thiacloprid analysis 48
4.3 Experimental 50
4.3.1 Reagents, chemicals and consumables 50
4.3.2 Nanoparticles and sorption isotherms 50
4.3.3 Biological exposure tests 50
4.3.4 Instrumental methods 51
4.3.5 Data analysis 51
4.3.6 Sample preparation 51
4.4 Results and discussion 52

4.4.1 Method development 52
4.4.2 Method validation 56
4.5 Application 58
4.5.1 Analysis of exposure medium samples 58
4.5.2 Toxicity test samples 59
4.5.3 Bioconcentration factor for ecotoxicological assessment 63
4.6 Conclusion 64
5 Determination of propiconazole and metabolites in mycelium from different fungi species from exposure studies in the presence of nanoparticles by HPLC-MS 65
5.1 Abstract 65
5.2 Introduction 65
5.2.1 Fungi species in ecosystems 65
5.2.2 Activity of the fungicide propiconazole 66
5.2.3 Analysis of pesticides in fungi mycelium 67
5.2.4 Nanoparticles 67
5.3 Experimental 67
5.3.1 Reagents, chemicals and consumables 67
5.3.2 Agar plate experiment with nanoparticles 68
5.3.3 Liquid culture experiments with nanoparticles 68
5.3.4 Quantification of propiconazole in fungi mycelium grown on agar plates 68
5.3.5 Quantification of propiconazole in fungi mycelium grown in liquid culture 69
5.3.6 Quantification of propiconazole in agar plates 69
5.3.7 Quantification of propiconazole and transformation products in liquid culture medium 70
5.3.8 Instrumental methods 70
5.3.9 Photochemical transformation 70
5.3.10 Ergosterol derivatization 71
5.3.11 Desorption of propiconazole from nanoparticles 71
5.3.12 Analysis of photo-transformation products 71
5.3.13 Statistics 71
5.4 Results and discussion 71
5.4.1 Method development 72
5.4.2 Identification of propiconazole transformation products 79
5.4.3 Evaluation of analytical procedures with regard to literature methods 84
5.5 Application 85
5.5.1 Sorption of propiconazole to nanoparticles 85
5.5.2 Analysis of exposure medium 85

5.5.3 Uptake and metabolism of propiconazole in *L. bicolor* grown on agar plates in the presence of nanoparticles 87
5.5.4 Liquid culture experiments 90
5.5.5 Mass balance 100
5.5.6 Bioconcentration factor 102
5.6 Conclusion 102
6 Analysis of wastewater and surface water samples fractionated by different Free Flow Electrophoretic techniques 104
6.1 Abstract 104
6.2 Introduction 104
6.2.1 Wastewater analysis 104
6.2.2 Electromigrative separation techniques 106
6.2.3 Project 108
6.3 Experimental 109
6.3.1 FFE-Experiments 109
6.3.2 Data analysis 110
6.4 Results and discussion 110
6.4.1 Development of target analysis method by HPLC-ESI-QTOF-MS 110
6.4.2 Application 112
6.5 Conclusion and outlook 125
7 Investigation of the influence of electrode geometry, temperature and convection on the transfer of ionic analytes by electromigrative enrichment processes 126
7.1 Abstract 126
7.2 Introduction 126
7.2.1 Preconcentration 126
7.2.2 Experimental setup 127
7.2.3 Experimental design 128
7.2.4 Electrochemical cell 130
7.3 Experimental 131
7.3.1 Reagents, chemicals and consumables 131
7.3.2 Enrichment Experiments 132
7.3.3 Electrochemical transformation by EC 132
7.3.4 Instrumental methods 132
7.3.5 Data analysis 133
7.4 Results and Discussion 133
7.4.1 Influence of pH 133
7.4.2 Assessment of experimental time and optimization strategy 134
7.4.3 Influence of electrode geometry, convection and temperature 135

7.4.4 Experiment with straight electrode and elevated temperature 138
7.4.5 Transformation experiments 139
7.4.6 Electrochemical transformation in literature 139
7.5 Conclusion 141
8 Discussion and conclusion 143
8.1 Analytical developments for pollutant analysis in biota 143
8.1.1 Method validation parameter of the HPLC-MS quantification methods 144
8.1.2 Quantification of pesticides and pharmaceuticals in different matrices 144
8.2 Ecotoxicological effects 147
8.2.1 Correlation of internal concentration and exposure concentration 147
8.2.2 Metabolism of pesticides 149
8.2.3 Effects of nanoparticles 149
8.2.4 Transfer of incorporated contaminants from aquatic to the terrestrial environment 152
8.3 Preconcentration and fractionation of ionic and ionizable analytes by electromigrative techniques 153
8.3.1 Water analysis and concentration 153
8.3.2 Water analysis 154
8.3.3 Investigation of effects on the electromigrative behavior of charged analytes 154
8.3.4 Electromigrative fractionation and preconcentration techniques 155
8.4 Conclusion 156
References 157
Supervision and project partners 179
Own contributions in the chapters: 181
Abbreviations 183
Acknowledgement 185
List of publications and scientific contributions 186

ABSTRACT

Anthropogenic contaminants are ubiquitous in the environment. These environmental pollutants enter the environment via various entry path for example wastewater treatment plants or pesticide application in agriculture. Released into the environment some are detected in surface waters. The environmental fate of contaminants depends on their physicochemical properties and on the local conditions. Contaminants may be transformed by biotic or abiotic processes like photo-transformation or hydrolysis. Aquatic organisms are exposed to contaminants which are present in water. To analyze trace amounts of contaminants in surface waters preconcentration and cleanup techniques are required to enable their detection with sensitive methods like mass spectrometry. Especially, the analysis of ionic and ionizable compounds is challenging and these compounds come more into focus as many metabolites are more polar than the parent compounds. In this thesis, electrophoretic techniques were investigated for their ability to fractionate and preconcentrate wastewater chemicals. Two different free flow modes were applied to surface water samples and an HPLC-MS method was developed for identification and quantification of 92 chemicals relevant in wastewater in the low ng/L range. In a second approach the impact of experimental conditions on the preconcentration of ionic analytes by electrophoretic techniques was investigated.

Aquatic organism are exposed to chemicals present in surface waters and in case of benthic living organisms additionally to sediment bound contaminants. Some of these chemicals are taken up by the organisms and may pose adverse effects. Incorporated chemicals might be metabolized, accumulated or excreted by the organisms. Bioaccumulation leads to higher concentrations in the organisms than in the surrounding water. Therefore, the analysis of xenobiotics in organisms is of special interest to detect compounds of concern as well as to more precisely predict the environmental behavior of compounds. In this thesis, extraction and quantification of the contaminants and their transformation products were achieved based on QuEChERS extraction and following analysis by HPLC-MS. The method was applied to different organisms from exposure experiments. For the pharmaceutical carbamazepine a quantification method was developed to analyze the internal concentration in *Chironomus riparius* larvae and adult midges in the low ng/g range. The method was applied to assess the transfer of the pharmaceutical from aquatic to terrestrial stages and thus the transfer to the terrestrial ecosystems. Analysis of larvae and midges from emergence studies indicated a transfer of 100 % carbamazepine body burden from larvae to midges. The developed method for analysis of the neonicotinoid thiacloprid was needed only 5 *Chironomus riparius* larvae and enabled quantification with a limit of detection of 12 ng/g wet weight. A third procedure was developed to analyze the fungicide propiconazole and three of its metabolites in fungi mycelium in the low ng/g range. To assess the impact of sorption to nanoparticles on the environmental fate of the chemicals, thiacloprid and propiconazole were quantified in larvae and fungi which were exposed to the pesticide in the presence of nanoparticles, which were demonstrated to sorb the pesticide. Analysis of thiacloprid residues larvae revealed equal concentrations in experiments independent from nanoparticles. Reduced transformation of propiconazole in fungi experiments was detected depending on the nanoparticle concentration in liquid culture experiments and reduced propiconazole uptake in experiments on agar plates. The analysis of different fungi species revealed strong inter species differences in and uptake and metabolism of the fungicide.

ZUSAMMENFASSUNG

Anthropogene Verbindungen sind omnipräsent in der Umwelt. Pestizide werden in der konventionellen Landwirtschaft im Tonnenmaßstab eingesetzt und gelangen während der Anwendung oder durch Starkregenereignisse in Oberflächengewässer. Haushaltschemikalien und Arzneimittel gelangen durch einen häufig unvollständigen Abbau in Kläranlagen über die Kläranlagenabflüsse in die Umwelt. Das Umweltverhalten der Stoffe wird durch ihre physikochemischen Eigenschaften und die lokalen Bedingungen denen sie ausgesetzt sind bestimmt. Stoffe sind in Oberflächengewässer biotischen und abiotischen Transformationsprozessen unterworfen oder adsorbieren an Partikel wie zum Beispiel Sediment- oder Schwebstoffpartikel. Um diese Stoffe, die häufig im Spurenbereich vorliegen, nachweisen zu können sind Anreicherungsprozesse vor der Analyse notwendig. Im Rahmen dieser Arbeit wurde erstmalig die Anwendungsmöglichkeit der Freiflusselektrophorese zur Fraktionierung organischer Spurenstoffe aus Oberflächengewässerproben untersucht. In einem anderen Ansatz wurde der Einfluss verschiedener Experimentaufbauten auf den Transfer und damit die Möglichkeit zur Aufkonzentrierung ionischer und ionisierbarer Verbindungen im elektrischen Feld untersucht. Die Anreicherung und Trennung ionischer Verbindung rückt in den Fokus der Umweltanalytik, da viele Metabolite polarer sind als ihre Ausgangsverbindungen.

Aquatische Organismen sind wassergelösten Stoffen ausgesetzt, benthisch lebende Organismen zusätzlich Sediment gebundenen Stoffen. Auch in sehr geringen Konzentrationen können Umweltchemikalien bereits negative Effekte in Organismen hervorrufen. Aufgenommene Stoffe können metabolisiert oder ausgeschieden werden oder sich im Gewebe anreichern. Eine Möglichkeit, bioakkumulierende Stoffe zu identifizieren, ist die Analyse von Biota aus Umweltproben oder Laborstudien. Im Rahmen dieser Arbeit wurden Extraktionsmethoden basieren auf Modifikationen der QuEChERS Extraktion entwickelt um organische Spurenstoffe und ihre Metabolite in verschiedenen Organismen mittels HPLC-MS zu untersuchen. Es wurde eine Nachweismethode entwickelt, die die Analyse des Arzneimittels Carbamazepin in Mückenlarven und Mücken im ng/g Bereich ermöglicht. Mittels dieser Methode wurde die interne Carbamazepin Konzentration in Larven und den aus den exponierten Larven geschlüpften Mücken untersucht. Die Ergebnisse zeigen einen vollständigen Transfer der Belastung vom Larven- in das Mückenstadium und damit zugleich einen Transfer von Schadstoffen aus der aquatischen in die terrestrische Umwelt. Mit einer weiteren Quantifizierungsmethode war die Analyse der internen Thiacloprid-Belastung bei einer Probengröße von nur 5 Mückenlarven mit einer Nachweisgrenze von 12 ng/g möglich. Um den Einfluss der Sorption von Pestiziden an Nanopartikel auf Aufnahme und Metabolismus zu untersuchen, wurden mit der entwickelten Methode zudem Larven aus Mortalitätsstudien bei Exposition mit dem Neonikotinoid Thiacloprid und Nanopartikeln analysiert. In der chemischen Analyse wurden vergleichbare Thiacloprid-Konzentrationen unabhängig von der Nanopartikel Konzentration gemessen. In ähnlicher Weise wurden Pilzmyzel-Proben aus Expositionsstudien mit dem Fungizid Propiconazol und Nanopartikeln untersucht. Die Methode ermöglichte die gleichzeitige Extraktion des Fungizides Propiconazol und drei seiner Metabolite. Die Analyse der Myzel-Proben aus Wachstumshemmstudien zeigte eine deutliche Spezies Abhängigkeit der Aufnahme und Metabolisierung des Fungizids in Flüssigkultur. Bei durch Sorption an Nanopartikel reduzierten Expositionskonzentrationen wurde eine Änderung der Metabolitkonzentration und -zusammensetzung in Flüssigkultur-Medium und Pilzmyzelextrakten nachgewiesen, sowie eine reduzierte Aufnahme des Fungizides in Experimenten mit Nanopartikeln auf Agarplatten.

1 INTRODUCTION

We use a growing number of chemicals in our day to day life, such as household chemicals, pharmaceuticals or pesticides in agriculture. The environmental fate and (eco)toxicological relevance of these chemicals is an issue of growing concern. Partially, these chemicals enter surface water directly for example via spray drift during pesticide application or they are discharged from wastewater treatment plant effluents. Many pharmaceuticals or chemicals used in personal care products are detected in treated wastewater, if they are not entirely removed.

Once they have entered surface waters the environmental fate and behavior of these chemicals depends on their physicochemical properties. Compounds might sorb to sediments or be transformed by abiotic processes like photo transformation and hydrolysis or by biotic processes. Aquatic organisms are exposed to chemicals present in the water directly via the surrounding water and sediments and possibly indirectly via ingestion of particles carrying sorbed chemicals. Incorporated compounds can be excreted, metabolized, accumulated and may negatively affect these organisms. To assess the risk of contaminants knowing their environmental fate is essential. Therefore, sensitive analytical methods are required to detect and quantify environmental contaminants and metabolites at low concentrations in surface waters and in biota.

On the one hand the bioavailability of chemicals can be reduced upon sorption of chemicals to sediments or particulate matter in surface waters e.g. by having sediments acting as a sink. On the other, hand sediments may act as a source by desorption of chemicals. Therefore, different effects have to be accounted for in assessment of the effects of the presence of particles: a) strong sorption could lower the bioavailability resulting in reduced toxic effects b) altered exposure conditions by particle bound transport into organisms may result in enhanced or reduced toxic effects, c) reduced transformation rates due to strong sorption may lead to a long-term exposure when desorption is slow. To evaluate these effects at environmentally realistic conditions, exposure experiments using the combination of particles and chemicals have to be conducted. The first part this thesis focuses on the development and application of methods for pollutant extraction from biota and quantification of contaminant residues. The developed extraction and quantification methods were applied to fungi, midges and midge larvae samples from laboratory exposure studies to combine knowledge on biological effects with internal concentrations as well as effects of nanoparticles on the bioavailability and transformation of pollutants. In the second part of this thesis, the development of separation and preconcentration techniques for ionic or ionizable analytes was investigated. These techniques are intended to especially concentrate and analyze ionic compounds, a group of contaminants increasingly gaining more attention. For a better understanding of their environmental relevance, new analytical techniques are required for this group of analytes.

1.1 ANALYSIS OF ENVIRONMENTAL CONTAMINANTS IN BIOTA

Organisms in the aquatic environment are exposed to micropollutants present in the water for their whole life and incorporate chemicals to a certain extent. Accordingly, organisms can be used as bioindicators. Although chemicals might occur at concentrations below the limit of detection in water, they potentially accumulate in organisms and may be detectable. Therefore, analysis of biota might help to identify compounds of concern and predict their environmental fate. Challenges in environmental biota analysis are limited sample amounts

available and large numbers of samples to be extracted. To meet these challenges fast, straightforward and cheap extraction techniques are required, which cover a broad range of physicochemical properties of analytes. Especially, the extraction of metabolites, which are often more polar than the parent compound [1] together with the development of multi-component methods comprising a broad range of physicochemical properties of analytes are challenging. The analysis of trace concentrations of analytes incorporated in biota is challenging even with sensitive methods due to high matrix load. Analysis by liquid chromatography coupled to mass spectrometry allows to separate and detect various analytes comprising a broad range of physicochemical properties in the ng/g range, however, sample preparation steps are still required to reduce matrix effects.

An extraction method which caused increasing attention in the last years is QuEChERS (Quick Easy Cheap Rugged and Safe) extraction procedure [2]. Originally invented for food analysis this procedure has spread to environmental analysis recently. The extraction method is based on a liquid-liquid extraction between water and acetonitrile phases, often followed by a dispersive solid phase extraction (dSPE) cleanup step. In the first chapter of this thesis, latest developments concerning applications of the QuEChERS extraction technique for pollutant analysis in biota were reviewed (Chapter 2). This first includes a summary on analytical aspects such as modifications of the original extraction procedure for contaminant analysis in biota are addressed and compared regarding the requirements of analytes (e.g. acid or base sensitive compounds and acidic analytes) and the removal of matrix components (e.g. extraction of organisms rich in lipid. In addition, examples of applications for the developed methods in environmental analysis are summarized.

Based on the QuEChERS extraction protocol and LC-MS analysis, quantification methods were developed for extraction of thiacloprid from midge larvae (Chapter 4), propiconazole and its metabolites from fungi mycelium (Chapter 5) and carbamazepine from midges and midge larvae (Chapter 3). For quantification of trace amounts of these contaminants incorporated by the organisms, different homogenization techniques and cleanup procedures were required and optimized depending on the matrix and the physicochemical properties of the analytes.

1.2 ECOTOXICOLOGICAL EFFECTS

1.2.1 Correlation of internal concentration and exposure concentrations

Aquatic organisms are exposed to various contaminants, e.g. pharmaceuticals originating from wastewater treatment plants or pesticides. These contaminants are often present at low aqueous concentrations where no biological effects may be expected. However, these contaminants might still be incorporated and accumulate in aquatic organisms. Incorporated concentrations of compounds may not always be directly linked to exposure concentrations, especially in case of bioaccumulating contaminants. Many organisms have aquatic larval stages and terrestrial living adult organisms, e.g. midges. Therefore, the question of transfer of these contaminants from larval to adult stages and thus from aquatic to terrestrial systems arises. Both are an important source of prey for insectivorous predators in the aquatic and terrestrial environment [3] and uptake of contaminants via prey is a relevant exposure route for predators [4]. Thus, they might act as a vector for bioaccumulating contaminants to terrestrial ecosystems. This transfer route has been observed for PCBs [5] and metals [6]. To address the question of midge larvae as possible vectors for pharmaceutical contaminations, the internal carbamazepine concentration in midge larvae and midges hedged from these larvae, which were exposed for 28 days, were determined (Chapter 3).

The pharmaceutical carbamazepine is used as an anticonvulsant. Due to its low degradation rate in wastewater treatment plants by less than 10 % it is used as a marker for wastewater [7]. Biological effects of carbamazepine on midge larvae at environmentally relevant concentrations were observed [8-9]. The method development of a modified QuEChERS extraction procedure and results of the analysis are presented in Section 5 in this thesis. To the best of my knowledge, this is the first time that the transfer of a pharmaceutical from larvae to midges was investigated.

In some cases internal concentrations are linked to biological effects more closely than the exposure concentration [10-11]. To investigate if the biological endpoint mortality of the non-target organism *Chironomus riparius* correlates with the internal concentration of the neonicotinoid thiacloprid larvae originating from exposure experiments were analyzed. The results and method development are shown in Section 2. Thiacloprid is an insecticide belonging to the group of neonicotinoids, which are applied due to their activity against insects and low toxicity for mammals [12]. Still, the EFSA advised to restrict the application of three compounds of this compound group, namely imidacloprid, thiametoxam and clothianidin in the EU, due to evidence of bee death caused by these compounds. For determination of the internal concentration in larvae, a modified QuEChERS extraction procedure was developed to analyze thiacloprid in 20 mg larvae, which were exposed in a 96 h mortality study.

In the environment not only acute effects are critical but also more sensitive long term effects on growth, reproductive and metabolic activity of organisms have to be considered. Fungi play an essential role in carbon and nitrogen cycles in soil ecosystems by degradation or modification of lignin and lignocellulose in forest soils [13]. Some fungi are able to degrade certain organic pollutants next to natural molecules [14]. They can then be used for soil remediation [14]. To cover uptake and possible transformation processes, a quantification method for propiconazole and three of its metabolites in mycelium was developed and applied to mycelium samples from growth inhibition experiments. The fungicide propiconazole was expected to reduce the growth rate of fungi mycelium by inhibition of natural lanosterol synthesis [15-16]. Therefore, a method to determine pesticide concentrations in agar plates, used as growth medium, was developed (Chapter 5). Comparison of internal concentrations in fungi grown on agar plates and in liquid culture enables to assess the impact of growth conditions on the uptake and transformation rate.

1.2.2 Effects of nanoparticles on pesticide bioavailability and transformation

Pesticides present in surface waters may interact with naturally born nanoparticles. Bioavailability of sediment bound contaminants is an issue of high importance with regard to long-term exposure or formation of contaminant sinks. Especially lipophilic contaminants tend to sorb to sediments, so that different exposure routes have to be taken into account to assess their ecotoxicological relevance. Benthic living organisms are exposed to contaminants via the water phase and the sediment [17]. Especially filter feeding organisms as midge larvae are exposed to sediment-bound particles via ingestion of these particles with their food and their pertubating activity [4, 17-18]. This might lead to altered physicochemical conditions in the gut of these organism which might change sorption/desorption equilibria. Nanoparticles are a special case of particles. Their large surface displaying a large reactive area [19] leads to higher sorption capacities of nanoparticles compared to larger particles. Nanoparticles may serve as sources or sinks of sorbed organic compounds including pollutants. Different effects of sorption to particles on the bioavailability were observed (1) enhanced bioavailability by carrier effects [20], (2) reduced bioavailability by strong sorption [17] and (3) reduced transformation rates.

To assess the effect of nanoparticles on the bioavailability of the neonicotinoid thiacloprid to midge larvae, the internal concentration in larvae exposed to the combination of both was determined. The analyzed larvae originated from exposure experiments with different kinds of nanoparticles (aluminum oxide and zeolite nanoparticles) and different thiacloprid concentrations. Approximately 5 larvae were available for extraction and analysis of thiacloprid residues with HPLC-QTOF-MS. Analysis of pollutants in midge larvae is challenging due to the small sample amounts available and only one paper, to my knowledge, analyzed contaminants in small numbers of midge larvae by nano-HPLC-MS/MS [21]. In this thesis, analysis was performed with HPLC-QTOF-MS in only 20 mg larvae homogenate (ca. 5 organisms).

Sorption to nanoparticles could alter the ability of organism to metabolize xenobiotics due to reduced bioavailability of the contaminants. A weak long-term exposure may result. This long-term release from sorbed compounds was observed in case of metal ions [22]. To assess effects of nanoparticles on the bioavailability and metabolic degradation of the pesticide propiconazole the internal concentration of propiconazole and three of its metabolites in fungi mycelium was analyzed with an optimized extraction procedure. Mycelium from three fungi species *Amanita muscaria*, *Cenococcum geophilum* and *Laccaria bicolor* was analyzed with a modified QuEChERS extraction procedure (Chapter 5). The fungi samples originated from growth inhibition experiments with the fungicide propiconazole and York-shell nanoparticles under different growing conditions.

1.3 APPLICATION OF ELECTROMIGRATIVE TECHNIQUES FOR CONCENTRATION OF IONIC AND IONIZABLE CONTAMINANTS

The second part of the thesis, focuses on the investigation of electromigrative techniques for preconcentration and separation of ionic or ionizable compounds in water analysis. Low concentrations of chemicals present in surface waters make their analysis challenging, even for analysis with sensitive detection methods like mass spectrometry [23]. Therefore, enrichment techniques are required to concentrate analytes and remove possibly impairing matrix components from environmental samples prior to separation and analysis. A common concentration technique for water samples is solid phase extraction combined with liquid chromatography for separation. However, especially ionic analytes are difficult to separate by liquid chromatography [24]. An alternative approach for fractionation of ionic analytes by electromigrative techniques is presented in Chapter 6 of this thesis: Two different modes of free flow electrophoresis (free flow isotachophoresis and free flow interval zone electrophoresis) were applied to wastewater and surface water samples. This method, commonly applied for biomolecule separation, separates ionic or ionizable compounds in an electric field based on the differences in their electrophoretic mobility [25]. Continuous fractionation of water samples would enable to simultaneously concentrate analytes in smaller volumes and obtain fractions and, thus, reduce sample complexity by separation of interfering analytes. Therefore, fractionation would lead to higher quantitative precision. To assess the applicability of these electromigrative techniques for fractionation of micropollutants in wastewater samples, fractionated and raw water samples were analyzed with a multi-component HPLC-QTOF-MS method. The method was developed to identify 92 chemicals relevant in water samples due to input from wastewater treatment plants.

In a second approach, a methodology based on long term electrokinetic sample injection was applied to preconcentrate ionic analytes. Electrokinetic sample injection is used for injection of ionic or ionizable compounds into capillaries. The transfer of solely charged

analytes at a specific pH value from a sampling to an analysis vessel would simultaneously remove non-charged compounds and those being oppositely charged, which remain in the sampling vessel, and preconcentrate the analytes of interest if different volumes are applied in inlet and outlet vials. An advantage of this electromigrative methodology is that only electrolytes are required as buffers and thus, by avoidance of organic solvents, this method is environmentally friendly. For electrokinetic sample injection, the migration behavior of analytes depends on the effective electric field strength which is determined by electrode geometry [26]. It can be optimized to ideally cover the sample volume in the inlet vial completely. To investigate the influence of pH, temperature, electrode geometry and convection on the electromigration behavior, a mixture of 19 analytes was tested in different experimental setups to improve analyte transfer in a proof-of-concept study, presented in Chapter 7.

2 QuEChERS Extraction in ecotoxicology: Analytical procedure and applications to biota - a review

2.1 Abstract

Emerging pollutants are a topic of growing concern. To evaluate associated risks of chemicals in the environment the knowledge about the chemicals' uptake in biota is crucial to predict effects and their environmental behavior. Classical extraction procedures such as solid phase extraction, mostly developed for target analysis, have several drawbacks such as costs and limited analyte coverage. A multi-residue extraction procedure originally developed for pesticide analysis in food is the QuEChERS extraction (Quick, Easy, Cheap, Effective, Rugged and Safe). Its low solvent consumption, the fast and easy sample preparation, the good matrix removal and the broad polarity spectrum of extractable analytes make it interesting for environmental analysis. In recent years it has been applied with different objectives in environmental analysis. For its use in ecotoxicology, several methodological adaptions such as miniaturization and extraction conditions were necessary and will be described in this review. However, most of the 33 publications included in this review concerning QuEChERS in environmentally relevant biota samples (including edible fish and mussels), focus on applications. These include investigating contaminant burden in biota from environmental samples to use organisms as bioindicators and assess the pollution of aquatic and terrestrial environments. In laboratory experiments, it was used to investigate the metabolism of pharmaceuticals and pesticides in organisms. In most publications, pesticides are investigated, but the focus currently widens to analysis of pharmaceuticals, wastewater chemicals and their metabolites. This review focuses on publications concerning examples of QuEChERS extraction for analysis of the body burden of chemicals in biota and metabolite studies especially method developments and adjustments to different organisms. Specifically extraction conditions of different papers are summarized with regard to certain requirements of biota and analytes for extraction and cleanup steps.

2.2 Introduction

To evaluate the associated risk of an increasing number of diverse chemicals present in the environment, among them pesticides, pharmaceuticals and chemicals from personal care products, new and optimized analytical methods, especially multi-residue methods covering many micropollutants and their metabolites are required. Analysis of pollutants in water, sediment, soil and biota is essential to assess the environmental behavior of chemicals used in our day to day life. To analyze high numbers of samples of different origins fast, cheap and straightforward extraction procedures for higher throughput are required. Since the first publication of QuEChERS extraction procedure in 2003 by Anastassiades this extraction method was increasingly applied in food analysis and quickly found its way also to environmental analysis [2]. QuEChERS extraction is an alternative to pressurized liquid extraction (PLE) and Soxhlet-extraction. A variety of kits containing mixtures of different salts and dSPE materials are now commercially available for food analysis and will facilitate automation and repeatability. The application of this method to the environmental matrices water, sediment and soil for analysis of pesticides and organic residues was reviewed by Bruzzoniti et al. in 2014 [27]. The review herein summarizes examples of organic pollutants

extraction from biota by QuEChERS extraction with particular focus on different modifications of the extraction conditions required by matrix and analyte properties. The first part focuses on the extraction conditions and the second part of this review focuses on applications of the extraction procedure to environmental samples.

2.2.1 Ecotoxicological interest

Organisms are exposed to chemicals via active uptake by food and passive uptake from the surrounding medium for example water or soil. Aquatic living organisms tend to accumulate pollutants present in water and sediment, therefore they can be used as bioindicators for pollutant burden of the ecosystem [28]. The analysis of biota samples exposed in the environment and analysis of water provides the possibility to assess the bioaccumulation and biotransformation potential of chemicals. Lipophilic compounds might occur in low concentrations in the environment but due to their eventually high bioaccumulation potential they might become enriched in biota even when water concentrations are lower than the limit of detection. In addition, biota analysis provides a mid- to long-term information on micropollutants compared to analysis of stream water.

Growing consumption of pesticides and their direct input from fields and of pharmaceuticals (both human and stock farming) with direct input or input due to insufficient removal rates of these compounds in wastewater treatment plants increases the complexity of the micropollutant mixture and effects. To identify the compounds of relevance from the thousands of chemicals present in the environment analysis of biota might be a useful tool, especially, because pharmaceuticals and insecticides are designed for uptake by organisms. To predict the environmental fate of chemicals, their quantification is required at different ecotoxicological levels: in sediment, water and biota, in soil and soil living organisms or at higher trophic levels in animals and their prey. To analyze large numbers of samples a fast and cheap methodology is required to obtain enough data to reach reliable predictions for risk assessment for effects on ecosystems.

2.2.2 Classical methods

Common extraction procedures in environmental analysis for quantification of pesticide and pharmaceutical residues in biota are liquid-liquid extraction (LLE) with different organic solvents, Soxhlet-extraction, sonication-assisted extraction, supercritical fluid extraction, microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), pulverized liquid extraction or matrix solid phase dispersion [29-32]. Their applications for analysis of persistent organic pollutants in biota were reviewed by Fidalgo-Used [29]. Most of them require additional cleanup by liquid adsorption chromatography, gel-permeation chromatography or solid-phase micro-extraction [29]. Applications of pesticide analysis in biota mostly by LLE followed by SPE were reviewed by Andreu [33].

2.2.3 QuEChERS extraction procedure

Recently QuEChERS extraction procedure initially applied in food analysis has been increasingly used in environmental analysis. Its use for sediments and soil analysis was reviewed covering the years 2010 - 2014 [34]. The extraction procedure was initially developed for pesticide analysis. Therefore, many publication still focus on pesticides and their metabolites. As pharmaceuticals are often in a similar polarity range like pesticides, the extraction procedure has also been applied for the analysis of pharmaceuticals and wastewater chemicals in different organisms. Publications using QuEChERS procedure for

extraction of contaminants from biota from field samples or laboratory experiments are summarized in Table 2.

The extraction procedure is based on a single phase LLE between two miscible solvents, water and acetonitrile. In the publication by Anastassiades et al. phase separation was induced by addition of sodium chloride and anhydrous magnesium sulfate [2]. The conditions relate to a miscibility gap between water and acetonitrile at these salting out conditions. Its applicability for a wide range of analyte polarities [2] stems from the ratios of acetonitrile to water or water to acetonitrile in the two phases, which can be influenced by the amount of salt added: Saturation of the water phase with (inorganic) salts reduces the acetonitrile content in the water phase and decreases the water content in the acetonitrile phase which leads to higher recoveries of polar analytes [2]. Therefore, multi-residue extraction is possible [2]. This advantage is especially useful for screening methods comprising 100 - 200 compounds [35-38]. The extract is cleaned and simultaneously residual water is removed in a dispersive solid phase extraction (dSPE) step by addition of anhydrous magnesium sulfate and a sorbent. During this step co-extracted matrix components are removed by mixing the acetonitrile phase with the appropriate sorbent. According to the requirements of the separation and detection methods, the extract can be analyzed in acetonitrile or the solvent changed to a more suitable one e.g. methanol or ethyl acetate in HPLC or GC separation. The method was originally developed for pesticide analysis in food and comprised 10 g sample extracted with 10 mL acetonitrile and the natural water content of 80 - 95 % for most vegetables or fruits was used as water phase [2]. For analysis of organic contaminants in environmental samples modifications of this procedure are required, mainly to enable extracting small sample amounts in the mg range or even single individuals such as gammarids or bees. Often, freeze dried samples or samples with low water content are in the focus of the work. In the following, different homogenization techniques, the use of additional non-polar extraction solvents, substitution of sodium chloride by acetate or citrate buffered conditions (for higher stability and recovery of labile metabolites and pesticides) and different dSPE materials are compared with respect to the extracted analyte and biota characteristics.

2.3 ANALYTICAL CONSIDERATIONS FOR SAMPLE PREPARATION

In food analysis the use of multi-component methods like LLE and subsequent SPE cleanup prior to the analysis is common [39]. Different sorbents used in dSPE and SPE cleanup are discussed in Section 2.3.5. Advantages and disadvantages of different extraction procedures in comparison to QuEChERS are summarized in Section 2.3.2 [30-31, 40]. Table 1gives an overview on extraction and cleanup conditions used for QuEChERS. Details on homogenization, extraction conditions, extraction solvents, matrix removal and the implementation of a dSPE step are discussed below.

Table 1: QuEChERS extraction conditions and cleanup procedures applied for extraction of pharmaceuticals, pesticides and wastewater chemicals from biota samples.

compound	QuEChERS extraction	cleanup	
46 compounds	70 µL water, 70 µL MeCN	none	[41]
PCBs	10 mL water, 10 mL MeCN	freeze out, dSPE (PSA, C18)	[42]
boscalid and metabolites	3 mL water, 3 mL heptane, 10 mL MeCN/triethylamine 2 %, citrate buffer	freeze out, dSPE (C18, PSA)	[43]
organophosphorus	15 mL MeCN, 12 mL water,	freeze out,	[44]

compound	QuEChERS extraction	cleanup	
insecticides	1 % glacial acetic acid	SPE C18 cartridges	
35 emerging pollutants	0.5 mL water, 0.5 mL MeCN, 0.2 mL hexane, citrate buffer	hexane	21
diclofenac and metabolites	5 mL water, 10 mL MeCN, 0.2 mL heptane acetate buffer	heptane	45
hormones pharmaceuticals	10 mL MeCN, 6 mL water, 3 mL hexane, acetate buffer	hexane dSPE (PSA and C18)	46
fluoxetine, carbamazepine	0.25 mL MeCN, 0.1 mL water, 0.15 mL hexane, citrate buffer	hexane dSPE (C18, PSA)	47
diclofenac and metabolites	5 mL water, 10 mL MeCN, 0.2 mL heptane, citrate buffer	heptane	48
UV filters, musk fragrances	10 mL water, 10 mL MeCN, citrate buffer	heptane, FA dSPE (Zsep+, PSA, C18,)	49
19 pesticides	10 mL MeCN, 10 mL water, 3 mL hexane	hexane, dSPE (PSA)	50
VMS	1 mL MeCN	0.4 mL pentane	28
80 pesticides	10 mL MeCN, 3 mL water, 3 mL hexane	hexane dSPE (PSA/C18)	51
13 pesticides	5 mL water, 5 mL MeCN	DMSO, ethyl acetate/ cyclohexane, DMF	52
19 neonicotinoids and fungicides	0.4 mL water, 0.5 mL MeCN	dSPE (PSA, C18, GCB) 150 µL MeCN/toluene	53
UV-filter	5 mL water, 10 mL MeCN	DLLME	54
oxazepam, carbamazepine, testosterone	0.5 mL MeCN, 0.5 mL water, citrate buffer	dSPE (PSA, C18)	55
carbamazepine, oxcarbazepine	10 mL water, 10 mL MeCN, citrate buffer	dSPE (PSA, C18, FA)	31
40 pesticides	8 mL water, 14 mL MeCN, 1 % FA, citrate buffer	dSPE (C18, activated charcoal)	56
52 pesticides	7.5 mL water, 10 mL MeCN	dSPE (C18 + PSA)	57
pesticides, pharmaceuticals, wastewater chemicals	2 mL water, 2 mL MeCN, 1 mL hexane	dSPE (C18)	40
halogenated pesticides pyrethroids and azole-fungicides	1.25 mL MeCN, 1.25 mL water/salt solution	dSPE (C18 + PSA , C18 + PSA + GCB)	58
diclofenac	3.5 mL water, 3.5 mL MeCN, 10 % FA	dSPE (Z-Sep)	59
9 pesticides	1 mL ethyl acetate	dSPE (PSA)	60
16 PAHs	10 mL MeCN	dSPE (PSA)	61
neonicotinoids	1.5 mL MeCN 1 mL water acetic acid + citrate buffer,	dSPE (PSA, C-18)	62
54 pesticides	1 mL water, 5 mL ethyl acetate 2 % FA	dSPE (C18)	63
26 endocrine disrupting chemicals	2 mL water, 20 mL MeCN	SPE (C18, cartridges)	64
200 pesticides and metabolites	10 mL water, 10 mL MeCN 1 % acetic acid	dSPE (PSA + Z-Sep+)	35
207 analytes	10 mL MeCN	dSPE (Z-Sep)	36
150 pesticides	10 mL MeCN, 5 mL water	dSPE (PSA, C18, GCB)	37
121 pesticides	27 mL MeCN	dSPE (PSA, C18)	38

MeCN: acetonitrile, PSA: primary secondary amines, GCB: graphiticed carbon black, FA: formic acid, SPE: solid phase extraction, dSPE: dispersive solid phase extraction, PAH: polycyclic aromatic hydrocarbons, PCB: polychlorinated biphenyls, VMS: volatile methyl siloxanes, DLLME: dispersed liquid-liquid micro extraction

2.3.1 Homogenization techniques and sample amounts

Typical sample sizes in environmental analysis are small compared to food analysis. In order to quantify the internal concentration in e.g. insect larvae miniaturization of the classical QuEChERS approaches from food analysis is necessary. Therefore, different homogenization techniques than chopping, as typically used for vegetables, were applied especially for analysis of single individuals of the species of interest. Homogenization has two impacts on the extraction procedure: (1) good homogenization and disintegration allow more effective and faster extraction of the analyte and thus higher recoveries. (2) However, often also increased co-extraction of matrix components may occur, possibly impairing the signal-intensity during HPLC-MS measurement [39]. Therefore, the applied homogenization techniques have to meet the requirements given by the matrix and by the stability of analyte binding. From the experience in laboratory, the most critical factor in selection of the homogenization technique is the sample amount available for analysis. For fish, samples aliquots in the g scale are available and a simple meat grinder was used to homogenize freeze-dried samples [30]. For samples available in smaller sizes from 0.8 to 5 g blending was used for homogenization of frozen matrices without prior freeze-drying for bees [37, 51], gammarids [40], fish [52] and mussels [61].

Freeze drying is a common procedure used before homogenization for samples available in g scale e.g. mussels [31, 45, 48-49, 54, 59], fish [30, 56], or bees [50]. In freeze-dried samples the water content was removed, which is in contrast to classical QuEChERS, where the native water content of e.g. vegetables is present for the formation of two phases. Therefore, for freeze-dried samples, addition of water as second phase during extraction is mandatory and in some cases water was added earlier in the sample preparation protocol to rehydrate the dried samples and enhance extraction efficiency [31].

A grinder was used for samples including e.g. 10 fish embryos or 20 daphnids or up to 5 g bees. Different kinds of beads for grinding were used: stainless steel beads for boscalid extraction from bees [43] or pharmaceuticals from single gammarids [55] and glass beads for fish embryos [41]. For the extraction of daphnids, beads were not further specified [58] .

In cases where only smaller sample amounts are available mortar and pestle applied after immersing the sample in liquid nitrogen were used to homogenize single snails [47], single bumble bees [53] or honey bees [35]. This method was also used in our laboratory to homogenize midge larvae and midges, but a grinder usable in Eppendorf tubes was used to reduce tissue loss during homogenate transfer. For temperature insensitive analytes homogenization with mortar and pestle of dried samples is an opportunity for example used in pesticide extraction from arthropods [60] and for fungi mycelium in our laboratory. Haroune et al. observed higher background noise due to higher contents of co-extracted matrix components from insect boluses and thus homogenized frozen samples without liquid nitrogen [63].

Homogenization with ultrasonic or blending devices allows to simultaneously combine homogenization and extraction in the same step. This was more closely investigated by comparison of the extraction efficiency for contaminants in fish obtained when using a pulse vortexer, a vibration shaker or a blender [36]. Sapozhnikova et al. observed, that 1 min extraction time in the blender was sufficient to obtain reproducible extraction rates, whereas 10 min were necessary in case of the vibration shaker [36].

Ultrasonic homogenization was used in one publication to extract neonicotinoids from eagle owl blood [62]. In this publication blood was extracted, whereas in the other publications

discussed in this review, whole organisms were extracted resulting in different challenges for sample preparation.

2.3.2 Comparison of extraction procedures

Extraction procedures have different impacts on the performance of the following analytical methods (e.g. LC or GC-MS). Higher extraction efficiencies for analytes are often accompanied by efficient co-extraction of matrix components interfering in MS analysis. In some publications, different extraction approaches were compared to find the most suitable one (see below). Another critical parameter to be considered is the minimal extraction time required to achieve reproducible extraction rates.

In a comparison of ultrasonic extraction (USE), pressurized liquid extraction (PLE) and QuEChERS of pharmaceuticals from fish tissue, highest recoveries and lowest matrix effects were observed for PLE, whereas in samples extracted by USE lowest recoveries were observed. Recoveries for pharmaceuticals were comparable for PLE and QuEChERS extracts [30]. Similar observations were made by Bueno et al. for extraction of pharmaceuticals from mussels. Comparing QuEChERS and PLE, QuEChERS was advantageous with regard to its lower solvent consumption, shorter extraction times and smaller amounts of sample that were required for comparable or higher recoveries [31]. Equal recoveries were observed in comparison of PLE and QuEChERS extraction capabilities for pharmaceuticals from fish tissue but less matrix effects and overall higher recoveries were observed by PLE [30].

Microwave-assisted extraction (MAE) and matrix solid phase dispersion (MSPD) were compared to QuEChERS concerning the extraction efficiencies of 54 contaminants from bird boluses [63]. In this study good recoveries were observed in MSPD extracts only for a small number of spiked analytes. For spiked QuEChERS extracts good recoveries for a broad range of physicochemical properties of analytes were observed. When the developed extraction methods were applied to environmental samples no contaminants were detected in QuEChERS extracts of exoskeleton samples, whereas in MAE extracted samples contaminants were detected. Therefore, the authors conclude, that MAE is capable of extracting analytes bound more tightly to samples, like in this case contaminants bound to the exoskeleton of insects [63].

Wiest et al. compared MSPD, SPE and QuEChERS procedure for extraction of 80 contaminants with a broad range of physicochemical properties from bees [51]. In this study, QuEChERS extraction was the only one allowing the extraction of the whole range of analytes in a single extraction step. In MSPD extracts poor recoveries were observed for most polar compounds and in SPE recovery was strongly depended on the chosen SPE material and thus only a part of the analyte polarity range was covered by one extraction step [51].

In comparison of QuEChERS, SPE and solvent extraction with acetone and dichloromethane to quantify 52 pesticides in honey bees, the lowest recoveries were observed for classical solvent extraction. For SPE and QuEChERS equal recoveries were observed [57]. Higher recoveries for analytes covering a wide range of physicochemical properties from gammarids were observed in QuEChERS extracts compared to SPE by Inostroza et al. [40].

The main advantage of QuEChERS extraction in comparison to other extraction techniques is that the simple procedure is less time and solvent consuming and thus more economical, especially when analytes of a broad polarity range are of interest and different matrices are addressed [57].

2.3.3 Extraction solvents

QuEChERS extraction procedures basically include LLE with two solvents: acetonitrile and water. Challenging matrices included those with high lipid content in fish or earthworms or waxes in bee samples (see Section 2.3.4). To remove these interfering substances, the extraction with acetonitrile and water can be extended by a further un-polar phase, for example adding hexane or heptane. This was used for the extraction of earthworm tissue [46] and gammarids [21, 40] or to remove wax, fatty acids, fatty acid esters and lipids from bee and pollen samples [50-51].

Instead of hexane, the less toxic heptane was used to combine extraction and cleanup in one step for the analysis of diclofenac and its transformation products from mussels and fish tissue with good results [45, 48]. Two extraction steps were required for fish tissue with the first extraction step with water/acetonitrile and the second extraction step with ethyl acetate/cyclohexane for cleanup and nonpolar pesticide extraction [52]. Pentane and acetonitrile were used for extraction of volatile methyl siloxanes from animal blood, and in this case the pentane phase was analyzed by GC-MS [28]. Stöckelhuber et al. used ethyl acetate instead of acetonitrile as it is cheaper, less toxic, and higher signal areas were achieved for pesticides extracted from arthropods [60].

Some challenging analytes, for example acidic pesticides or labile compounds, require controlled pH conditions during the extraction procedure. To enhance the recovery of boscalid and stabilize its metabolites containing an acidic functional group from bee samples heptane and triethylamine were added during the extraction process [43].

For samples of high matrix load, additional cleanup steps may be necessary: Cunha et al. added a dispersed liquid-liquid micro extraction (DLLME) step to clean the organic phase from QuEChERS extraction and enhance recoveries of UV filters from mussel extracts. For this, the acetonitrile phase from QuEChERS extraction was added to a mixture of trichloroethylene and water at pH 3 [54]. An additional extraction step with toluene after dSPE with graphitized carbon black (GCB), primary secondary amines (PSA) and C18 allowed to elute analytes bound to GCB. The combined extracts were used for analysis which enhanced the recovery of planar analytes and maintained the advantage of reduced matrix effects due to dSPE of pesticides in bumble bee extracts [53].

In all extraction procedures, 0.5 - 20 mL of combined solvents were used for extraction, except in case of a miniaturized version of the extraction procedure for extraction of 600 µg fish embryo samples with only 70 µL water and 70 µL acetonitrile [41].

2.3.4 Removal of critical matrix components: lipids and waxes

In QuEChERS extraction the lipid content of analyzed matrix is a critical parameter as contents of 1 % lipid already show a significant influence on the extraction results [39]. An additional hexane phase was shown to remove lipids from QuEChERS extracts, as discussed in Section 2.3.3. Also dSPE with C18 or Zsep$^+$ sorbent removes lipids to a certain extent. However, freezing out of QuEChERS extracts for 1 - 12 hours, is another simple and effective protocol to remove waxes or lipids. This method was applied to clean extracts of bees [43] and earthworms [37, 42, 50] for pesticide analysis and for extraction of PCBs from earthworms [42]. This method has also been used to clean eagle owl blood extracts for analysis of neonicotinoids [62].

2.3.5 Dispersive solid phase extraction materials

In this step, different sorbents are added to remove interfering matrix components from the extracts. The added sorbent should retain co-extracted matrix components, which otherwise impair downstream analytical methods such as GC or LC-MS. Consequently, different materials have been applied for different matrices. When using MS for detection, which is common given the low concentration in biota, biological co-extracted and co-eluting matrix components often impair the ionization process of the analytes in the ion source of the MS [65]. Both signal enhancement or suppression were observed [66]. Dilution of samples equally dilutes analyte and matrix components. Therefore, less signal-suppression and higher analyte signal intensities may occur [67]. This was observed for pesticide signals in diluted bee extracts [51] comparing different amounts of sample used for extraction. In the range of 0.25 - 1 g extracted mussel tissue highest recoveries of pharmaceuticals and their transformation products were observed in 0.25 g tissue [31].

Only a few methods were published analyzing the raw extract without further cleanup, e.g. for fish embryo extracts, which is a relative simple matrix compared to other organisms like bees. Here, the authors wanted to minimalize analyte loss during workup and reproducible quantitative results were obtained also for concentrations close to the limits of detection in the ng/g range [41]. Volatile methyl siloxanes were determined in raw extracts of blood without further cleaning steps [28].

To reduce matrix effects by removal of co-extracted matrix components a dSPE step can be implemented. In this step interfering matrix components are removed by transferring the acetonitrile phase to a sampling vessel containing the sorbent and dispersing the sorbent in the organic solvent. Interfering matrix components eventually bind to the sorbent and can be removed. However, it is possible that also analyte is (partially) removed. Therefore, the choice of sorbent is essential and has to be optimized with regard to matrix removal and prevention of analyte losses. Common dSPE materials often applied in environmental analysis are PSA, GCB, aluminum N, Z-sep and Z-sep+ and C18. PSA is used to remove high amounts of proteins, organic acids, polar pigments, sugars and fatty acids by interaction of acidic functions from compounds and amine functions from PSA in the QuEChERS raw extract [35, 48]. Z-sep and Z-sep+, zirconium dioxide coated silica sorbents, are especially useful for removal of proteins and lipids [35, 68]. The interaction of PSA is pH-dependent, as compounds are removed by ionic interaction, whereas Zsep and Zsep+ remove compounds by hydrophobic interaction independently from the applied pH [35]. This was demonstrated, when differences in analyte recoveries were observed in PSA removal steps in acetate buffered systems compared to citrate buffered systems [35]. Aluminum N has similar selectivity as PSA and removes matrix components by interaction with H-bonding processes. C18 selectively retains nonpolar compounds [48] and GCB removes planar compounds like pigments or chlorophyll [2, 53]. Another sorbent removing polar matrix components is silica [69].

For most matrices and analytes a single sorbent was sufficient for dSPE (see Table 1). PSA was used for analysis of pesticides in bees [50], PAHs in mussels [61] and pesticides in arthropods [60]. C18 was used for removal of interfering matrix components from extracts of insect boluses [63], and C18 cartridges in an SPE step were used to clean bee extracts for pesticide analysis [44] or endocrine disruptors from fish [64]. Z-sep was used to clean mussel extracts to analyze diclofenac [59] and fish extracts to determine pesticide contaminations [36].

Especially for multi-component analysis the combination of two or more sorbents was chosen for sample cleanup. For extraction of pesticides from bees the combination of PSA/Z-sep+ was compared to PSA/C18 and PSA/C18/GCB. The removal of lipids and bees

wax was more effective by Z-sep+ than by the other tested sorbents but recoveries of analytes with acidic functional groups and triazoles were reduced. This effect was overcome by combining Z-sep and PSA sorbents in acetate buffered extraction conditions [35]. For analysis of analytes covering a broad range of polarities the combination of PSA and C18 or GCB was often used. In comparison of PSA/GCB and PSA/C18 dSPE of honey bee extracts, PSA/GCB retained apolar compounds to a great extent leading to poor recoveries and thus, PSA/C18 was chosen for dSPE [51]. Higher recoveries were also observed for pharmaceuticals extraction from earthworm extracts cleaned by the combination of PSA/C18 compared to PSA-only cleanup [46]. The combination PSA/C18 was used to clean extracts from earthworms for analysis of PCBs [42], boscalid and its metabolites in bee extract [43] and neonicotinoids from eagle owl blood samples [62]. Oxazepam was shown to be completely retained by Z-sep [55], whereas the combination of C18/PSA was successful in cleanup of gammarid extracts. In comparison of C18/PSA/GCB vs. PSA/C18 (endcapped) dSPE higher recoveries from daphnid extracts were observed for azole and pyrethroid insecticides, which are molecules with a planar structure [58]. The combination of PSA/C18/GCB was used to clean bumble bee extracts for pesticide analysis [53] and for the analysis of 150 pesticides in bee extracts [37]. PSA/C18/activated charcoal was used to clean fish extracts for pesticide analysis [56].

For extraction of pharmaceuticals from mussel tissue addition of formic acid (FA) enhanced the recovery of carbamazepine and oxcarbamazepine [31]. The same was observed for recoveries of UV filter from mussel tissue extracts in C18/PSA + FA cleaned extracts compared to extracts without addition of acid [49].

An alternative to remove lipophilic components is the addition of a hexane phase, as demonstrated for example for gammarids, bivalves or honey bee tissue [21, 45, 50], as discussed in Section 2.3.3. In case of single invertebrates, higher recoveries were achieved for extracts cleaned by hexane compared to extracts cleaned by dSPE with PSA/C18, especially for the hydrophobic analytes [21, 47]. The combination of hexane during extraction plus dSPE with PSA for cleanup enabled the quantification of broad range of contaminants [40]. A similar cleanup strategy was developed for extracts of fish and mussels: In these sample preparation methods, hexane and heptane were used for cleanup of QuEChERS extracts. Treatment with PSA was described not to affect the recovery of diclofenac but recoveries of its more polar transformation products, whose concentrations were reduced during the dSPE step [45, 48].

2.3.6 Acetate and citrate buffered extractions

In many cases, the addition of sodium chloride and the use of non-buffered conditions revealed sufficient selectivity and recoveries for extraction of various analytes e.g. from gammarids [40], daphnids [58], mussels [61] and animal blood [28]. However, when a defined pH is required to ensure stability of compounds which are prone to hydrolysis or when repeatable conditions are envisaged, different buffers can be applied during the extraction process. For compounds with neutral species at distinct pH values binding effects to dSPE sorbents e.g. PSA are possibly enhanced or suppressed. Selection of a buffered or non-buffered extraction procedure depends on both analyte and matrix [21]. Instead of sodium chloride two buffered systems with acetate or citrate were proposed. Because water and acetonitrile are miscible a mixture of salts has to be added to promote phase separation and shift the partitioning of analytes into the organic phase and eventually reduce the water content in the organic phase. In the initial publication sodium chloride and magnesium sulfate were used [2]. Substitution of sodium chloride by acetate or citrate buffered systems improved the stability of pH-sensitive analytes [47]. The method for pesticide analysis in food published by AOAC

(Association of Official Agricultural Chemists, belonging to the United States Department of Agriculture) used 1 % acetic acid and sodium acetate [70]. In the European Norm citrate buffers are included [71]. In case of polar analytes the addition of acetic acid [35, 44, 62] or formic acid [56, 59, 63] sometimes enhanced analyte recoveries.

Citrate salts were added for extraction of environmental contaminants from bee samples [37, 51, 57], for boscalid and metabolite extraction from bees [43] and for pharmaceuticals and UV filters from mussels [31, 54]. Acetate buffer extraction was used for pharmaceutical analysis in earthworms [46] or extraction of pesticides from bumble bees [53] and bees [44]. The more acidic acetate extraction was chosen for extraction of triazole and acidic pesticides from bees. At these low pH values most acidic pesticides were neutral and thus not retained by PSA [35].

The choice of the buffering ion is strongly matrix dependent. Thus, for diclofenac higher recoveries for the extraction from bivalves were obtained when acetate instead of citrate was added for buffering [45]. The opposite was observed for extractions from fish [48], whereas for mussels addition of formic acid to acetate buffered conditions revealed increased recoveries for diclofenac [59]. For extraction of UV filters from mussels non-buffered conditions were sufficient [54]. Similarly, higher recoveries were observed for acetate buffered extraction of neonicotinoids from eagle owl blood compared to citrate buffered extraction [62] and for citrate buffered extractions (compared to acetate buffered systems) in the analysis of pharmaceuticals in invertebrates and snails [21, 47].

2.3.7 Analytical methods compatible with QuEChERS extracts

Common separation and detection methods are LC-MS/MS and for less polar compounds GC-MS/MS [39]. Mass spectrometry provides the sensitivity, selectivity and matrix tolerance required for trace analysis of emerging pollutants in environmental samples. Therefore, mass spectrometry was used in all publications discussed in this review except for the analysis of pyrethroid insecticides in daphnids which were conducted by electron capture detection after GC separation [58], see Table 3: Method validation parameters, LOD, LOQ, recovery and concentration in samples extracted with optimized method.. In studies comprising a broad range of pesticides of different physicochemical properties the QuEChERS extracts were analyzed by both GC and HPLC-MS, for example for contaminants and pesticides in bees [38, 51]. GC has been used for separation of less polar contaminants in case of pesticides in bees [37] or pyrethroid pesticides in daphnids [58]. Silylation was used for derivatization of UV filter compounds in mussel extracts for GC separation [54].

The application of nano-LC-MS/MS systems enabled the quantification of body-burden in single individuals. This was used for analysis of emerging pollutants in snails [21] and invertebrates [47] or single gammarids [55]. Triple quadrupole methods reduced the background noise and thus, enhanced limits of detection. However, sample preparation remains a step required in environmental analysis (see Section 2.3.5 and Table 1).

2.4 APPLICATION

2.4.1 Environmental monitoring

QuEChERS was used for environmental monitoring purposes or studies on transformation and environmental fate of pesticides and wastewater chemicals in terrestrial and aquatic organisms. The analysis of neonicotinoid concentrations in bees and bee products but also

in birds was of particular interest also due to discussions and following European decision to restrict the use of the neonicotinoids imidacloprid, clothianidin and thiamethoxam [72].

Pesticides

QuEChERS has been developed for pesticide analysis in food and is thus designed for a specific polarity range common to many pesticides. QuEChERS was used to analyze concentrations of pesticides in different fish species sampled along the Jucar river to assess the environmental behavior and biological effects of these pesticides [56]. Lazartigues et al. developed a method to quantify pesticides in fish muscle [73].

More applications were published concerning pesticide analysis in terrestrial organisms than in aquatic organisms. Pesticides applied in vine cultivation were analyzed in insects, spiders and snails sampled in vineyards [60]. Insect boluses from birds were analyzed for residues of 54 pesticides but only detected after microwave-assisted extraction (MAE) and not in QuEChERS extracts [63]. The authors assumed a reduced extraction efficiency of QuEChERS extraction compared to MAE. The burden of neonicotinoids in eagle owls, representing a species of higher trophic level and a predator of insects, was assessed by blood analysis with QuEChERS extraction [62].

With the bee death being a topic of concern several publications used QuEChERS extraction for analysis of pesticides in bees collected after poisoning incidents [35, 37, 50] but also in bee products like pollen and honey. In addition, bees were investigated as potential bioindicators for contaminant burden [38, 51]. E.g. the fungicide boscalid and its metabolites were analyzed in honey bees [43]. Honey bees, bumble bees, honey and pollen were screened for pesticides taking advantage of QuEChERS multi-residue capabilities. Screening methods included 19 to 200 compounds [35, 37-38, 44, 50, 53, 57]. Furthermore, with a method comprising 80 veterinary drugs and other environmental contaminants honey bees, honey and pollens were analyzed [51].

Wastewater chemicals and pharmaceuticals

Gammarids originating from the river Danube were analyzed with a method comprising 74 compounds including wastewater chemicals, pharmaceuticals and pesticides [40]. Diclofenac and some of its metabolites were analyzed in bivalves [45, 59] and fish [48]. Carbamazepine and its metabolites are another example of pharmaceuticals analyzed in marine mussels as bioindicators [31]. Carbamazepine and fluoxetine were analyzed in molluscs exposed in laboratory experiments [47]. Different extraction techniques were compared for pharmaceutical extraction from fish tissue [30]. A method for quantification of 26 endocrine disruptors in fish was developed and applied to zebra fish samples [64]. Emerging pollutants were analyzed in marine mussels among them UV filters and musk fragrances [49] and in mussels, clam and mullet [54]. Zebra fish embryos were exposed to 46 volatile organic compounds and the uptake was analyzed [41]. 35 emerging pollutants were analyzed in gammarids, chironomids and snails exposed downstream of a wastewater treatment plant effluent [21]. Volatile methyl siloxanes were analyzed in the blood of turtles, cormorants and seals from Canada [28]. A method including 207 contaminants was developed to analyze them in fish and evaluated with regard to different extraction techniques [36]. PAHs were analyzed in wild bivalves [61].

Two publications focused on uptake kinetics: The kinetic of bioconcentration of carbamazepine, oxazepam and testosterone was investigated in gammarids [55]. Likewise, the time-dependence of internal azole and pyrethroid concentration in daphnids was determined [58].

For terrestrial organism only PCBs in earthworms and soil were extracted with QuEChERS for spiked samples [42]. Earthworms were used for the assessment of the bioaccumulation potential of hormones and pharmaceuticals [46].

2.4.2 Analysis of transformation products

Transformation products are an issue of growing concern, especially for pharmaceuticals. In some cases, transformation products are more active than the parent compound or inactive compounds are metabolized to active transformation products [1]. Metabolites are mostly more polar than their parent compound to enhance their excretion [1]. To capture metabolites and parent compounds in one extraction step a method is required covering a broad range of polarity. QuEChERS extraction has been applied to extract diclofenac together with its transformation products from fish and [48] mussel tissue [45] and for extraction of pesticides and their metabolites [35] as well as of boscalid and its metabolites from bees [43].

Table 2: Application of QuEChERS extraction procedure in biota with focus on compounds of interest and objective of the studies.

compound	organism	method	objective	
pesticides	fish	HPLC-MS/MS	pesticides in water and fish	56
pesticides	fish	LC-MS/MS	13 pesticides in sediment, water and fish	52
pesticides	insects, snails, spiders	GC-MS/MS	micro-QuEChERS of biota sampled in vineyards	60
pesticides	boluses	HPLC-MS/MS	exposure of insects	63
neonicotinoids	eagle owl blood	HPLC-TOF	neonicotinoids in free-ranging birds	62
fungicides and metabolites	honey bees	UHPLC-HRMS	fungicides in bees	43
pesticides	bees	HPLC-MS/MS	comparison of SPE and QuEChERS for bees and honey	57
pesticides	bees	HPLC- and GC -MS/MS	method development to quantify 200 pesticides and metabolites in bees	35
pesticides	honey bees	HPLC-MS/MS	internal concentration in bees from beekeepers	50
neonicotinoids and fungicides	bumble bees	UHPLC-MS/MS	neonicotinoids in single bees	53
organophosphor-insecticides	bees	HPLC-MS/MS	insecticides in honey bees and pollen	44
pesticides	bees	HPLC- and GC-MS/MS	pesticides in the environment	51
pesticides	bees	GC-MS/MS	pesticides in bees	37
pesticides	bees	HPLC- and GC -MS/MS	pesticides and metabolites in bees	38
pesticides, waste water chemicals, pharmaceuticals	gammarids	HPLC-MS/MS	bioindicators	40
pharmaceuticals and metabolites	bivalves	HPLC-MS/MS	diclofenac and transformation products in the environment	45
diclofenac	fish	HPLC-MS/MS	diclofenac in the environment	48
diclofenac	mussels	HPLC-MS/MS	mussels as bioindicators	59
pharmaceuticals	bivalves	HPLC-MS	pharmaceuticals in aquatic	31

carbamazepine and metabolites			organisms	
pharmaceutic	molluscs	nano-HPLC-MS/MS	bioaccumulation	47
endocrine disruptors	fish	UHPLC-MS/MS	water and fish	64
UV filters and musk fragrances	bivalves	GC-MS/MS	sea pollution and biota	49
UV-filters	bivalves and algae	GC-MS/MS	personal care products, waste water chemicals in biota	54
volatile organic compounds	zebrafish embryo	LVI-GC-MS/MS	exposure	41
pollutants	gammarids, snails and midge larvae	nano-HPLC-MS/MS	bioaccumulation in cage experiments	21
methyl siloxanes	seal, turtle, cormoran, blood plasma	GC-MS	internal concentration in blood of wild animals	28
PCBs, PAHs und pesticides	fish	LPGC-MS/MS	evaluation of extraction parameters	36
PAHs	bivalves	GC-MS/MS	edible bivalves	61
pharmaceuticals	fish	UHPLC-MS/MS	comparison of extraction techniques	30
PCBs	earthworms	GC-MS	method development	42
micropollutants	gammarids	nano-HPLC-MS/MS	kinetic of bioconcentration	55
pesticides	daphnids	GC-ECD	uptake and kinetics in daphnids	58
pharmaceuticals	earthworms	HPLC-MS/MS	bioaccumulation in lab experiments	46

HPLC: high performance liquid chromatography, GC: gas chromatography, UHPLC: ultra-high performance liquid chromatography, TOF: time of flight mass spectrometer, HRMS: high resolution mass spectrometry, LVI: large volume injection, ECD: electron capture detection, PCB: polychlorinated biphenyl, PAH: polycyclic aromatic hydrocarbons, LPGC: low pressure gas chromatography

2.4.3 Quantification strategies

Different quantification approaches are common in biota analysis. (1) Matrix-matched calibration: in this approach extracts are spiked before extraction and matrix effects occurring during detection (mainly ion suppression/enhancing effects for MS detection) are considered. (2) Using internal standards with stable isotopes (e.g. deuterated forms of the analyte of interest) is advantageous for quantification in complex matrices [74]. The deuterated internal standard has similar physicochemical properties like the analyte and thus, it undergoes the same loss during workup and changes in ionization efficiency as the analyte. Care has to be taken that no chromatographic separation between analyte and internal standard is reached which is less likely using ^{13}C-labeled compounds. (3) External calibration: in this approach a calibration curve for standards in a suitable solvent is recorded and matrix effects and recovery are assessed in additional experiments.

The signal intensity of analytes in the final extract is determined by two different factors: recovery and matrix effects. The combination of both gives the signal intensity or absolute recovery of an analyte. In Figure 1 analyte signal intensities are shown for a mixture of 13 compounds in a midge larvae extract. Signal intensities in extracts spiked before extraction

are in the range of 10 - 50 % compared to the signal area of analyte in methanol at the same concentration.

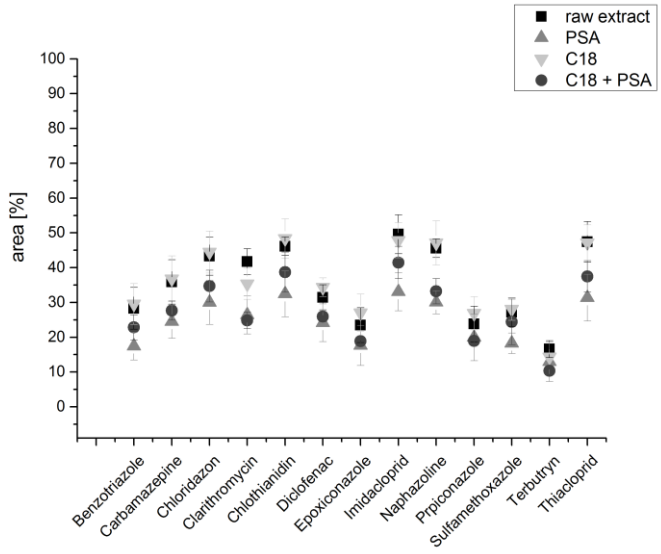


Figure 1: Signal intensities of analytes in midge larvae extracts spiked before QuEChERS extraction with 10 µg/L analyte, compared to signals intensities of analytes of the same concentration in MeOH (herein referred to as absolute recoveries). For extraction to 20 mg larvae tissue 10 µL standard mixture (c = 100 µg/L) were added and the sample extracted with 0.5 mL acetonitrile and 0.5 mL water. Phase separation was induced by addition of 25 mg NaCl and 75 mg $MgSO_4$ and for cleanup the acetonitrile phase was transferred to a vial containing 25 mg sorbent and 70 mg $MgSO_4$. After evaporation of the solvent the residue was redissolved in 0.5 mL MeOH and analyzed by HPLC-MS. For more detailed information see Section 2.

The signal intensities in extracts spiked before analysis range from 48 - 95 %, compared to the signal intensities of analytes in methanol (shown in Figure 2). Thus, the matrix effect can be calculated as difference in signal area in matrix compared to signal area in solvent, which was 5 - 52 % in the example given in Figure 2.

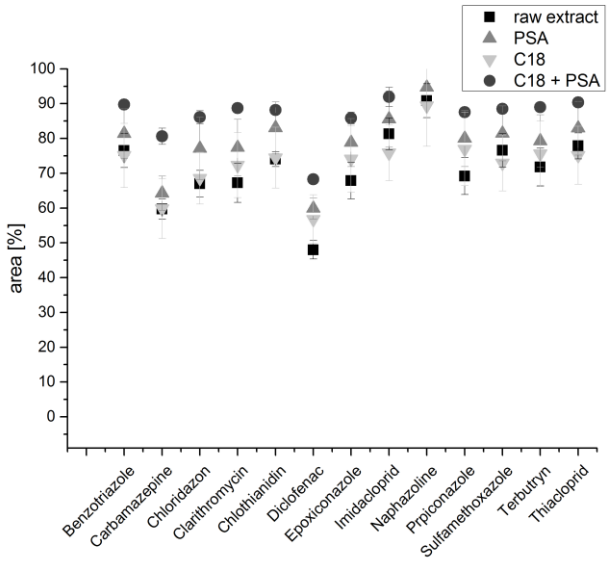


Figure 2: Signal intensities of analytes spiked to midge larvae extracts after extraction and before analysis. Signal intensities in % are calculated compared to analyte signals in MeOH at the same concentration.

The difference between the absolute recovery (Figure 1) and the matrix effect (Figure 2) is the relative recovery (Figure 3) or the loss of compound during extraction. This relative recovery shows the highest standard deviation ranging from 16 - 65 %, in the example

shown in Figure 3. The combination of analyte loss during extraction and matrix effects during analysis may result in an underestimation of body-burden.

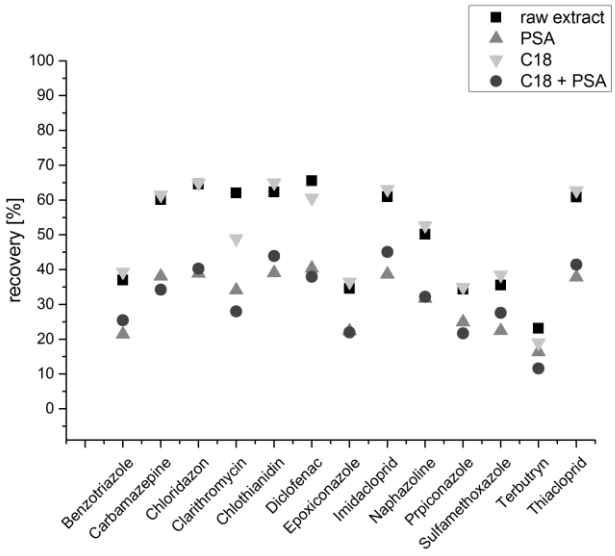


Figure 3: Relative recoveries of analytes were calculated as the ratio of signal intensity in extracts spiked before extraction to signal intensity in extracts spiked before analysis (Figure 1)/(Figure 2).

Quantification by internal standards can take into account possible analyte loss during workup and signal suppression or enhancement during analysis. Matrix matched calibration takes signal suppression during analysis into account but losses during recovery have to be determined in additional experiments. Most applications used internal standards e.g. for quantification of pesticides in bees and fish [37, 44, 50, 52], pharmaceuticals in snails [47], carbamazepine, diclofenac and UV filter in mussels [31, 45, 49, 54, 59], PCBs in earthworms [42] and VMS in animal blood [28].

Matrix-matched calibration was used to correct for matrix effects in pesticide analysis in bee extracts [51], for pesticide analysis in fish tissue, where matrix effects were below 20 % [56], for analysis of contaminants in gammarids [40] and analysis of pesticides in daphnids [58]. Two different approaches were made by Kurth et al. to analyze contaminants in fish embryos by solvent spike calibration and method-matched calibration [41]. For method-matched calibration QuEChERS was conducted with spiked solvent but without matrix and solvent spiked calibration was performed as external calibration.

2.4.4 Recoveries and limit of detection

Recoveries were in the range of 70 - 120 % for most analytes in various matrices, from pesticides in fish [50] or bees [53] to pharmaceuticals in earthworms [75] or mussels [31], compare Table 3. Low recoveries were observed for base sensitive compounds and hydrophobic compounds in honey bee extracts [51]. Limits of detection were in the ng/g range for all analytes in all publications listed in Table 3.

Table 3: Method validation parameters, LOD, LOQ, recovery and concentration in samples extracted with optimized method.

organism	LOD	LOQ	recovery [%]	sample amount	measured concentration	
honey bees				5 g	0.2 - 36.3 ng/g	[43]
bees		0.3 - 10 ng/g	34 - 96	5 g		[57]

organism	LOD	LOQ	recovery [%]	sample amount	measured concentration	
bees	10 ng/g	1 - 100 ng/g	70 - 120	5 g		35
honey bees	0.95 - 25 ng/g	3.4 - 75 ng/g	70 - 120	1 g	4 - 95 ng/g	50
bumble bees	0.01 - 0.96 ng/g	0.04 - 2.9 ng/g	71 - 102	1 individual (98 ± 30 mg)	14 - 67 ng/g	53
honey bees	0.1 – 24 ng/g	3 – 66 ng/g	60 – 120	5 g	30 – 2419 ng/g	51
bees		10 - 500 ng/g	70 - 120	2 g	10 - 4864 ng/g	37
bees		0.1 - 30 ppb		3 g	2 - 13780 ppb	38
bees		0.02 - 10.7 ng/g	86 - 106	3 g	0.3 - 32.7 ng/g ww	44
boluses	0.1 - 3 ng/g	0.4 - 7 ng/g	49 - 106	0.05 g dw	2 - 404 ng/g	63
eagle owl blood		2 - 10 ng/mL	68 - 134	500 µL blood	3.28 ng/mL	62
seal, turtle cormoran blood plasma	0.01 - 0.035 ng/g	0.033 - 0.116 ng/g	88 - 99	1 g	0.143 - 7.39 ng/g	28
insects, snails and spiders	0.05 - 0.2 ng/g	0.2 - 1 ng/g	84 - 110	500 mg	1.68 ng/g	60
earthworms	< 14 ng/g	1,6 - 40 ng/g	44 - 98	250 mg ww	43 - 195 ng/g	46
earthworms		0.01 - 0.05 ng/g	70 - 120	5 g	n.d.	42
daphnids	58 - 168 ng/g ww	119 - 571 ng/g ww	95 - 111	20 individuals	50-114 ng/g ww	58
zebrafish embryo	1 - 25 ng/g		63 - 133	10 embryos	5 - 175 ng/g	41
invertebrates	ng/g	ng/g	40 - 98	1 - 12 individuals, 12 - 20 mg ww	105 ng/g	21
gammarids		0.01 - 2.16 ng/g ww		900 mg	0.1 - 4 ng/g	40
gammarids	0.1 - 2.2 ng/g	0.3 - 4.7 ng/g	0.8	20 mg ww	1-5 ng/g	55
molluscs	16 - 127 ng/g	18 ng/g, 128 ng/g	> 85	1 gastropod	1 - 6 µg/g	47
bivalves	0.1 - 0.3 ng/g dw	0.2- 1 ng/g dw	67 - 110	2 g	3.5 ng/g dw[2]	31
bivalves	0.5 - 50 ng/g dw	0.5 - 50 ng/g dw	91 - 112	2 g	833 - 3992 ng/g dw	49
bivalves and algae	2 - 30 ng/g	5 - 100 ng/g	70 - 110	3.5 g dw	n.q.	54
bivalves	0.2 ng/g	0.5 µg/g	69 - 99	0.5 g dw		59
bivalves	0.01 - 0.99 µg/L	0.02 - 2.99 µg/L	89 - 112	10 g	52.91 ng/g ww	61
bivalves	1 ng/g	1 ng/g	78 - 117	100 mg	6 ng/g	45
fish	0.01 - 5 ng/g	0.03 - 11.25 ng/g	70 - 100	2 g	0.18 - 518.9 ng/g	56
fish	0.15-0.6 ng/g dw	0.2-1 ng/g dw	76 - 120	200 mg	0.05 - 4.1 µg/L	48
fish				10 g		36
fish	0.01 - 9.04 ng/g	0.01 - 30.12 ng/g	69 - 120	5 g	5.7 ng/g	64

ww = wet weight, dw = dry weight

2.5 DISADVANTAGES AND LIMITATIONS

QuEChERS extraction was invented for extraction of pesticides from vegetables and fruits. Care has to be taken for the transfer of QuEChERS methodology as analytes may not always be present in a free form but bound to bio(macro)molecules which may render QuEChERS extraction less effective compared to other extraction procedures. This has been shown in extraction of contaminants bound to the exoskeleton of insects, where the extraction performance of QuEChERS was less efficient in comparison to microwave-assisted extraction [63]. This binding also changes bioavailability to higher organisms and possibilities of internal metabolism.

The extraction has to be optimized for analysis of new combinations of matrix and analytes. This labor intensive method development is a drawback, especially if salts used in dSPE and for phase separation are weighed in manually. The necessity of manual weighing in extraction protocols, where kits are not commercially available, is a further labor intensive step. Strong ion suppression in MS analysis occurring in biota experiments is another limitation, especially in case of samples rich in lipid or wax [51]. Clearly, the development of new dSPE materials opens the way to new fields of application by enhanced of more efficient removal of interfering matrix components to overcome these limitations.

QuEChERS is a LLE between at least two phases (water and acetonitrile) this results in an equilibrium partitioning of analytes between these two phases. Especially polar compounds tend to stay in the more polar aqueous phase so that recoveries are low. A possibility to overcome this limitation in food analysis was to use different extraction protocols, e.g. QuPPE (Quick Polar Pesticides Extraction) [76]. This extraction protocol uses acidified methanol and has been applied e.g. for extraction of paraquat and diquat from stark rich food such as grain [77]. To the best of our knowledge, applications explicitly addressing the QuPPE extraction in environmental analysis have not been published yet.

2.6 CONCLUSION AND FUTURE PERSPECTIVES

In this review applications of QuEChERS extraction procedure in environmental analysis were summarized. Various modifications were described: the introduction of another organic solvent (hexane or heptane) for extraction of lipid or wax rich samples like bees or earthworms to remove these interfering components during extraction. Acidic conditions were used to enhance the recovery of acidic analytes or compounds sensitive to high pH values. The high compatibility of QuEChERS extracts to GC and HPLC and the analysis of different solvent phases by different separation techniques make the procedure interesting for a broad range of chemicals. Coupling to MS allows to quantify substances in trace amounts as required for small samples and low micropollutant uptake.

The advantage of QuEChERS extraction is that it covers a broad range of polarity of analytes. This enables the simultaneous extraction of pharmaceuticals or pesticides and their metabolites as shown for diclofenac and boscalid. This feature makes QuEChERS extraction an interesting sample preparation technique also for screening methods. The extraction procedure has been applied for analysis of contaminants in biota sampled after poisoning incidents of bees or to determine the pollution of aquatic environments by analysis of invertebrates or fish. This demonstrates the broad applicability of QuEChERS to assess the contamination of aquatic and terrestrial environments by analysis of a broad range of contaminants and their transformation products in biota. Modifications of solvent volumes and homogenization procedures allow to analyze individual organisms e.g. bees or

gammarids or small numbers of daphnids and fish embryos. This opens the way to analyze small numbers of environmental samples or organism from laboratory experiments but also from the field. A review of figures of merit in several publications revealed that the extraction method has matured and reached acceptable robustness.

3 DETERMINATION OF CARBAMAZEPINE TRANSFER FROM EXPOSED MIDGE LARVAE TO ADULT MIDGES BY QUECHERS EXTRACTION AND HPLC-MS/MS ANALYSIS

3.1 ABSTRACT

In this study, the fate of carbamazepine was investigated during the metamorphosis from *Chironomus riparius* larvae to imagines. The aim of this project was the development of a method for the quantification of carbamazepine in midge larvae and midges. The developed extraction procedure was based on QuEChERS extraction and analysis performed by HPLC-MS/MS. Quantification limit for carbamazepine was 5 µg/L in 10 mg extracted tissue corresponding to approximately 3 midges or 2 larvae and a concentration of 12 ng carbamazepine per g tissue. Quantification based on deuterated internal standard was performed with a recovery of 95 ± 15 %. The developed method was applied to samples originating from emergence tests. For the first time, quantification of the internal concentration in larvae and midges allowed the investigation of pollutant transfer during the pupation process. Results indicate a transfer of 100 % body burden from larvae to midges. This shows a potential transfer of aquatic trace contaminants to terrestrial ecosystems due to uptake in aquatic larvae.

3.2 INTRODUCTION

Pharmaceuticals released into surface water from insufficient removal in wastewater treatment plants are of growing concern. These compounds exhibit the risk of uptake and bioaccumulation by aquatic invertebrates. Possible bioaccumulation in insect larvae give rise to the question of transfer along the food chain in terrestrial environments. A comprehensive understanding of this transfer has to include an understanding of pollutant fate during metamorphosis of for example aquatic living midge larvae to midges, a potential food source for terrestrial organisms. Midges, as well as midge larvae, are an important source of prey for birds, fish and other insectivorous predators in the aquatic and terrestrial environment [3]. Therefore, larvae and midges are of interest with respect to contaminant transfer in food webs. Effects of pharmaceuticals and pesticides on insect larvae have been studied, but their uptake and the internal concentration has been investigated much less. Especially the link between body burden and toxicity is not studied intensively. In this study, the internal concentration of the pharmaceutical carbamazepine (CBZ) in midge larvae and the transfer to midges was investigated.

3.2.1 Chironomids and contaminant transfer

Benthic living chironomid larvae are a dominant species in aquatic sediments [6]. The nonbiting midge *Chironomus riparius* is established as a model organism for ecotoxicological tests in several OECD guidelines to assess the impact of chemicals on aquatic invertebrates [78-81]. The important role in food webs of midge larvae makes them interesting as a vector for contaminant burden. The transport via body burden pollutants from the aquatic to the terrestrial environment after emergence by holometabolic insects with aquatic larvae and terrestrial imagines, has been investigated for trace metals [6] and PCBs [5].

Transfer of polychlorinated biphenyls (PCBs) from midge larvae, which were exposed via contaminated sediments, to tree swallows was observed in field studies [82]. Metamorphosis can affect the contaminant concentration in midges in two ways: by increasing or decreasing concentrations. For example, 3-fold higher PCB concentrations were detected in adult midges compared to concentrations in larvae [5]. The biomass of adult midges is approximately one third of the biomass of midges and thus the PCB burden changes, when calculated to biomass but not when calculated per individual. For polycyclic aromatic hydrocarbons and metals, predominantly lower concentrations (2 to 125-fold) were observed in midges compared to the one in larvae [83]. The impact of midges to work as vectors from aquatic to terrestrial ecosystems has been investigated for different metals in *Chironomus riparius*. Trace metals like zinc, copper and cadmium are known to accumulate in larvae, with different fate during metamorphosis. In case of copper, the metal was almost completely excreted from larvae over pupae to imagines and in case of zinc, the body-burden was decreasing from larvae over pupae to imagines [6]. For arsenic similar results were observed in laboratory experiments. 72 % of the arsenic burden was excreted between larval and adult stages [84].

For pharmaceuticals in midge larvae very little is known in general, especially with regard to metamorphosis. In this study, the uptake and transfer of the antiepileptic drug carbamazepine were closely investigated by analysis of larvae and midges, exposed in emergence studies, conducted by Katharina Heye at the Department of Aquatic Ecotoxicology at the University in Frankfurt.

3.2.2 The pharmaceutical carbamazepine

Carbamazepine (CBZ) is used in chronic pain therapy and against epilepsy [85], with an estimated consumption of 87 tons in Germany in 1999 [86]. The anticonvulsant acts by modulating Na^+ channels [87]. In humans, it is metabolized in the liver by cytochrome P450 by oxidation processes [85]. The main metabolites are the oxidized compound 10,11-dihydro-10,11-epoxycarbamazepine and its products 10,11-dihydro-10,11-dihydroxycarbamazepine from further hydroxylation [88]. 29% of carbamazepine orally administered by humans is excreted unchanged via feces and urine [86]. The removal rate of carbamazepine in sewage treatment plants can be as low as or even below 10 % [7] . Low removal rates in wastewater treatment plants are a result of carbamazepine's resistance towards biodegradation and low sorption rates to sludge [86]. Therefore, carbamazepine enters surface waters usually via treated wastewater. The high concentrations in surface waters make it a suitable indicator for the presence of municipal sewage contaminations [7]. In a monitoring study in Northern Germany in surface waters and wastewater treatment plant effluents concentrations of 84 - 790 ng/L were detected [74]. Another monitoring study carried out in Poland revealed carbamazepine residues in waste water and drinking water samples [89]. Presence in surface waters might lead to contamination of aquatic living organisms. An influence of 0.2 – 2 µg/L carbamazepine on ecosystems by alteration of organic litter degradation rates was observed in mesocosm studies [90]. In Indian rivers, a change in species abundance was observed with increasing carbamazepine concentrations [91]. In gammarids sampled in the UK, carbamazepine was detected in streams at low ng/g concentrations [32].

3.2.3 Effects of carbamazepine in chironomids

The toxicological impact of carbamazepine was under investigation. Carbamazepine was shown to cause effects in growth and mortality tests with *C. tenans* at environmentally relevant concentrations [8]. In sediment contact tests with *C. dilutus*, lower toxicity was observed, compared to tests without sediment. This was explained by reduced bioavailable

concentrations due to sorption to sediment particles [17]. An impact on hedging rate of *C. riparius* was observed for increasing exposure concentrations of CBZ [9].

3.2.4 Quantification of carbamazepine in biota

Carbamazepine residues and uptake rates were analyzed in different aquatic species. Bioconcentration factors were investigated in *Gammarus pulex* and *Notonecta glauca* based on radioactively labeled experiments [92]. For extraction of carbamazepine from fish tissue, different approaches were made. For example, pulverized liquid extraction with gel permeation chromatography for cleanup and analysis by UHPLC-MS/MS [30] or liquid-liquid extraction with hexane and ethyl acetate with subsequent analysis by HPLC-MS/MS [87].

Quantification of pollutants in chironomid larvae is challenging due to limited biomass available for analysis. For investigation of matrix effects liquid extraction of carbamazepine from chironomids was performed with acetone and samples processed by SPE. In HPLC-MS analysis the limit of detection for carbamazepine was at 0.86 µg/L in extracts from 9 – 12 chironomid larvae [65]. The extraction method used in this study was based on QuEChERS extraction (Quick Easy Cheap Effective Rugged and Safe) and optimized to quantify carbamazepine in small numbers of chironomids. QuEChERS extraction procedure was originally developed by Anastassiades for pesticide quantification in fruits and vegetables [2] and has recently been applied to environmental samples and biota including invertebrates. E.g. emerging pollutants were extracted from individuals of *Potamopyrgus antipodarum*, *Valvata piscinalis, Gammarus fossarum* and small numbers of *Chironomus riparius* larvae with this method and subsequently analyzed by Nano-LC-MS/MS [21, 47, 55]. QuEChERS was applied for detection of carbamazepine in earthworms [46]. The developed method required 10 mg tissue and analysis was performed with HPLC-MS/MS.

3.3 EXPERIMENTAL

3.3.1 Reagents, chemicals and consumables

HPLC solvents methanol hypergrade LC-MS (chromasolv), water hypergrade LC-MS (chromasolv), acetonitrile LC-MS grade (chromasolv) and formic acid (98%, eluent additive for LC-MS) were purchased by Sigma-Aldrich (Steinheim, Germany).

Carbamazepine, carbamazepine-$^{13}C_6$, sodium chloride and magnesium sulfate were purchased by Sigma-Aldrich (Steinheim Germany). PSA bulk sorbent and C18 bulk sorbent were purchased by Agilent Technlogies (Waldbronn, Germany).

The micro-homogenizer PP was supplied by Carl Roth (Karlsruhe, Germany) and PTFE syringe filter 0.45 µm, 3 mm by Macherey-Nagel (Düren, Germany).

3.3.2 Biological exposure tests

Biological tests are briefly described here, as test parameters are published elsewhere by Heye et al. [93]. The emergence test was performed according to OECD Guideline 233 for a 28 days life cycle test. Fourth instar larvae were used for analysis and adult midges hedged from exposed larvae. Samples were frozen in liquid nitrogen and stored at -70 °C until workup. Mortality, hedge and fertility were observed as endpoints. More detailed information was published elsewhere [93]. Larvae were exposed to carbamazepine in nominal concentrations of 0.025 - 3.2 mg/L for 28 days. 10 - 15 fourth instar larvae were collected per exposure level the day before hatching and stored at -20 °C until analysis. 5 - 30 midges per

concentration level from the same exposure studies were collected within 24 hours after hatching and stored at -20 °C. Potential sorption of propiconazole to sediment was determined by analysis of medium at the beginning of the exposure time after 14 days and at the end of the experiment after 28 days.

3.3.3 Analysis of exposure medium samples

Exposure medium samples were taken at the beginning of the emergence test, after two weeks and at the end of the treatment after 4 weeks. Samples were stored at -20 °C. Aliquots of 2 mL were centrifuged for 3 min at 10 000 rpm, filtered and analyzed by HPLC-MS/MS.

3.3.4 Extraction procedure of midges and larvae

25 mg frozen larvae were homogenized in liquid nitrogen with a micro-homogenizer. Isotopically labeled internal standard carbamazepine-$^{13}C_6$ in methanol was added resulting in a final concentration of 20 µg/L in 0.5 mL extract. After 1 h at room temperature, 0.5 mL acetonitrile and 0.5 mL water were added. For extraction, samples were shaken with a vortex device for 30 sec and 25 mg sodium chloride and 75 mg anhydrous $MgSO_4$ were added and the sample shaken for 30 sec. After 3 min centrifugation at 10 000 rpm 0.4 mL of the organic acetonitrile phase was either directly analyzed or cleaned by a dispersed SPE (dSPE) step.

Analysis of raw extracts: The organic layer was evaporated to dryness and the residue resolved in 0.25 mL methanol and filtered for analysis.

Cleanup by dSPE: The organic layer was transferred to an Eppendorf tube containing dSPE sorbent for cleanup. For dSPE 1) 12 mg PSA and 90 mg anhydrous $MgSO_4$ or 2) 12 mg C18 (non-endcapped) and 90 mg anhydrous $MgSO_4$ or 3) 12 mg PSA, 12 mg C18 and 90 mg anhydrous $MgSO_4$ were used. The sample was shaken for 30 sec, and after centrifugation for 3 min at 10 000 rmp, the acetonitrile phase was evaporated to dryness in a stream of nitrogen and the residue was reconstituted in 0.25 mL methanol. After filtration samples were analyzed by HPLC-MS/MS.

For calibration in matrix and method validation samples were spiked after homogenization and before extraction with carbamazepine. To investigate signal suppression, samples were treated as described above without addition of reference compounds in methanol before the extraction. Instead, carbamazepine and internal standard were added to the final extract before injection at a final concentration of 20 µg/L.

3.3.5 Instrumental methods

For LC-MS/MS analysis, a 1260 Infinity LC system coupled to a 6550 iFunnel QTOF HPLC-MS/MS system (Agilent Technologies, Waldbronn, Germany) was used. Aliquots of 10 µL sample were injected onto a Zorbax Eclipse Plus C18 column (2.1 x 150 mm, 3.5-Micron, narrow bore, Agilent Technologies, Waldbronn, Germany) at a column temperature of 40 °C. A jet stream electrospray ionization (ESI) source was operated with a nebulizer pressure of 35 psig, drying gas temperature of 160 °C, at a flow rate of 16 L/min and a fragmentor voltage of 360 V. In the positive ionization mode capillary voltage was set to -4000 V, skimmer voltage to 65 V and nozzle voltage to -500 V. The mass range was 100-1200 m/z with a data acquisition rate of 1 spectrum/s. For MS/MS spectra the acquisition time was set to 200 ms/spectrum and the masses (m/z = 243.1236 and 237.1022) were isolated in a range of m/z = 4 in a retention time window of 11 ± 1 min. The collision energy was set to 24

V and the mass range to 100 – 1000 m/z. The sheath gas temperature was set to 325°C with a flow rate of 11 L/min. For internal calibration purine and HP0921 (Agilent Technologies, Waldbronn, Germany, m/z = 121.0508, 922.0097) were used. A gradient elution at a flow rate of 0.3 mL/min using water, containing 0.1 % formic acid, and methanol was used. The initial content of 95 % water was decreased after 1 min to 5 % water over 7 min and after another 7 min at 5 % increased to 95 % water over 0.5 min. Data analysis was performed with MassHunter software (Agilent Technologies, Waldbronn, Germany).

3.3.6 Data analysis

MassHunter Workstation software quantitative analysis and qualitative analysis both Version B.06.00 (Agilent Technologies, Waldbronn, Germany) were used for analysis. Retention time of carbamazepine was 11.2 min in scan mode with target m/z = 237.1022 ± 100 ppm (carbamazepine) and m/z = 243.1236 ± 100 ppm (isotopically labeled carbamazepine).

Measured values were tested for normal distribution by Shapiro-Wilk-test with Origin 9.1.0 (OriginLab, Northampton, USA) at a 0.05 level. If normal distribution was proven, significant differences of variances were tested with one-way ANOVA with the software Origin 9.1 at a level of 0.05. The same software was also used for linear regression and fitting.

3.4 Results and Discussion

3.4.1 Method development

Samples were analyzed with HPLC-MS/MS as described in literature for carbamazepine residues in gammarid, fish, earthworm and sediment extracts [30, 46, 55, 65]. To account for matrix effects and analyte recovery during workup isotopically labeled internal standard (ISTD) was used for quantification. Matrix effects can lead to signal suppression or enhancement caused by interfering co-eluting matrix components. Analyte and internal standard have the same physicochemical properties exhibiting nearly the same retention times and signal response during separation and analysis. Molecular structures and labeling positions of the internal standard are presented Figure 4.

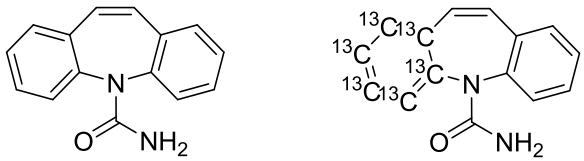
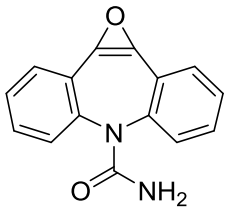
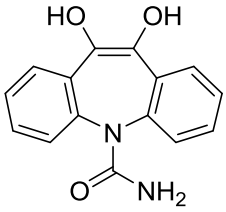


Figure 4: Molecular structure of the fungicide carbamazepine ($C_{15}H_{12}N_2O$, M = 236.27 g/mol) the isotopically labelled internal standard carbamazepine-$^{13}C_6$ ($^{13}C_6C_9H_{12}N_2O$, M = 242.22 g/mol).

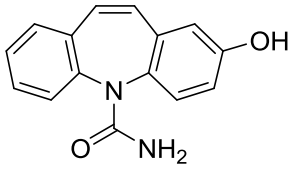
Three metabolites known from literature [93-94], structures and exact masses are given in Figure 5 were searched in the HPLC-MS data, but neither detected in larvae nor in midge samples.



10,11-epoxi-carbamazepine
$C_{15}H_{10}N_2O_2$
$[M+H]^+ = 251.0815$



10,11-dihydroxy-carbamazepine
$C_{15}H_{12}N_2O_3$
$[M+H]^+ = 269.0921$



3-hydroxy-carbamazepine
$C_{15}H_{12}N_2O_2$
$[M+H]^+ = 253.0972$

Figure 5: Metabolites known from literature [93-94], were analyzed but not detected in samples.

For separation, a C18 column was used and a gradient elution with methanol and water as solvent both containing 0.1 % formic acid was optimized (see Section 3.3.5). Extracted ion chromatograms of 237.1022 → 194.0971 and 243.1226 → 200.1171 were plotted with an extraction window of 0.1 m/z, an example is shown in Figure 6. The retention time for carbamazepine was 11.27 ± 0.04 min and for the internal standard 11.27 ± 0.04 min (n = 70 samples).

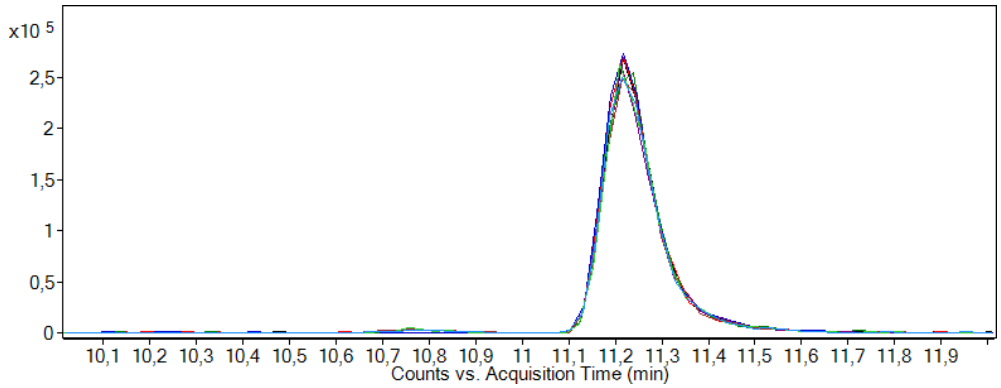


Figure 6: Extracted ion chromatograms in the MS/MS mode of carbamazepine 237.1022 → 194.0971 m/z and the internal standard 243.1226 → 200.1171 m/z in four measurements at a concentration of 20 µg/L in midge larvae extract.

MS/MS was used in order to enhance selectivity and signal to noise ratio. The MS/MS mode of the QTOF was optimized with respect to fragmentation voltage (300 – 400 V), collision energy (CE 20 - 40 V), nebulizer voltage (30 – 40 V), nozzle voltage (400 – 500 V) and octopole voltage (700 – 800 V). Best results were achieved for CE 24 V and a fragmentation voltage of 360 V in the positive ESI mode, the optimized method is described in Section 3.3.5. With these para meters, the most abundant fragment ion was formed by loss of the amide group, Figure 7 Hardly any other fragments were observed with these parameters, which is advantageous for limits of detection.

Masses of analytes and fragments used as quantifier are summarized in Table 4 together with retention times.

Table 4: Parameter for carbamazepine and isotopically labelled internal standard carbamazepin-$^{13}C_6$ in MS/MS analysis.

analyte	**formula**	**[M]**	**$[M+H]^+$**	**t_R [min]**	**fragment $[M\text{-}CONH_2+H]^+$**
carbamazepine	$C_{15}H_{12}N_2O$	236.27	237.1022	11.27 ± 0.04	194.0971
ISTD	$^{13}C_6C_9H_{12}N_2O$	242.22	243.1236	11.27 ± 0.04	200.1171

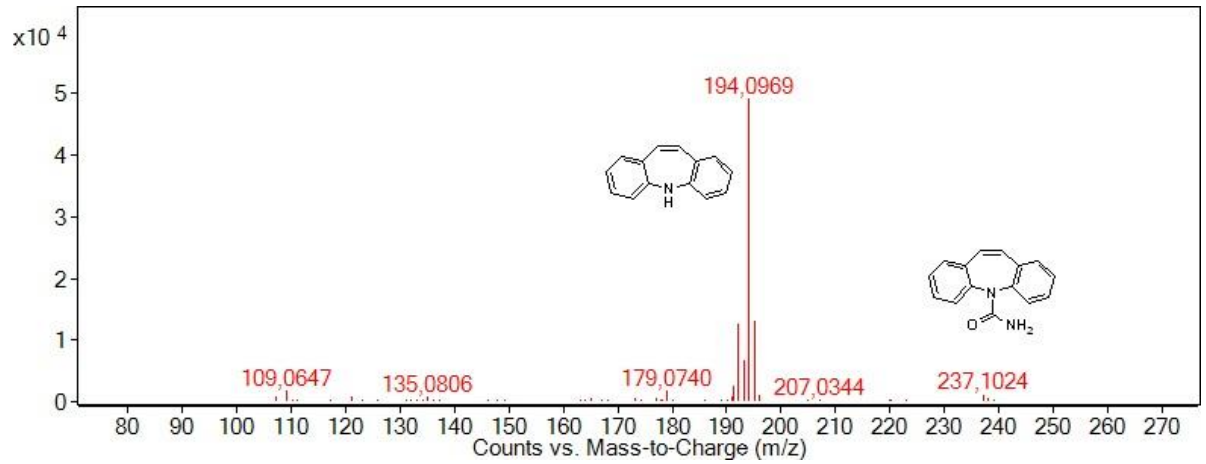


Figure 7: MS/MS mass spectrum of carbamazepine and its most abundant fragment ion at the retention time of 11.2 min, with CE 24 V and a fragmentation voltage of 360 V. Further parameters in Section 3.3.5.

3.4.2 Optimization of QuEChERS extraction procedure

Extraction was based on a QuEChERS extraction similar to the one applied for carbamazepine extraction from earthworms and aquatic invertebrates [21, 46]. For method development, different dSPE materials were tested for matrix removal after extraction. For this purpose, dSPE with C18 non-endcapped, PSA, the combination of both and the raw extract were compared. The comparison did not reveal significant differences for the signal area of extracts spiked before injection. However, the signal intensity of carbamazepine was always 1.18 times the signal intensity of internal standard at the same concentration. This effect may occur from different ionization efficiencies of both compounds. This was considered in the following measurements by correction of signal areas by this factor for calculation of the carbamazepine concentration. Evaluation based on signal intensity showed C18 as treatment with highest overall signal intensity and recovery; results are presented in Table 5. The relative standard deviation in spiked sample extracts was 2.8 % (n = 5) for signal areas.

Table 5: Comparison of effects of dSPE materials on signal intensity and recovery of carbamazepine in midge larvae. Signal area in sample + spike is the area detected in samples spiked before the extraction (this includes losses during workup and matrix effects), signal area in extracts + spike are extracts spiked before injection (this value only accounts for matrix effects). Signal suppression is calculated by 100 % - extract + spike. Recovery was calculated by signal area in extracts to signal area in extracts spiked after the extraction.

dSPE step	signal area sample + spike [%]	signal area in extract + spike [%]	recovery [%]	signal suppression [%]
PSA	34	78	43	22
C18	50	77	65	23
C18 + PSA	39	88	44	12
raw extract	46	76	61	24

Signal suppression was calculated by signal area of extracts spiked after extraction and before injection compared to signal area of standard in methanol at the same concentration.

$$\text{Signal suppression [\%]} = \frac{\text{area (CBZ in extract)}}{\text{area (CBZ in MeOH)}}\ x\ 100$$

Recovery was calculated by comparison of signal area in extracts spiked before extraction to the one in extracts spiked prior to measurement.

$$\text{Recovery } [\%] = \frac{\text{area (spiked before extraction)}}{\text{area (spiked before measurement)}} \; x \; 100$$

The greatest signal suppression was observed in raw extracts. Signal suppression is caused by interfering matrix components, and therefore this result for the uncleaned extract is likely. The highest signal intensity was detected in the extract cleaned with the combination of both dSPE materials, simultaneously removing polar and non-polar matrix components. The highest signal intensity of 88 % and 78% in the sample cleaned with C18 + PSA and PSA, however, were accompanied by the lowest recovery rates of 44 % and 43 % (see Table 5). In literature, comparable signal suppression of approximately 20 % was reported for carbamazepine signals in invertebrate extracts cleaned by hexane [47], but no effect on recovery by PSA/C18 cleanup [47].

The dSPE step partly removes analyte together with matrix components. Therefore, if enhancement of the signal area is greater than analyte loss during the extraction the implementation of a cleanup step results in overall higher signal intensities and lower limits of detection. In this experiment, the extracts cleaned by C18 revealed the highest overall signal intensity followed by the raw extract, although signal suppression was higher than in the other treatments. This indicates that interaction of PSA with carbamazepine is greater than with non-endcapped C18. In the final method the cleanup with C18 was implemented.

3.4.3 Method validation

Method validation for carbamazepine analysis in exposure medium

HPLC-MS/MS analysis was performed for filtered medium samples without preconcentration via SPE with cartridges as described in literature [65]. Calibration for carbamazepine in water was performed in the range of 0.1 – 32 µg/L with 10 calibration points, with an R^2 value of 0.9996 for linear regression, see Figure 8. Measurements revealed a limit of detection of 0.1 µg/L and a limit of quantification at 0.2 µg/L. Limit of detection was calculated as the concentration revealing a signal three times larger than the background noise (S/N = 3) and the limit of quantification by the DIN 32645.

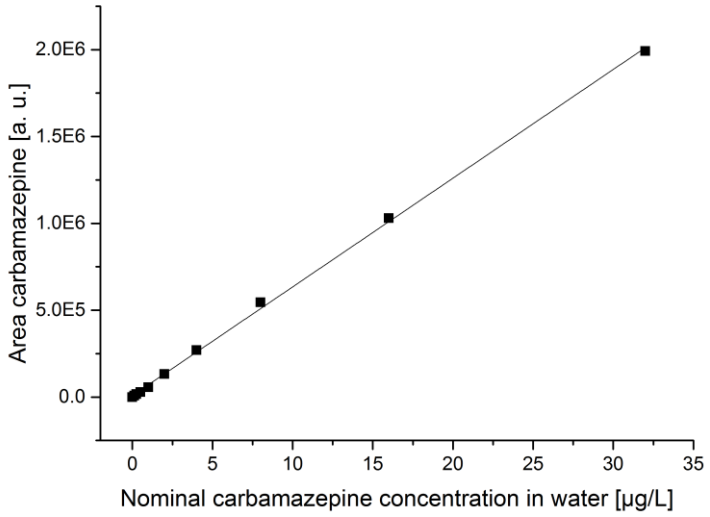


Figure 8: Calibration curve for the analysis of carbamazepine in medium revealing a limit of detection of 0.1 µg/L and limit of quantification of 0.2 µg/L. Calibration was performed in the range of 0 - 32 µg/L with an R^2 = 0.9996 analyzed in duplicates.

Method validation for carbamazepine analysis in larvae extract

Method validation was carried out with 20 mg tissue homogenates of uncontaminated larvae spiked with carbamazepine. Concentrations used for calibration were in the range of 1 - 40 µg/L in the extract to cover the typical concentrations in larvae. 3 replicates for each concentration level were extracted and treated with dSPE independently (see Section 3.3.4). Samples were spiked with 1, 2, 5, 10, 20 and 40 µg/L and 20 µg/L ISTD.

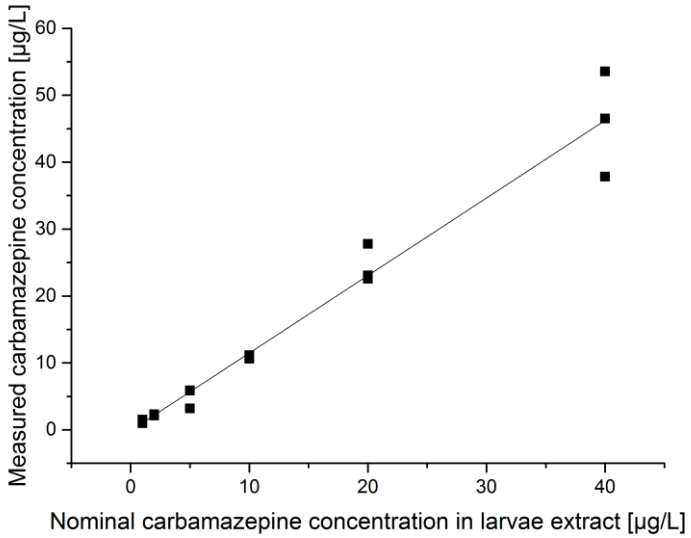


Figure 9: Calibration of carbamazepine in midge larvae extract performed in three independently treated replicates and five concentrations, analysis by HPLC-MS/MS. Linear regression with an R^2 value of 0.9947 and a limit of detection of 1 µg/L and limit of quantification of 5 µg/L, recovery in average was 95 ± 15 %. The detected carbamazepine concentration was calculated based on the signal area of isotopically labeled carbamazepine.

Using isotopically labeled standards has the major advantage, that analytical results can be corrected for losses during sample preparation and instrumental analysis, even if signal

suppression occurs [77]. LOD and LOQ are based on the signal to noise approach. Quantification was based on matrix-matched calibration using isotopically labeled internal standards. Samples revealed a satisfactory recovery of 95 ± 15 % based on the internal standard. Limit of detection and limit of quantification were calculated by S/N = 3 and S/N = 10. The method exhibited values of 1 µg/L and 5 µg/L, respectively. LOD and LOQ of carbamazepine in *C. riparius* larvae extracts are comparable with the ones reported by Dussault et al. They reported a LOD of 1.1 µg/L and a LOQ of 2.8 µg/L in biota [65].

3.5 APPLICATION

3.5.1 Analysis of exposure medium

Carbamazepine concentration in water samples was analyzed according to the method described in Section 3.3.3 at the beginning of the experiment at day 0, after two weeks at day 14 and four weeks at day 28.

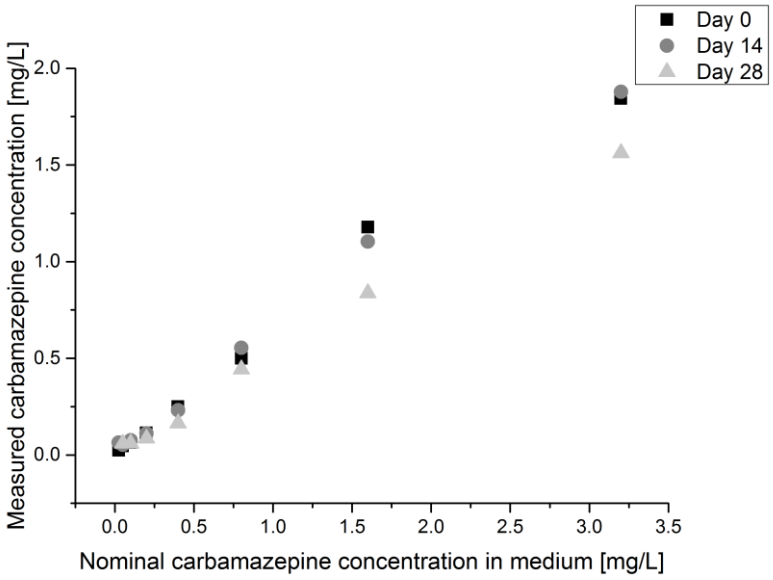


Figure 10: Carbamazepine concentration measured in exposure medium analyzed by HPLC-MS/MS at the beginning of the experiment, after 14 days and at the end at day 28.

The water concentrations at day 0 and day 14 were 63 ± 5 % of the nominal concentrations, presumably due to sorption to sediment. At day 28 the concentrations further decreased to 50 ± 6 % of the nominal concentration, pointing to minor degradation processes. This process was pronounced for higher exposure concentrations, especially for 1.6 and 3.2 mg/L. These findings confirm an almost steady exposure concentration during the treatment.

3.5.2 Analysis of carbamazepine transfer from larvae to midges during exposure studies

The developed QuEChERS extraction procedure, described above in Section 3.3.4, was applied to larvae and midges originating from emergence tests. Only limited numbers of individuals (larvae or midges) were available from these tests, therefore the number of individuals pooled for analysis differ between exposure concentration levels. Especially at high exposure concentrations, only small amounts of midges were available. Midge larvae were exposed to 0.025 – 3.2 mg/L carbamazepine in water for 28 days and larvae sampled

one day before pupation. Analyzed midges were sampled within 24 hours after hatching. In case of midges, 3 – 70 individuals and in case of larvae, 1 -15 individuals, were analyzed per replicate. For every exposure concentration organisms were pooled and divided into two replicates, which were treated independently during workup and analysis. Carbamazepine burden in tissue is given as ng carbamazepine per g wet weight tissue and quantification was based on spiked internal standard, Figure 11. Larvae of the negative control did, as expected, not contain any carbamazepine contamination.

With increasing level of exposure an increase in body-burden for larvae and emerged midges was observed in all treatment groups. In the exposure range tested, an increasing carbamazepine concentration for both midges and larvae was detected. For midges exposed to 0.8 mg/L carbamazepine different values for the two replicates were detected. The reasons for this are unknown. The signal area of internal standard differed in these measurements from other samples.

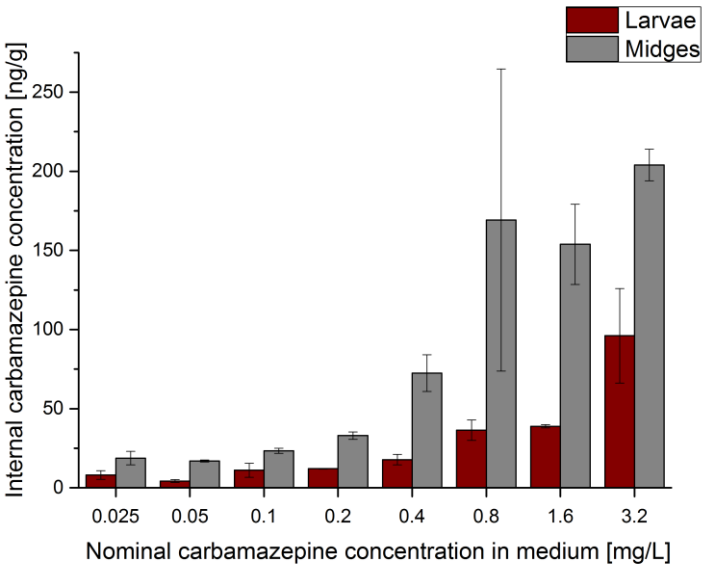


Figure 11: Internal carbamazepine concentration in larvae (average value from n = 4) and midges extracted with QuEChERS extraction and analyzed by HPLC-MS/MS.

The adult midges emerging from exposed larvae had a significantly higher body-burden by approximately 3-fold regardless of the exposure concentration. When interpreting the body-burden of larvae and midges, it has to be considered that biomass reduced by approximately 70 % during metamorphosis. An average weight of 2 mg was determined for midges and of 6 mg for larvae. If all carbamazepine taken up by larvae is retained upon metamorphosis, a 3-fold bioconcentrated burden in midges is expected. An increase by 3-fold has been observed in experiments with PCBs [83].

Exponential fitting showed an acceptable correlation of internal concentration analyzed in tissue extracts and exposure concentrations, shown in Figure 12. For larvae, a linear increase in incorporated concentrations could also be argued from the data.

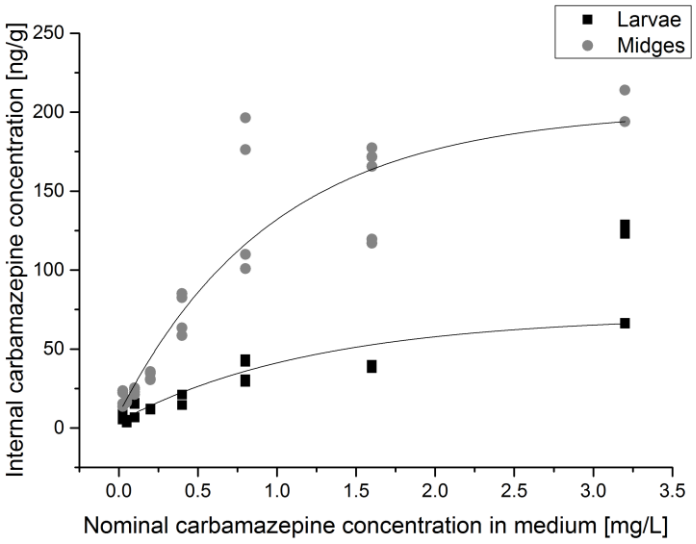


Figure 12: Exponential fit of internal concentrations in larvae and midge tissue extracts, shown in Figure 11.

To link the contaminant burden to the individual in contrast to biomass, the internal concentration per individual was calculated. An average weight of 2 mg was determined for midges and 6 mg for larvae. The data are plotted in Figure 13.

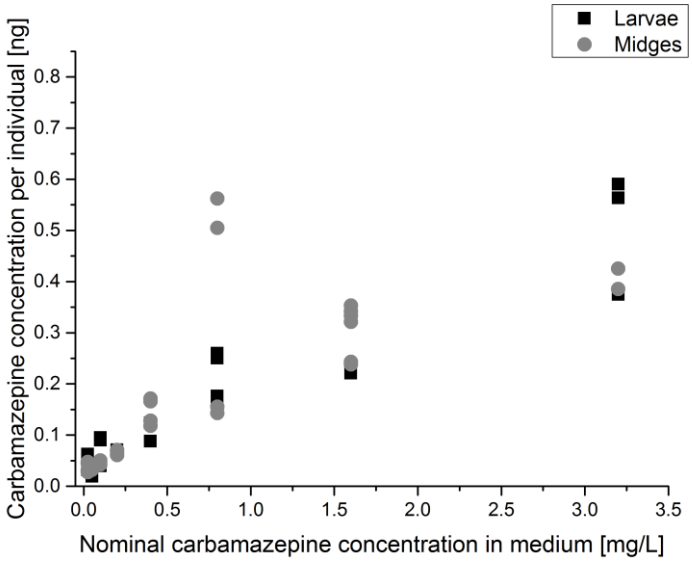


Figure 13: Body burden of carbamazepine in ng per individual larvae and midge, data from Figure 11. Average mass of 2 mg (midges) or 6 mg (larvae).

The results show that at each exposure level, the body-burden in larvae and hedged midges was equal when referring to individual insects and their development stage. No significant differences were observed. Reduced body weight upon metamorphosis concentrates carbamazepine and the body burden correlated to biomass thus increases in dose. This can be explained by the larvae lipid content which is in the range of 3 % dry weight [95] or 1.5 % wet weight [96] with increasing content for midges of 3 and 3.5 % wet weight for male and female midges [96].

The results confirm that for *C. riparius* no reduction in carbamazepine burden occurs during metamorphosis, when taking increasing lipid content or lowered weight into account. This may be explained by the hydrophobicity of carbamazepine (log K_{ow} = 2.45). This indicates that larvae do not have efficient excretory mechanisms for carbamazepine.

3.5.3 Bioconcentration factor

The bioconcentration factor (BCF), the relation of exposure concentration to the detected concentration in larvae was calculated. Taking into account body weight, the BCF values are:

$$\mathrm{BCF}\ [\frac{\mathrm{L}}{\mathrm{kg}}] = \frac{\text{concentration in biota } [\frac{\mathrm{mg}}{\mathrm{kg}}]}{\text{concentration in medium } [\frac{\mathrm{mg}}{\mathrm{L}}]}$$

BCF of carbamazepine was 0.056 ± 0.03 L/kg for larvae and 0.18 ± 0.08 L/kg for midges. The internal concentrations for the exposure concentration of 0.025 mg/L were not included for BCF calculation, because the internal concentrations were too low in this exposure experiment. The factor of 3 in bioaccumulation factors (BAF) between larvae and midges results from their 3-fold lower body weight, as shown in Figure 11 and Figure 12.

3.6 CONCLUSION

A quantification method for carbamazepine in both stages midge larvae and midges was developed. The miniaturized QuEChERS extraction procedure including a dSPE cleanup step with C18 enabled the detection of carbamazepine in small numbers of individuals. For analysis, only 2 larvae or 3 midges corresponding to approximately 10 mg were required. With the optimized method, carbamazepine was detectable at an LOQ of 5 µg/L corresponding to 12 ng carbamazepine per g wet weight tissue. Recovery based on ISTD was 95 ± 15 %. The method was applied to midges from emergence toxicity studies. The findings indicate a quantitative transfer of carbamazepine from larvae to adult *C. riparius* midges, and thus a potential for pollutant transfer from aquatic to terrestrial ecosystems and along the food web.

Due to a lack of literature data concerning micropollutants in chironomids, the results are compared to BCF calculated in other organisms: In *Gammarus pulex* Meredith-Williams et al. reported a BCF of 7.1 L/kg [92]. For the pharmaceutical formoterol with a log K_{ow} similar to that of carbamazepine (2.2 vs. 2.4 for carbamazepine) BCF values ranging from 32 to 42 L/kg were calculated for gammarids exposed to radiolabeled pharmaceuticals [97]. For fish *Pimephales notatus* and *Ictalurus punctarus* bioaccumulation factors of 2.5 - 3.8 in field experiments for carbamazepine were calculated [87]. Our results showed internal carbamazepine concentrations and thus BCF, which were 10 to 100-fold lower than in higher organisms. Hendriks et al. reported decreasing BCF values with increasing size of organisms in model calculations assuming on faster elimination rates by vertebrates [98].

Larvae accumulated considerable amounts of carbamazepine which were transferred to midges during metamorphosis. By bioaccumulation due to higher body burden pollutants are incorporated into the food web. The results presented here confirm the transfer of pharmaceutical wastewater pollutants by bioaccumulation in aquatic organisms to terrestrial ecosystems.

4 QUANTIFICATION OF THIACLOPRID IN CHIRONOMIDS EXPOSED TO THE NEONICOTINOID IN THE PRESENCE OF NANOPARTICLES WITH A MINIATURIZED QUECHERS PROCEDURE BY HPLC-MS

4.1 ABSTRACT

Toxicity studies often focus on active substances alone whereas in the environment natural and artificial nanoparticles are additionally present. This coexistence might lead to changes in the substances' bioavailability and uptake due to sorption processes. The uptake of the pesticide thiacloprid by *Chironomus riparius* larvae simultaneously exposed to the insecticide and nanoparticles was investigated. The sorption isotherms allowed the calculation of the bioavailable concentration of the pesticide in the exposure medium. In order to study the uptake by quantification of thiacloprid in *Chironomus riparius* larvae, an analytical workflow was developed including a miniaturized QuEChERS extraction procedure. The optimized method was applied to chironomid larvae from thiacloprid laboratory toxicity studies. Quantification was achieved with HPLC-MS. The developed method is based on the extraction of approximately 5 larvae and thus enables the quantification in few individuals. Thiacloprid concentrations were detected in larvae tissue at low ng/g wet weight range. This is the first time internal-concentrations of thiacloprid are directly linked to bioavailability and mortality of midge larvae.

4.2 INTRODUCTION

Pesticides are ubiquitous in the aquatic environment and might cause effects on non-target organisms. Insecticides are applied in conventional agriculture in the range of tons per year. In 2014 100 - 250 tones thiacloprid were sold for use in Germany additional 250 – 1000 tons were exported [99]. Upon application on the field, pesticides may enter surface waters via spray drift or runoff, and expose aquatic organisms [100].

4.2.1 The pesticide thiacloprid

One class of insecticides are neonicotinoids, which are dominated by the four compounds imidacloprid, thiacloprid, clothianidin and thiamethoxam. The use of neonicotinoids is strongly rising and they are among the five major classes of insecticides. In 2007 their market share comprised 24 % [101]. In 2010 256 tons of the active substances were applied in Germany, the second most commonly used among them was thiacloprid [102].

Advantages of neonicotinoids are their easy application and their high selectivity to target insects, combined with lower toxicity towards mammals and birds compared to insects. Neonicotinoids bind to the nicotinoidic receptor [12]. They are used for wood preservation and sprayed against sucking and chewing insects. Their neurotoxic effect on insects stems from irreversibly blocking the nicotinoidic acetylcholine receptor (nAChRs) and disrupting the central nerve-system of the insects [103]. The high persistence of these compounds leads to long lasting protection of field crops [103]. With regard to regulation, all four neonicotinoids are potential antagonists to the glucocorticoid receptor due to their molecular structure, containing an aromatic ring and bear agonistic groups [104]. Therefore and because of its endocrine disrupting properties, thiacloprid is a candidate for substitution [105]. In February

2018 the European Food and Safety Authority confirmed a high risk for honey bees and bumble bees by imidacloprid, clothianidin and thiametoxam, considering 1500 studies, and thus recommended to prohibit usage of these compounds for seed and soil treatments of crops attractive to bees [72]. Thiacloprid, the molecular structure is given in Figure 14, is more closely investigated in this study.

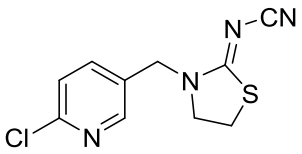


Figure 14: Molecular structure of the neonicotinoid thiacloprid ($C_{10}H_9ClN_4S$, molar mass 252.72 g mol^{-1}).

Disadvantages and ecotoxicological concern of neonicotinoids are their high toxicity towards non-target organisms. The problem is further augmented as neonicotinoids are systemic pesticides, which means the active substance is in the xylem and in dust and nectar. Therefore, bees are exposed to high concentrations during pollen collecting time [100]. With regard to neonicotinoids' fate in the environment, different observations have been made: in one study neonicotinoids were observed to be mobile in soil [99]. In aquatic ecosystems thiacloprid sorbs to sediments very fast and in field applications a DT50-value, the time during which 50 % of the initial concentration are degraded, up to 1000 days was measured [100]. In literature persistence against photolytic transformation under environmental conditions is reported [106] whereas photo-transformation products were detected in laboratory studies and vegetables [107-108]. Another environmentally relevant transformation path by microbes has been observed in soil [109-110]. Imidacloprid is the most frequently detected neonicotinoid in environmental samples [103]. Thiacloprid concentrations up to 4.5 µg/L were measured in German rivers in in monitoring studies [111]. Monitoring results in biota are discussed below.

4.2.2 Relevance of nanoparticles in the environment

Every particle with a size of 1 – 100 nm is characterized as nanoparticle [112]. Accordingly, the diversity of nanoparticles' physicochemical characteristics is very high. Natural nanoparticles are often biological organic matter like netted biomolecules for example peptides, proteins or polysaccharides. Also, nanoparticles from minerals emerge from weathering processes of stones [22] and can be found in the environment. In the aquatic environment, various naturally-born nanoparticles were detected [112]. In contrast to naturally-born nanoparticles synthetic particles from anthropogenic input are often functionalized, often causing different environmental behavior and toxicity compared to natural nanoparticles [113]. Their large surface is the most important property of nanoparticles, displaying a large reactive area [19]. In the environment particles undergo alteration processes, which may lead to aggregation of nanoparticles to larger particles and thus altered mobility and surface properties. To assess the environmental fate and relevance of nanoparticles, these processes have to be taken into account [19].

4.2.3 Effects of nanoparticles

Due to their small size, nanoparticles can be transported into cells and tissues via passive and active pathways [22]. Cell walls act as a barrier for nanoparticles or as primary interaction site [19]. Stable colloidal suspensions of nanoparticles in water can promote the uptake in organisms and toxic effects. In pore water in soil, fungi hyphae interact with water and compounds solved in the water. Nanoparticles can enter algae and fungi and sorb to organic material like polysaccharides and glycoproteins [19]. The impact of synthetic and natural-born

nanoparticles on organisms and the environmental fate has been investigated in several studies in the last years [19, 113]. In cells, oxidative stress and cell-membrane damage were reported as adverse effects, caused by the interaction of nanoparticles (e.g., different modifications of Al_2O_3) and proteins as well as lipid-saccharides [114]. Protection mechanisms against engineered nanoparticles are also reported, (e.g. they can act as antioxidants or nutrient stock) [19]. Effects on the behavioral level were observed in case of feeding and reproduction rate of *L. variegatus* in experiments with Ag nanoparticle concentrations of 1.1 g/kg [115].

4.2.4 Ecotoxicological impact of micropollutants bound to nanoparticles

Due to their high surface to volume ratio nanoparticles may serve as vectors or sinks for organic compounds including pollutants. A comprehensive view of the fate of micropollutants in the environment has to include particle-facilitated transport. The higher sorption capacities of smaller sediment particles in contrast to larger particles has been observed [116]. These effects observed for sediments can generally be expected to be even stronger in case of nanoparticles.

From an ecotoxicological point of view, sorption not only alters the transport and thus possibilities of exposure of micropollutants, but also the bioavailability of compounds may be changed significantly. Different ways of altered uptake can be considered for particulate matter (both nano- and macro-particles): (1) Nanoparticles could act as a vector when taken up by organisms. Filtering invertebrates, for example, ingest particles according to their size or carbon content [116]. Thus, adsorbed pollutants might be transported into organisms. For metal and metal-oxide nanoparticles uptake and distribution in terrestrial and aquatic organisms has been investigated [22]. The carrier effect has been observed in case of diuron bound to carbon nanotubes. In a toxicity study, the photosynthetic activity of algae was reduced, although sorption to carbon nanotubes reduced the bioavailable concentration of diuron. The higher toxicity was explained by increased local concentrations of diuron bound to carbon nanotubes [20]. Another example is an incident in a monitoring station, where effects on daphnids were observed without correlation to increasing pollutant concentrations in the river. In this case, particle-bound exposure was suspected [117]. (2) The exposure concentration may be lowered due to reduced bioavailability upon strong sorption. (3) A reduced bioavailability (internally or externally) could also lead to a reduced metabolism inside the organisms or physical or chemical degradation in the environment. These changed equilibrium conditions of the sorption processes can lead to chronic exposure as demonstrated for metal ions [22]. In equilibrium conditions, combined sorption and desorption processes resulted in a steady release of metal ions, leading to a chronic exposure to aquatic organisms [22]. (4) Especially hydrophobic compounds sorb to sediment particles rather than staying in the aqueous phase and thus lead to increased uptake and bioaccumulation in benthic macroinvertebrates [18]. For nonylphenols, it is suggested from laboratory experiments with *L. variegatus* tissues, that the ingestion of sediment plays an important role in accumulation and exposure [4]. For carbamazepine reduced bioavailability caused by sorption to sediment particles and thus reduced toxicity to chironomids was observed in exposure studies comparing tests with and without sediment present during exposure [17]. In this thesis, the bioavailability of the pesticide is investigated in detail in the presence of different nanoparticles. Different organisms are then exposed to mixtures of nanoparticles and thiacloprid for a deeper investigation of the interplay of toxicological effects and bioavailability.

4.2.5 Ecotoxicological studies with chironomids

In aquatic sediments, chironomids are the dominant species [6]. They are important for food web as prey to higher organisms like fish or birds [3]. Bioaccumulated pollutants in prey, for example, midge larvae are an important way of exposure for their predators [4]. The lipid content of organisms is often crucial for bioaccumulation, because lipophilic pollutants tend to accumulate in the lipid. Larvae lipid content is in the range of 3 % dry weight [95]. Incorporation of pollutants can lead to their transfer to higher trophic levels like fish or the terrestrial environment by transfer to birds. In case of sediment-bound metals and hydrophobic compounds, bioaccumulation in chironomids was reported [18, 118]. For this reason, chironomids are a model organism for ecotoxicological tests [78-81].

Chironomids are benthic living organisms and therefore are exposed to pollutants both present in water and bound to sediment with an equilibrium between sorption and desorption. This combined exposure might lead to higher pollutant concentrations in organisms than what may be expected from the pollutant concentration determined in water [17]. Further reasons for higher exposure concentrations are perturbating activities and filtration by benthic organisms, enhancing desorption processes and thus increasing bioavailability [17]. However, also other environmental factors such as presence of organic matter have to be considered [10]. The burrowing activity of midge larvae, known as bioturbation, induces transport of sediment-bound pollutants even from lower sediment levels to the water phase [5]. Aquatic insect larvae might ingest pesticides adsorbed onto sediment as part of their diet [119].

Pesticides are applied in agriculture and partly enter surface waters via runoff or spray-drift. Especially insecticides affect non-target organisms in rivers or streams. To assess the effect, laboratory toxicity studies are often carried out, for example, the toxicity of neonicotinoids to midge larvae has been investigated in numerous studies in recent years [3, 120-125]. In these publications, water and sediment concentrations are reported, but internal concentrations of neonicotinoids were not analyzed, although the internal concentration might give a better indicator of uptake than exposure concentrations [11]. Laboratory experiments with *C. riparius* revealed that actual body residues of the investigated compounds correlated more accurately with dose-response concentrations than water concentrations [10].

4.2.6 Analytical aspects of thiacloprid analysis

Extraction methods

Very few methods exist for emerging pollutant determination in midge larvae. The main disadvantage is their small size, necessitating miniaturization compared to related food analysis. To overcome low concentrations per individual, organisms need to be pooled (similarly to analysis of fish embryos [41]) to increase pollutant concentration in the extract. In addition sensitive analytical methods are mandatory. As conducted in this thesis, thiacloprid is mostly analyzed using HPLC-MS. To account for the high matrix load from the extraction of full organisms, optimized extraction protocols as well as sample cleanup is required. Thiacloprid as an insecticide has widely been analyzed in different kinds of vegetables and processed food like wine [126-132]. All studies used the multi-residue extraction method QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe). This is a liquid-liquid extraction procedure involving acetonitrile and water, originally invented for polar pesticide residue analysis in food by Anastassiades [2].

In ecotoxicology, neonicotinoids have been analyzed in different organisms and environmental matrices in recent years, because they are suspected to have adverse effects

on non-target organisms like bees. Depending on matrix complexity different extraction methods were used. In case of fish embryos, sonication and methanol extraction were sufficient, due to low matrix burden [133]. For more complex matrices, QuEChERS was applied with its advantage of low solvent and time consumption. Some applications for the analysis of neonicotinoids in environmental samples are given in Table 6.

Table 6: QuEChERS applied to environmental samples to analyze neonicotinoids in aquatic organisms, birds, bees and soil.

matrix	extraction	detection	compound	LOD	reference
fish embryos	methanol	HPLC-MS/MS	polar pesticides	ng/mL	[133]
gammarids	QuEChERS Hexane dSPE	HPLC-MS/MS	micro pollutants	ng/g	[40]
eagle owl blood	QuEChERS dSPE	HPLC-MS/MS	neonicotinoids	ng/mL	[62]
boluses	QuEChERS dSPE	HPLC-MS/MS	pesticides	ng/g	[63]
bumble bees	QuEChERS dSPE	UHPLC-MS/MS	neonicotinoids	0.02 ng/g	[53]
honey bees	QuEChERS dSPE	HPLC-MS/MS	pesticides	ng/g	[35]
honey bees and honey	MeCN/EA SPE	HPLC-MS/MS	neonicotinoids	100 ng/g	[134]
bees wax	Oxalic acid	CE-MS	neonicotinoids	ng/mL	[135]
honey	DLLME and QuEChERS	HPLC-DAD	neonicotinoids	ng/g	[136]
bee bread	QuEChERS	UHPLC-MS/MS	insecticides	pg/g	[137]
soil	QuEChERS dSPE	HPLC-DAD	imidacloprid and metabolites	ng/g	[138]

dSPE: dispersive solid phase extraction, MeCN: acetonitrile, SPE: solid phase extraction, EA: ethyl acetate, DLLME: dispersive liquid-liquid micro-extraction, HPLC: high performance liquid chromatography, MS: mass spectrometry, UHPLC: ultrahigh performance liquid chromatography, DAD: diode array detector

QuEChERS extraction was applied to analyze thiacloprid in terrestrial and aquatic organisms as well as honey and soil. Numerous publications concerning honey bees or bee products like honey, pollen or bee bread were published in recent years because neonicotinoids are suspected to cause bee death [134-135]. QuEChERS has been used in some of these publications to analyze individual bees or bumble bees [35, 53, 136-137]. To investigate the environmental distribution and bioaccumulation in the food chain, boluses and eagle owl blood were analyzed for neonicotinoids [62-63]. Method detection limits and concentrations detected in environmental samples are in the range of ng/g. Neonicotinoids and their degradation products have been extracted with the QuEChERS extraction protocol from soil [138-139] or pharmaceuticals from sewage sludge [140]. This technique was also used to determine emerging pollutants, among them thiacloprid in gammarids [40].

The only publication analyzing micropollutants, among them the pesticides diuron and spinosad, in midge larvae using QuEChERS extraction procedure with subsequent nano-LC-MS/MS analysis was published in 2014 by Berlioz-Barbier [21].

With respect to the limit of detection, different optimization approaches were applied. The impact of different extraction devices on extraction efficiency was investigated. Comparing the different techniques, vortexing was sufficient for analyte extraction from fish tissue [36]. Removal of coeluting and interfering matrix components was optimized by addition of a

dispersive solid phase extraction (dSPE) step after extraction. In this step, different sorbents are available for optimization. The sorbent should retain co-extracted matrix components, which might cause matrix effects, consequently, different materials are applied to different matrices. Common dSPE materials often applied in environmental analysis are PSA (primary secondary amines), graphitized carbon black (GCB), aluminum N and C18. PSA removes matrix components as fatty acids, organic acids, saccharides and some pigments from the QuEChERS raw extract [2]. Aluminum N acts comparable to PSA and removes matrix by interaction with H-bonding processes. GCB is potent for planar molecules like pigments or chlorophyll [2]. Another sorbent is silica removing polar matrix components [69]. For matrices containing lipophilic components addition of a hexane phase was implemented in some cases to remove nonpolar substances from gammarid, bivalve or honey bee tissue extracts [21, 45, 50].

In this thesis, a method is developed to quantify the internal concentration of thiacloprid in midge larvae exposed under laboratory conditions. Aluminum oxide and zeolite nanoparticles were used in the acute toxicity experiments with midge larvae.

4.3 EXPERIMENTAL

4.3.1 Reagents, chemicals and consumables

HPLC solvents methanol hypergrade LC-MS (chromasolv), water hypergrade LC-MS (chromasolv), acetonitrile LC-MS grade and formic acid (98 %, eluent additive for LC-MS) were supplied by Sigma-Aldrich (Steinheim, Germany). Thiacloprid was purchased by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Magnesium sulfate, sodium chloride, thiacloprid-d_4 were purchased by Sigma-Aldrich (Steinheim Germany). PSA bulk sorbent was purchased by Agilent Technologies (Waldbronn, Germany). Y30 nanoparticles were purchased by Thermo Fisher Scientific (Waltham, USA).

The micro-homogenizer polypropylene was supplied by Carl Roth (Karlsruhe, Germany) and PTFE syringe filter 0.45 µm, 3 mm by Macherey-Nagel (Düren, Germany).

4.3.2 Nanoparticles and sorption isotherms

Nano sized zeolite nanoparticles were investigated in sorption studies with different thiacloprid concentrations carried out by the Center of Geosciences, University of Tübingen, Germany. Free thiacloprid concentrations were reduced in exposure studies by addition of 5.2, 18.2 and 391.7 mg/L. More detailed information can be found elsewhere [141]. Aluminum oxide nanoparticles with 410 nm were synthesized and investigated in sorption studies with thiacloprid, but no sorption was observed. More detailed information can be found elsewhere [142].

4.3.3 Biological exposure tests

Toxicity tests were performed by the Institute of Evolution and Ecology, University of Tübingen, Germany. Briefly, mortality tests with fourth instar *Chironomus riparius* larvae were conducted with thiacloprid and nanoparticles and the combination of both. In zeolite experiments, the free thiacloprid concentration was reduced by sorption of thiacloprid to zeolite nanoparticles. In experiments with aluminum oxide particles, nanoparticles and thiacloprid were present without sorption. Every test vessel contained 30 mg quartz sand

and 100 mL test solution. Five fourth-instar larvae were exposed for 96 hours at 21 °C. More detailed information can be found elsewhere [141-142].

4.3.4 Instrumental methods

For HPLC-MS/MS analysis, a 1260 Infinity LC system coupled to a 6550 iFunnel QTOF LC/MS system (Agilent Technologies, Waldbronn, Germany) was used. Aliquots of 10 µL sample were injected onto a Zorbax Eclipse Plus C18 column (2.1x150 mm, 3.5-Micron, narrow bore, Agilent Technologies, Waldbronn, Germany). A jet-stream electrospray ionization (ESI) source was operated with a nebulizer pressure of 35 psig, drying gas temperature of 160°C, a flow rate of 16 L/min and a fragmentor voltage of 360 V. In the positive ionization mode capillary voltage was set to -4000 V, skimmer voltage to 65 V and a nozzle voltage to -500 V. The mass range was 100-1200 m/z with a data acquisition rate of 1 spectrum/s. The sheath gas temperature was set to 325°C with a flow rate of 11 L/min. For internal calibration solutions of purine and HP0921 (Agilent Technologies, Waldbronn, Germany, m/z = 121.0508, 922.0097) in methanol/water (95/5) were used and sprayed via a reference nebulizer. For HPLC separation, a gradient elution at a flow rate of 0.3 mL/min using water, containing 0.1 % formic acid, and methanol was chosen. The initial content of 95 % water was decreased after 1 min to 5 % water over 7 min and after another 7 min of 5 % water, it was increased over 5 min to 95 % water again.

For MS/MS analysis the compounds thiacloprid (m/z = 253.0309) and the deuterated internal standard (m/z = 257.0553) were isolated in a mass range of 4 m/z. A collision energy of 24 V was applied and a retention time window of 1 min. Spectra were acquired with 200 ms/spectrum at 50 – 400 m/z.

4.3.5 Data analysis

Data analysis was performed with MassHunter Workstation software quantitative analysis and qualitative analysis, Versions B.06.00 (Agilent Technologies, Waldbronn, Germany). Thiacloprid was characterized by m/z with a mass peak width of 50 ppm and by retention time with a window of 1 min.

Measured values were tested for normal distribution by Shapiro-Wilk-test with Origin 9.1.0 (OriginLab, Northampton, USA) at a 0.05 level. If normal distribution was proven, significant differences of variances were tested with one-way ANOVA with the software Origin 9.1.0 (OriginLab, Northampton, USA) at a level of 0.05. Not-normally distributed samples were tested with Kruskal-Wallis-test for differences with the software R (3.3.1, R Foundation for statistical computing). For linear regression in calibration experiments, the software Origin 9.1.0 (OriginLab, Northampton, USA) was used.

4.3.6 Sample preparation

Pre-studies on homogenization and extraction method

In pre-studies, the homogenization method was optimized using 70 mg frozen larvae as a starting point. For extraction and analysis, QuEChERS and HPLC-MS were used.

A) Homogenization in liquid nitrogen using mortar and pestle: 70 mg frozen larvae were homogenized in liquid nitrogen using mortar and pestle and 10 µL isotopically labelled standard in methanol was added. Homogenized samples were extracted with 0.5 mL water and 0.5 mL acetonitrile. After vortexing for 30 s, 50 mg sodium chloride and 150 mg anhydrous $MgSO_4$ were added. After vortexing for 30 s and centrifugation at 10 000 rpm for

3 min, the organic phase was transferred to an Eppendorf tube containing 25 mg PSA and 100 mg anhydrous $MgSO_4$. Samples were vortexed for 30 s and centrifuged for 3 min at 10 000 rpm. The organic phase was evaporated and the residue reconstituted in 250 µL methanol and filtered through 0.45 µm PTFE filters prior to HPLC-MS analysis.

B) Homogenization in liquid nitrogen using a micro-homogenizer: The same method as in A) was used, but homogenization was achieved using a micro-homogenizer (Carl Roth, Karlsruhe, Germany)

C) Homogenization with sand and mortar and pestle: For homogenization with sand 70 mg larvae were homogenized with mortar and pestle with addition of 0.5 g sand and 1 g Na_2SO_4. The homogenized sample was transferred to 15 mL Falcon tubes and a) 10 mL acetonitrile or b) 5 mL water and 5 mL acetonitrile were added. After vortexing for 30 s 750 mg anhydrous $MgSO_4$ and 250 mg sodium chloride were added to extract b, containing 5 mL water. Both samples were vortexed for another 30 s and centrifuged for 3 min at 10 000 rpm. For cleanup the organic layer was transferred to a Falcon tube containing a) 1 g anhydrous $MgSO_4$ and 250 mg or b) PSA 500 mg anhydrous $MgSO_4$ and 125 mg PSA. After vortexing for 30 s and centrifugation for 3 min at 10 000 rpm the supernatant was transferred, evaporated to dryness in a nitrogen stream at room temperature and reconstituted in 250 µL methanol, then filtered through a 45 µm PTFE filter prior to HPLC-MS analysis.

Final protocol for the processing of larvae samples for HPLC-MS analysis

For the final and optimized protocol using liquid nitrogen and a micro-homogenizer (Carl Roth, Karlsruhe, Germany) in Eppendorf tubes 20 mg frozen larvae (ca. 5 larvae in total) were needed. After homogenization 10 µL isotopically labeled thiacloprid standard (100 µg/L) in methanol was added and after evaporation at room temperature for 30 min in nitrogen stream, 0.5 mL water and 0.5 mL acetonitrile were added to the homogenized larvae. After vortexing for 30 s, 25 mg sodium chloride and 75 mg anhydrous $MgSO_4$ were added to induce phase separation. The sample was again vortexed for 1 min and centrifuged at 10 000 rpm for 3 min. The organic phase was transferred to an Eppendorf tube containing 25 mg PSA and 90 mg anhydrous $MgSO_4$. Samples were vortexed for 30 s and centrifuged for 3 min at 10 000 rpm. The acetonitrile supernatant was evaporated to dryness in nitrogen stream at room temperature and redissolved in 250 µL methanol. Prior to analysis by HPLC-MS the extract was filtered through a PTFE syringe filter.

Water sample preparation

For each concentration tested in exposure studies, four replicates, each containing 5 larvae, were analyzed. Overlaying water samples were taken at the beginning of the test and after 96 h of exposure and stored in 2 mL plastic tubes at -8 °C. Prior to analysis, samples were centrifuged at 3 000 rpm for 3 min and 1 mL supernatant was filtered through a PTFE syringe filter (pore size 0.45 µm, Chromafil) and analyzed by HPLC-MS.

4.4 RESULTS AND DISCUSSION

4.4.1 Method development

An extraction procedure based on QuEChERS extraction with HPLC-MS for analysis was developed. Coupling of HPLC and MS provides the sensitivity, selectivity and matrix

tolerance required for trace analysis of emerging pollutants in small sample volumes like midge larvae extracts.

HPLC-QTOF-MS method

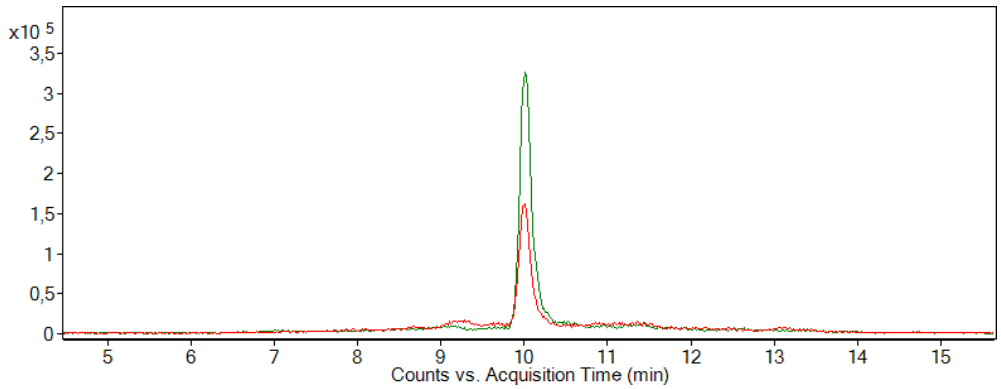


Figure 15: Extracted ion chromatogram of 50 µg/L thiacloprid (235.0309 in green) and 30 µg/L internal standard (257.0553 in red) each extracted with expansion of ± 0.01 m/z in larvae extract.

Thiacloprid in methanol was detected by HPLC-ESI(+)-QTOF in MS and MS/MS mode. The limit of detection (LOD) for both the MS and MS/MS mode was 0.2 µg/L. For quantification, deuterated thiacloprid-d_4 was used as internal standard (ISTD) to take into account possible signal suppression by co-eluting matrix components.

To avoid mistakes by external calibration and increase precision for extracts a deuterated analog of thiacloprid was used as internal standard (ISTD). With this ISTD analyte losses during the extraction process and impacts from signal suppression in the measurement can be corrected. The use of internal standards for quantification in a complex matrix like wastewater analysis is well studied [74].

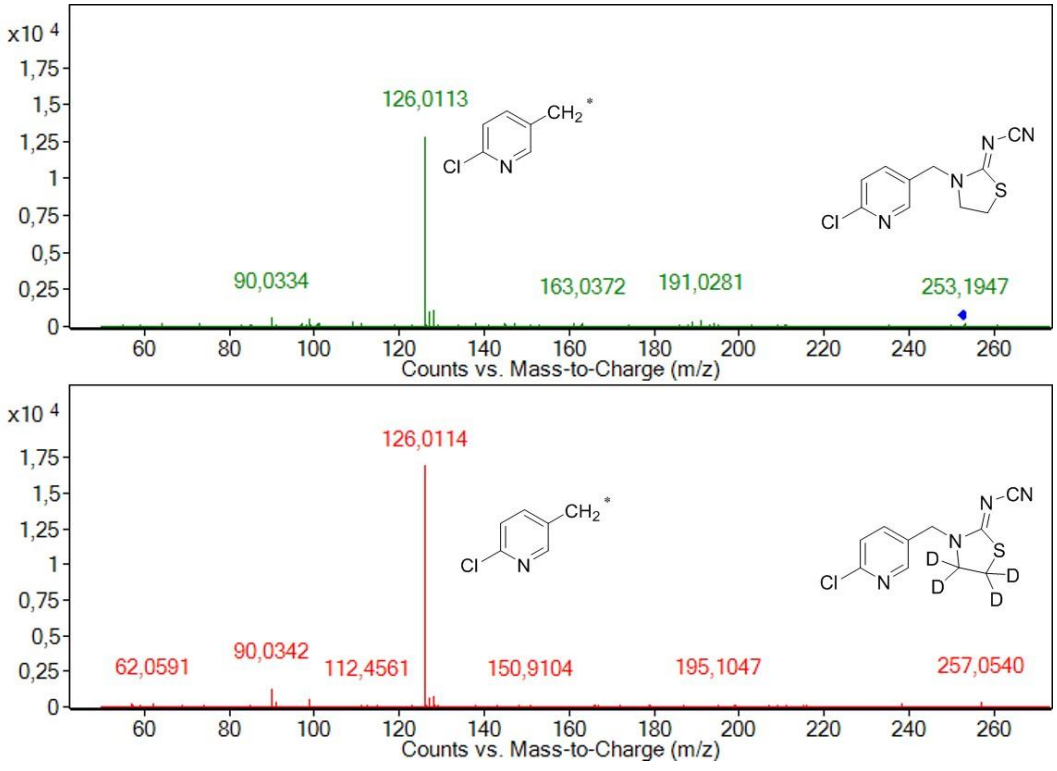


Figure 16: Signal intensity of thiacloprid precursor ion m/z = 253.0309 and isotopically labeled thiacloprid-d_4 m/z = 257.0553 and their most abundant fragment ion m/z = 126.0114 in MS/MS mode. 10 µL sample were injected

with 10 µg/L thiacloprid in methanol. For fragmentation CE 25 V, precursor m/z = 253.0309 and a fragmentation voltage of 360 V were used. The signals of the compounds co-eluted at a retention time of 10.1 min.

For thiacloprid and ISTD the most intense fragment ion in the MS/MS mode was at m/z = 126.0105 (Figure 16) and tenfold lower than that of the precursor ion in the MS mode. Therefore, signals of thiacloprid and ISTD could not be distinguished in the MS/MS mode, other fragments had much lower intensities and their use would have increased the limit of detection (LOD). Because of these reasons the MS mode was chosen and optimized for analysis in larvae extract.

Pre-studies on homogenization and extraction methods

Homogenization has two impacts on the extraction procedure: (1) more effective and faster extraction of the analyte and thus higher recovery but (2) often also increased co-extraction of matrix components possibly impairing the signal-intensity during HPLC-MS measurement [39]. Three procedures were evaluated for homogenization. Frozen larvae were homogenized with A) mortar and pestle in liquid nitrogen, B) a micro-homogenizer directly in the Eppendorf vessel where the extraction was carried out later on, and C) with mortar and pestle in combination with Na_2SO_4 as a drying agent and sand [143]. The subsequent liquid-liquid extraction with water and acetonitrile was carried out according to the method published by Anastassiades [2] with some adaptions (see Section 4.3.6).

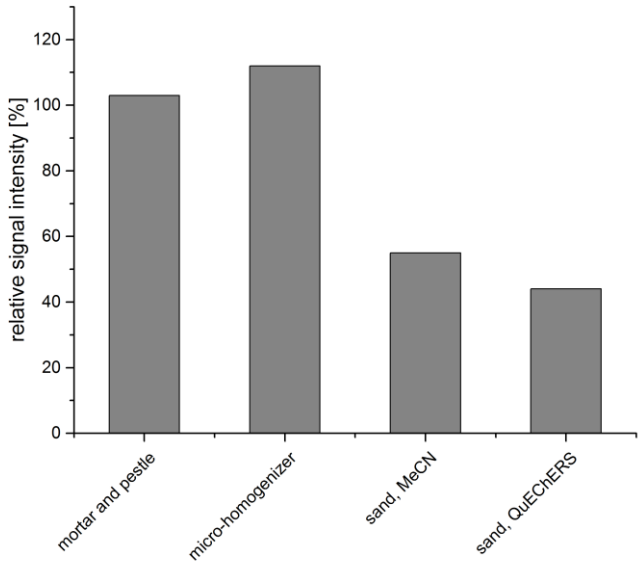


Figure 17: Relative signal intensities for thiacloprid in chironomid extract, processed following different preparation protocols a) homogenized with mortar and pestle or micro-homogenizer and b) extracted with acetonitrile or QuEChERS (protocol described in Section 4.3.6).

Signal intensities of extracts were compared to signal intensities of thiacloprid in methanol in the same concentration as the theoretical calculation for the sample injection solution. Figure 17 shows these relative signal intensities in % for different sample preparation protocols given in Section 4.3.6. In extracts homogenized with mortar and pestle and by micro-homogenizer in liquid nitrogen, relative signal intensities are in the same range of 100 – 110 %, whereas homogenization with sand leads to significantly reduced relative signal intensities below 60 %, Figure 17. The lower signal intensities in the two samples extracted with sand can be explained by higher solvent consumption, as in these samples 5 instead of 0.5 mL were used and 10-fold higher loads of salt, which might have removed analyte. The application of sand and anhydrous Na_2SO_4 required higher solvent volumes in the last

extraction protocol compared to other homogenization methods. Reduction of solvent consumption might have increased recovery and resulted in comparable results like the other protocols. However, due to easier handling, excellent relative signal intensities in the range of 100 % and expected small sample volumes in the following, the homogenization by micro-homogenizer and liquid-liquid extraction with acetonitrile and water was optimized.

Sample cleanup by dSPE

In the first step of QuEChERS extraction analyte is extracted from the matrix. As water and acetonitrile are miscible phase separation has to be induced by addition of salts, NaCl and $MgSO_4$. In a second step, the organic extract can further be cleaned in a dSPE, implemented to remove interfering matrix components. Matrix effects often result in signal-suppression by changes in the ionization process during the measurement by biological co-extracted matrix components [65]. The effect of co-eluting compounds arising from the matrix can result in signal enhancement or suppression [66]. Dilution of samples equally dilutes analyte and matrix components. Therefore less signal-suppression and higher analyte signal intensities may occur [67].

To investigate the possible signal suppression, samples were spiked before extraction or before analysis. Chromatographic signals were then compared to standard measurements injected at the same concentration in methanol. PSA was reported in the literature to reduce matrix effects in gammarid and honey bee samples analyzed for thiacloprid residues [35, 40]. Therefore, PSA was chosen for dSPE of larvae extracts.

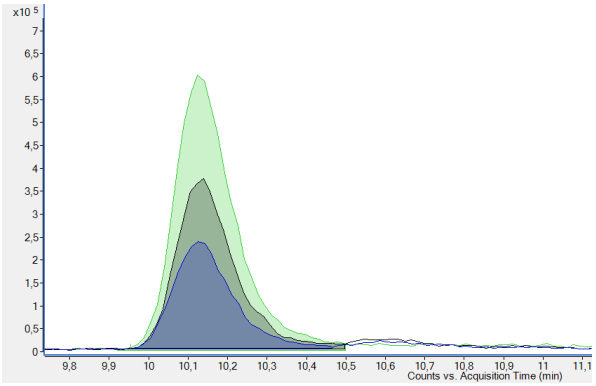


Figure 18: Signal intensities of thiacloprid m/z = 253.0309 in methanol (green), thiacloprid in PSA cleaned extract (brown) and raw extract (blue). Samples extracted as described in Section 4.3.6.

As shown in Figure 18 signal intensity of thiacloprid in raw extracts was 34 ± 4 % compared to thiacloprid injected in methanol. An additional cleanup step with PSA enhanced signal intensity to 67 ± 2 %. Thus, cleanup with PSA led to 100 % higher signal intensities, compared to measurements of the raw extract. The addition of a cleanup step was mandatory to remove interfering co-extracted matrix components and thus enhance signal intensities also in other publications. E.g. Kiljanek reported matrix effects for thiacloprid in honey bee QuEChERS extracts; after dSPE 2-fold higher signal intensities were achieved [35].

In all sample measurements, significant signal suppression for the target analyte was observed despite the cleanup step with PSA. Further corrections were implemented using the ISTD to account for persisting matrix effects and analyte losses during extraction [144].

The impact of the amount of co-extracted matrix components on signal suppression was briefly investigated by measuring extracts with increasing amounts of larvae from 10, 30 to 50 mg per extracted sample. Each sample was spiked with 10 µg/L thiacloprid after

homogenization and before extraction. The concentrations detected in the dilution experiment were 11 ± 0.5 µg/L, 8 ± 0.3 µg/L and 10 ± 0.5 µL for 10, 30 and 50 mg tissue, respectively. The results show that the method is robust with regard to co-extraction of the matrix in the sample range of 10 – 50 mg, as no correlation between matrix amount and signal-suppression was observed.

4.4.2 Method validation

Precision, recovery and matrix effect

The inter-day repeatability of the signal area of five measurements at 5 µg/L was 3 % without calculation on internal standard. Recovery is defined as the ratio of the measured peak-area of thiacloprid to the peak-area of the analyte in spiked samples in %. In this thesis, a recovery based on deuterated internal standard (ISTD) was 100 ± 5 % (n = 12). For thiacloprid, a retention time of 10.005 ± 0.005 min (n = 12) and for the internal standard of 10.001 ± 0.007 min (n = 12) was measured with HPLC.

The matrix effect is quantified via the ratio of the analyte peak intensity of a spiked sample to the one of the analyte standard in solvent [145]. A matrix effect of 27 ± 2 % signal suppression of thiacloprid in matrix compared to methanol was detected. The matrix effect was calculated as the ratio of the signal area of thiacloprid in larvae extract, spiked before analysis and after the extraction, and signal area of thiacloprid standard in methanol as a reference.

Limit of detection and linear range

Determination of LOD and LOQ in matrix were based on external calibration experiments. Unburdened larvae material, purchased as fish feed, was spiked with concentrations of 0.5, 1, 2, 5, 7, 10, 15 and 20 µg/L thiacloprid in triplicates. Sample homogenization, extraction and cleanup were performed according to the optimized procedure, described in Section 4.3.6. Limit of quantification (LOQ) was determined as the analyte concentration producing a peak intensity ten times larger than background noise (S/N = 10). The limit of detection was determined based on a signal intensity three times larger than background noise (S/N = 3) with 0.5 µg/L. Limit of quantification was 1 µg/L corresponding to 12 ng/g wet weight using 20 mg midge larvae (ca. 6 individuals). This corresponds to a limit of quantification of 1 µg/L in a sample volume of 250 µL.

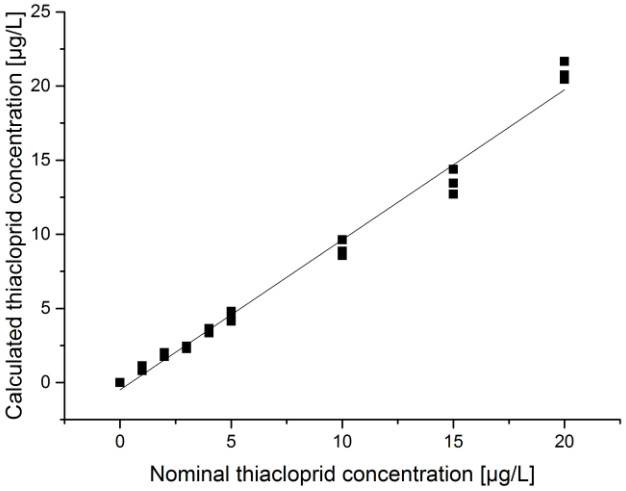


Figure 19: Calibration curve of thiacloprid in larvae extract, with R^2 = 0.98623 and a slope of m = 1.01189. Signal areas are corrected based on ISTD. For each concentration, three independent samples were extracted with the optimized QuEChERS protocol and cleaned by dSPE with PSA.

For method linearity, correlation coefficients of R^2 = 0.98623 were achieved for triplicate experiments with spiked matrix in the concentration range of 0.5 – 20 µg/L. The calibration curve is shown in Figure 19.

Table 7: Method validation parameters for thiacloprid in water and larvae extract.

	water	**larvae extract**
LOD	0.2 µg/L	0.5 µg/L
LOQ	0.5 µg/L	1 µg/L
R^2	0.9991	0.98623
m	201668	1.01189
linear range	0.2 – 10 µg/L	0.2 – 10 µg/L
matrix effect		27 ± 2 %
recovery		100 ± 5 %

Method validation parameters are listed in Table 7. LOD and LOQ are in the order of literature values. Berlioz-Barbier et al. analyzed 3 - 4 pooled chironomid larvae and reported an LOD in the ng/g range using a nano-LC-MS/MS method. They were able to detect concentrations in larvae of 1 - 50 ng/g wet weight (ww) [21]. The LODs obtained in this thesis are in the same range with 500 ng/L, corresponding to 12 ng/g ww, and the analysis is possible with standard equipment by HPLC-ESI-QTOF. Three publications analyzing thiacloprid in bees, bumble bees and gammarids reported a LOD in the low ng/g or pg/g range, but in these publications 100 - 200 mg sample were consumed [35, 40, 53]. Thus 5 to 10 times more, than in the method developed in this thesis.

The optimized method was applied to analyze thiacloprid residues in midge larvae and their exposure medium from toxicity tests under laboratory conditions.

4.5 APPLICATION

4.5.1 Analysis of exposure medium samples

For toxicity studies, the bioavailable thiacloprid concentration in water was reduced by sorption to nanoparticles. In every test vessel, thiacloprid was added at a concentration of 5 µg/L in case of aluminum oxide and 1 µg/L in case of zeolite nanoparticle experiments. To reduce the bioavailable concentration nanoparticles were added in different concentrations. The water concentration was analyzed at the beginning of the test (t_0) and end of the test after 96 hours (t_{96}). Within test duration, no transformation or degradation processes of thiacloprid in water were visible. The vessel contained water, sediment and nominal thiacloprid concentrations. The nominal concentrations were calculated for the water phase without considering sorption processes to sand. Therefore, reduced water concentrations are due to sorption processes of thiacloprid to sediment. Measured thiacloprid concentrations are shown in Figure 20.

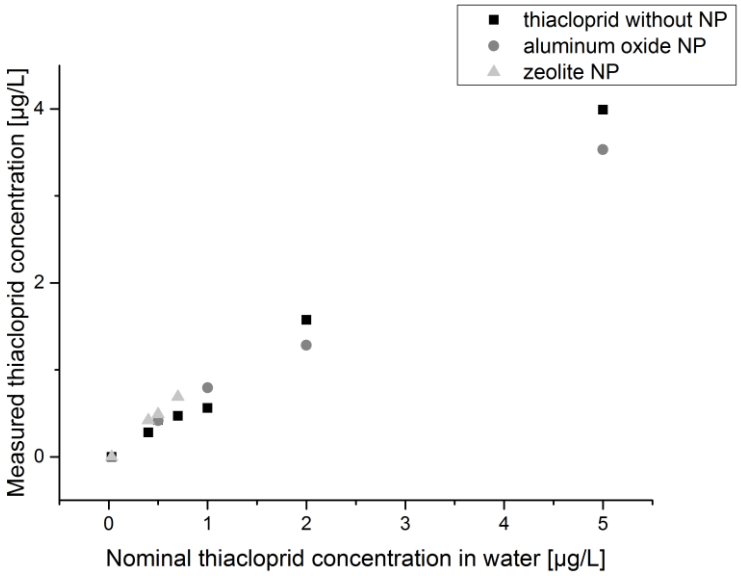
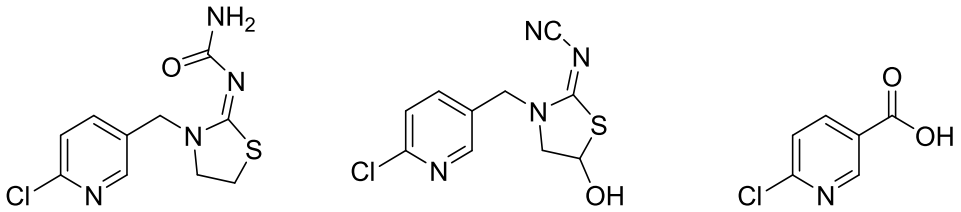


Figure 20: Detected thiacloprid concentrations in water samples measured in two replicates per concentration. Different nominal thiacloprid concentrations were applied (■) or thiacloprid concentrations were reduced by addition of nanoparticles from 1 µg/L in zeolite experiments (▲) and 5 µg/L in aluminum oxide experiments.

The measured concentration in the experiments without nanoparticles was 81 ± 2 % of the nominal concentration, most likely due to sorption to sand of about 20 %. In case of aluminum nanoparticles, the detected thiacloprid concentration was 74 ± 7 % of the nominal concentration. In these experiments the same nanoparticle concentration of 300 mg/L was applied to every thiacloprid concentration. The higher sorption rates and higher variability in experiments with aluminum oxide nanoparticles might be due to changes in sorption behavior of particles and thus altered sorption processes during the experiments. In experiments with zeolite nanoparticles, the measured concentration in water was 81 ± 17 % of the nominal concentration, so it was in the same range for experiments without nanoparticles, indicating that additional sorption to sand is about 20 %. Degradation of thiacloprid can be excluded, because degradation products known from literature [146] were not detected. Chemical structures of degradation products are shown in Figure 21.



$C_{10}H_{11}ClN_4OS$ 270.74 | $C_{10}H_9ClN_4OS$ 268.72 | $C_6H_4ClNO_2$ 157.55

Figure 21: Degradation products analyzed in tissue and water extracts, thiacloprid-amide, 4-hydroxy-thiacloprid and 6-chloro-nicotinic acid, known from literature [146].

Hydrophobic compounds tend to sorb on sediments and organic material rather than being dissolved in the aqueous phase, therefore, in toxicity studies the bioavailable concentration in water correlated with organic carbon content [147]. In a study with *Chironomus riparius* larvae exposed to herbicides (ioxynil, pendimethalin and bentazone) and sediment with different characteristics, particle size and organic content affected the environmental fate of the chemicals and the bioaccumulation potential [148]. Coarse particles with higher organic content led to higher bioaccumulation potential than experiments with sediment containing higher inorganic matter e.g. clay [148].

4.5.2 Toxicity test samples

Analyzed larvae originated from toxicity studies. Larvae were exposed to different thiacloprid concentrations in single substance experiments (only thiacloprid or only nanoparticles) for 96 h. Midge larvae are filter feeder and filter the water with nanoparticles for food particles. Incorporation of nanoparticles was observed for both kinds of particles [141-142]. Samples with nanoparticles only, showed, as expected, no internal concentration and no interference with the analyte during LC-MS analysis. In combination tests, the bioavailable concentration (nominal after sorption) of thiacloprid was calculated and reduced by addition of thiacloprid-sorbing nanoparticles with known sorption isotherms (see Section 4.3.2). Midge larvae were exposed in 5 replicates, collected after 96 h and frozen at -20 °C until extraction. For analysis, all 5 replicates had to be pooled and were divided in 1 or 2 samples for each concentration to obtain the required number of organisms.

Larvae exposed to thiacloprid

Midge larvae were exposed to solely thiacloprid at concentrations ranging from 0.03 – 5 µg/L. After 96 h of exposure, all living larvae were pooled and divided in duplicates. These duplicates for each concentration, were possible as only 20 mg sample were required for the method. In case of 1 and 5 µg/L high mortality did not allow to split the sample.

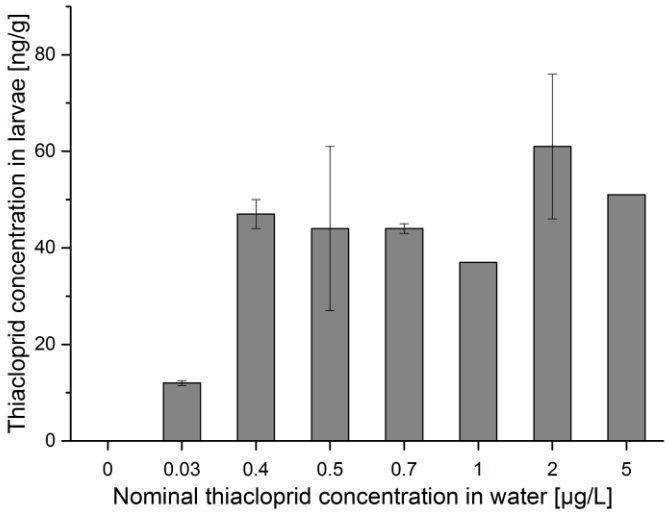


Figure 22: Internal thiacloprid concentrations in QuEChERS extract originating from midge larvae which were exposed to different thiacloprid concentrations. Thiacloprid concentrations were calculated depending on the signal area of internal standard and extracted following the developed protocol described in Section 4.4.1. Thiacloprid was analyzed by HPLC-MS in 10 µL sample volume with the method described in Section 4.3.5.

Larvae exposed to 0.4 – 5 µg/L thiacloprid showed no difference in the internal-concentration with 47 ± 7 ng/g wet weight larvae. Only larvae exposed to a nominal concentration of 0.03 µg/L thiacloprid showed a significantly ($F = 113$, $p = 0.05$) lower internal concentration of 12 ± 0.5 ng/g, data given in Figure 22.

These results can be explained by the fact that larvae were gained from a mortality test. Mortality is a harsh endpoint, which is observed at the relatively high exposure concentrations used. The doses calculated via the internal concentrations might not lead to differences in the response for an endpoint like mortality. In the second lowest concentration of 0.4 µg/L, which is more than 10-fold higher than the lowest concentration, already 30 % mortality was observed. This indicates that the experiments were carried out in a concentration range, where all larvae cope with the same internal concentration. Sublethal effects might occur at lower concentrations, and the internal concentration might be of relevance for lower exposure concentrations. To further support the hypothesis of differences in uptake and thus bioaccumulation at lower concentrations and a maximum internal concentration of ca. 50 ng/g, further investigations at lower exposure concentrations are required.

Larvae exposed to thiacloprid and zeolite nanoparticles

In contrast to exposure studies with thiacloprid only (Figure 22) exposure experiments with nanoparticles revealed the same internal concentration for all samples (see Figure 23). The analyzed internal concentration varied between the treatments from 36 - 53 ng/g wet weight, but the mean values correlated well with the results from exposure studies without nanoparticles. Within duplicates corresponding to one exposure concentration, the variability of detected internal concentrations was 0.5 – 3 % for zeolite experiments.

For the lowest exposure concentration this finding might be due to the incorporation of nanoparticles by the larvae, followed by desorption processes of thiacloprid in the body. Another possibility is that nanoparticles incorporated by the larvae kept thiacloprid sorbed in the gut, but the extraction conditions with acetonitrile and water led to desorption of

thiacloprid from the zeolite particles and detection in HPLC-MS. Incorporation of zeolite nanoparticles was observed in the toxicity experiment [141].

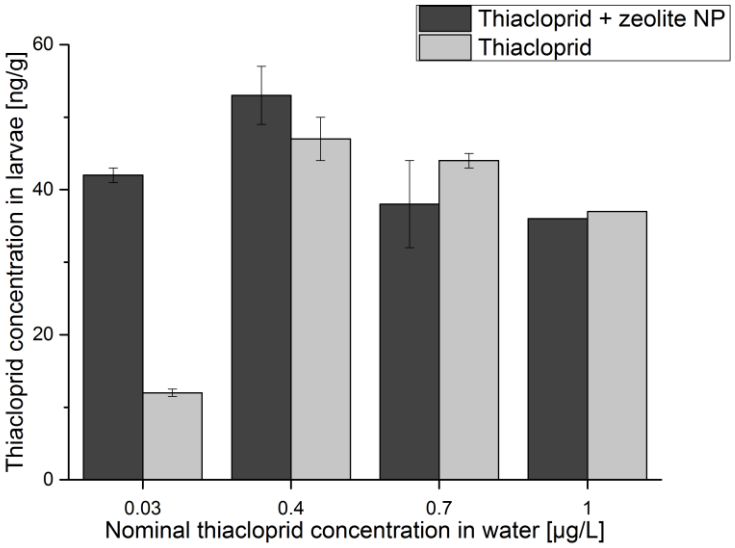


Figure 23: Internal concentration of thiacloprid in larvae exposed to zeolite nanoparticles and thiacloprid (black) and different thiacloprid concentrations without nanoparticles (grey) as reference. Bioavailable thiacloprid concentrations were reduced from 1 µg/L by addition of zeolite nanoparticles at concentrations of 5.2, 18.2 and 391.7 mg/L resulting in 0.03, 0.4 and 0.7 µg/L free thiacloprid, respectively.

Nanoparticles with a higher affinity to organic matter than water rather tend to stay in the larvae gut than to be excreted. In order to verify this hypothesis, larvae were placed in new vessels after exposure with thiacloprid and zeolite nanoparticles for 96 h and were exposed to pure water for further 24 h with the expectation of excretion of nanoparticles. However, after 24 hours in thiacloprid free medium, the nanoparticles were still present, which was proven by microscope analysis [141].

Larvae exposed to thiacloprid and aluminum oxide nanoparticles

The analysis of larvae originating from exposure studies for 96 h to aluminum oxide nanoparticles and thiacloprid showed similar results as in the previous experiments with zeolite nanoparticles. Comparison of data of the experiment with nanoparticles and without nanoparticles are shown in Figure 24.

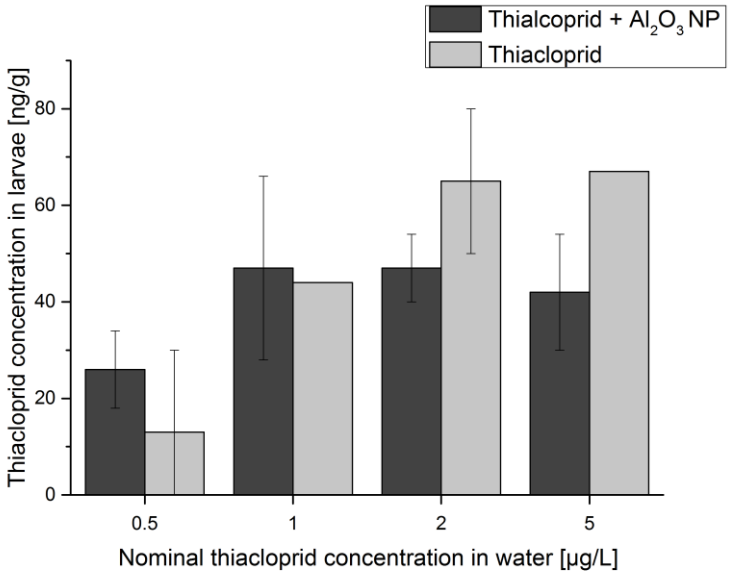


Figure 24: Internal thiacloprid concentration of larvae exposed to 300 mg/L Al_2O_3 nanoparticles and thiacloprid (black) and thiacloprid solely (grey) as reference. Larvae were pooled and analyzed in duplicates corresponding to the same exposure concentration. Samples were analyzed with the developed extraction procedure and quantification based on internal standard.

The internal concentration in larvae exposed to the lowest nominal thiacloprid concentration of 0.5 µg/L showed slightly lower internal concentrations of 26 ng/g in average than larvae exposed to higher thiacloprid concentrations. In experiments with aluminum oxide nanoparticles in average thiacloprid concentrations of 53 ± 4 ng/g within a range of 26 - 76 ng/g were detected in larvae. The variability within duplicates of 7 - 19 ng/g is greater than in experiments with zeolite nanoparticles. This might be due to higher exposure concentrations, because the variability in experiments without nanoparticles is also higher at higher thiacloprid concentrations. Another reason could be, that the variability in detected exposure concentrations compared to nominal concentrations expected by calculation according to sorption isotherms, was higher than in the zeolite nanoparticle experiment (see Section 4.5.1).

Analysis of larvae died during exposure experiments

To find the lethal internal concentration of thiacloprid to chironomids, larvae which died during the exposure experiments were analyzed. Most analyzed larvae died between 72 and 96 hours of exposure. Samples originating from all three treatments, without nanoparticles, with zeolite and with aluminum oxide nanoparticles were analyzed. Results are presented in Figure 25. The average internal concentration overall larvae was 54 ± 11 ng/g wet weight, independent of the treatment during exposure.

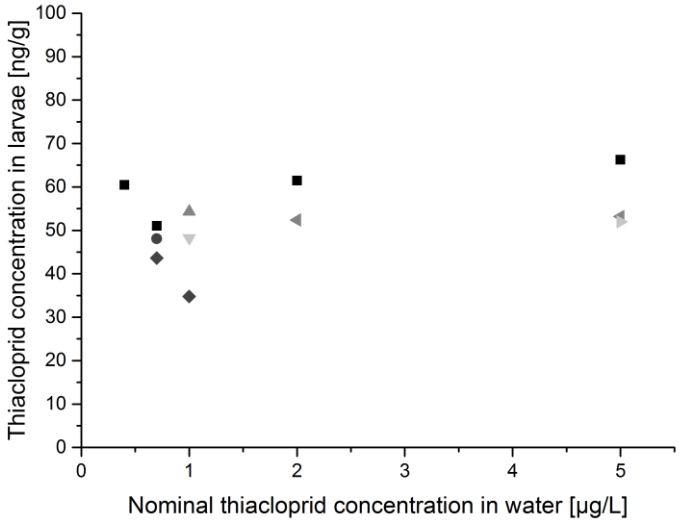


Figure 25: Internal concentration of thiacloprid in larvae which died during the 96 h exposure time. Exposure to different nanoparticles and thiacloprid solely (■ thiacloprid solely, ▲ thiacloprid and zeolite nanoparticles, ● thiacloprid and aluminum oxide nanoparticles, black 96 h, grey 72 h, light grey 48 h.

The internal concentration in dead larvae was in the range of the one in larvae from the exposure studies. This indicates that the internal concentration of approximately 50 ng/g corresponds to the lethal concentration of thiacloprid to *Chironomus riparius*.

4.5.3 Bioconcentration factor for ecotoxicological assessment

The ratio between an analyte concentration in water and the incorporated concentration in organisms is defined as bioconcentration factor (BCF). It is calculated by the concentration in exposure medium and the concentration in organisms at a steady state.

$$BCF = \frac{\text{concentration in larvae}}{\text{concentration in water}}$$

In kinetic models estimating the bioaccumulation potential, of a compound calculation is often based on hydrophobicity and the main uptake route taken into account is passive sorption. Therefore, the bioaccumulation potential for active substances like pharmaceuticals which follow partially active uptake mechanisms might be underestimated [32]. For bioaccumulation, uptake via food and other uptake routes as well as toxicokinetics are taken into account, which are not considered in the bioconcentration. Bioconcentration includes passive uptake of contaminants through the body surface from the water. The second uptake route of contaminants is via dietary uptake from food and sediment. In bioaccumulation, both processes are taken into account [119]. For midges, the uptake via food has been shown to be predominant, contributing 86 – 87 % to the uptake of hydrophobic contaminants [119].

Lipophilicity is expressed by the octanol-water partitioning coefficient (log K_{ow}). A low log K_{ow} value indicates high water solubility and thus a low bioconcentration potential. This model gives good results for example in case of atrazine and chlorpyrifos uptake in *Lumbriculus variegatus.* In a toxicity study with sediments spiked with both compounds chlorpyrifos (log K_{ow} = 4.96) revealed a higher bioconcentration potential than atrazine (log K_{ow} = 2.61). With a log K_{ow} of 1.26 thiacloprid has a low potential for bioconcentration [105]. The estimated BCF for thiacloprid, calculated based on log P data, is 2. Nevertheless, bioaccumulation

experiments with sediments spiked with Cd indicated, that *Chironomus riparius* are able to mobilize sediment-bound Cd, because bioaccumulation increased in correlation to bound Cd but not in correlation to the Cd concentration in overlying water [149]. This indicates, that active uptake via food and simultaneous ingestion of these particles, is a major uptake route compared to passive uptake from the water. Therefore, bioaccumulation is more accurate to predict thiacloprid fate in sediment-water tests than bioconcentration. Bioconcentration and bioaccumulation of different pesticides in aquatic insect larvae have been reviewed by Katagi and Tanaka [119]. Comparing BCF (bioconcentration factor), BAF (bioaccumulation factor) and BSAF (biota-soil accumulation factor) values for pesticides in different aquatic insects, a high diversity was found between insect species and different pesticides [119].

In kinetic studies with herbicides and uptake in *L. variegatus,* a steady state was achieved for many cases within 72 h [148]. The stable internal concentration observed in this study within three treatments and different exposure concentrations indicates a fast equilibrium, present within the 96 h of acute toxicity test, for thiacloprid in *C. riparius* larvae. For calculation of BCF, the lowest exposure concentration resulting in the average internal concentration was used. The calculated BCF from experiments is 70 ± 10 L/kg.

The results from this study, however, reveal a bioaccumulation potential for thiacloprid in midge larvae. It is suggested, that uptake is not only based on hydrophobicity taking mainly passive partitioning into account but also on active uptake processes and particle-bound analytes as food. For insecticides and insects, uptake mechanisms might be more specific, resulting in higher uptake rates than estimated. For *Chironomus riparius* larvae no studies are reported to my knowledge.

4.6 CONCLUSION

In this project, an extraction and quantification method of thiacloprid in midge larvae was developed and applied to samples from exposure studies. The extraction procedure is based on QuEChERS extraction with an additional cleanup step with PSA in a miniaturized format using only 0.5 mL water and 0.5 mL acetonitrile. The LOD by HPLC-MS was determined to be 0.5 µg/L in 250 µL extract requiring 20 mg sample or approximately 5 larvae and the LOQ was determined to be 1 µg/L corresponding to 12 ng/g wet weight. Quantification was based on deuterated internal standard with a recovery of 100 ± 5 %. The method offers the possibility to quantify thiacloprid residues in the low ng/g range in small amounts of 3 - 5 larvae, which makes it interesting also for field studies.

With the applied procedure the internal concentration of thiacloprid in *Chironomus riparius* larvae was analyzed, showing a significantly lower internal concentration at the lowest exposure concentration in experiments with solely thiacloprid comparted to samples from higher exposure concentrations. In experiments with nanoparticles, the internal concentration was the same in all larvae. This might be caused by higher concentrations required in mortality tests compared to studies investigating sublethal effects. The developed method can be used to assess the internal concentration in midge larvae serving as an indicator organism.

5 DETERMINATION OF PROPICONAZOLE AND METABOLITES IN MYCELIUM FROM DIFFERENT FUNGI SPECIES FROM EXPOSURE STUDIES IN THE PRESENCE OF NANOPARTICLES BY HPLC-MS

5.1 ABSTRACT

In toxicological studies the interaction of possible toxic substances with nanoparticles is only rarely considered, although in soil natural and artificial particles are present, which might alter the bioavailability of fungicides in pore-water and soil upon sorption. The objectives of this study were (1) to develop a quantification method for propiconazole in fungi mycelium and exposure medium, (2) to determine the bioaccumulation and metabolic fate by analysis of the parent compound propiconazole and its metabolites in mycelium and medium and (3) to investigate differences in uptake and metabolism between different fungi species. The applied fungicide was propiconazole, which is used as biocide and pesticide. Analyzed samples originated from growing studies in liquid culture and on agar plates with propiconazole and York-shell nanoparticles reducing the bioavailable concentration by sorption. The developed method was based on a miniaturized QuEChERS extraction procedure, requiring only 20 mg mycelium per extraction. A method detection limit of 1 µg/L and a limit of quantification of 2 µg/L allowed quantification at a concentration of 20 ng propiconazole per g dried fungi mycelium. In extraction of mycelium originating from agar plates a limit of quantification of 5 ng/g was achieved. In exposure studies the impact of nanoparticle sorption on biodegradability of propiconazole and distinct inter-species differences in metabolism were observed. Bioconcentration factors ranged from 5 to 56 L/kg for different species. A clear influence of growing conditions on the uptake and metabolism of propiconazole in *Laccaria bicolor* was observed with a bioconcentration factor of 56 L/kg in liquid culture experiments and with a bioconcentration factor of 4600 L/kg in agar plate experiments. To my knowledge this is the first time the influence of nanoparticle sorption and the differences in metabolism of propiconazole in different fungi species and growing conditions were investigated.

5.2 INTRODUCTION

5.2.1 Fungi species in ecosystems

The interaction of plants with mycorrhizal fungi is fundamental for sustainable plant productivity with vital functions in the carbon and nitrogen cycles [13]. Fungi play a key role in ecosystems for degradation, soil formation, soil aggregation and resistance to stress, drought or heavy metals [150]. The ability to degrade or modify lignin and lignocellulose is essential for carbon, nitrogen and phosphate recycling in forests [151]. Mycorrhizal fungi form a symbiosis with plants, by provision of mineral elements and water in exchange of up to 20 % of the plants' photosynthetic products [152-153]. For plants growing next to fungi also interaction with surrounding bacteria between the fungus, microbes and plants are essential, e.g. for nutrient provision [154-155]. Microbial biomass contains up to one third ectomycorrhizal mycelium [156]. In agriculturally used soil, the occurrence of ectomycorrhizal fungi is reduced in contrast to forest soil. This is due to heavy fertilization, cultivation of non-mycorrhiza crops and soil disturbance [150]. Ectomycorrhizal fungi are a significant component of forest ecosystems [157] and the three dominant divisions are basidiomycetes, ascomycetes and

zygomycetes [158]. Fungi are not only able to degrade natural organic compounds, but even some organic pollutants and pesticides. The degradation potential of fungi for remediation of contaminated soil has been reviewed by Meharg and Cairney [14].

Fungi used in this study are *Laccaria bicolor*, *Amanita muscaria* and *Cenococcum geophilum*. *L. bicolor* and *A. muscaria* belong to the basidiomycetes and *C. geophilum* to the ascomycetes. *C. geophilum* is not host-specific and grows in symbioses with a wide range of tree species worldwide [159] for example, *Picea abies* [160] or *Pinus sylvestris*. In experiments on the sensitivity of different forest fungi towards the fungicide propiconazole *C. geophilum* was observed to be one of the more sensitive mycorrhizal fungi [161]. The ability of *C. geophilum* to degrade different PCBs was observed [14]. *A. muscaria* is famous for its poisoning syndromes when consumed by humans [162]. *A. Muscaria* was observed to be able to degrade phenanthrene, anthracene, fluoranthene and pyrene [14]. *L. bicolor* has been used in several laboratory experiments and its genome is completely sequenced [13 163-164] . Therefore, this fungus was used in experiments to give the opportunity to link internal concentration and growth inhibition to genetic changes. *Laccaria bicolor* forms symbioses with different trees among them *Picea mariana* and *Pseudotsuga menziesii* [13, 165]. Degradation of phenolic compounds produced by plants and thereby detoxification of soil was observed for *L. bicolor*, which used degraded products as carbon source [165]. Wood degrading fungi excrete extracellular enzymes into the surrounding soil to degrade large biomolecules like lignincellulose [166-167].

5.2.2 Activity of the fungicide propiconazole

The fungicide propiconazole is applied in agriculture and for wood preservation as a mixture of four biocidally active isomers [168]. The molecular structure is shown in Figure 26. It is used in agriculture as pesticide and in house paint and wood oil in outdoor applications as biocide [169]. In experiments with ectomycorrhizal fungi a strong inhibitory effect of propiconazole was observed for 64 fungi species [161]. It is applied in forests against *Gremmeniella abietina* and *Lophodermim seditiosum* in concentrations of 0.125 kg/ha [161]. 75 % degradation of propiconazole by fungi in treated wood was observed within 21 days [170]. Degradation was also observed by bacteria isolated from fungi mycelium [171-172].

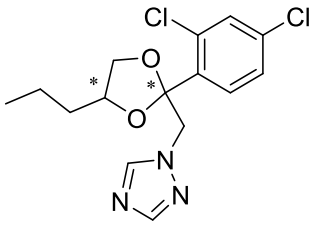


*Figure 26: Molecular structure of the fungicide propiconazole, stereo centers marked with *, applied as mixture of four biocidally active isomers as pesticide and biocide ($C_{15}H_{17}Cl_2N_3O_2$ m/z = 342.0771).*

Propiconazole activity consists in the inhibition of ergosterol biosynthesis by inhibition of C-14 demethylation of lanosterol [15-16]. Ergosterol belongs to the vitamin D2 compounds and is structurally related to cholesterol, which is present in mammals. Ergosterol is part of cell membrane in fungi and can reach up to 0.2 – 8 mg/kg dry weight. 60 – 70 % of sterols in fungi are ergosterol [173]. In vertebrates triazole fungicides inhibit Cytochrome P450 depending mono-oxygenases [174]. Therefore, propiconazole is suspected to be an endocrine disruptor. In experiments estrogenic and anti-androgenic activity, as well as influence on the aromatase activity, were observed [175-176].

Propiconazole, a prioritized substance in Germany [177], was monitored in surface water and groundwater [169]. In 2017 the compound was declared as sufficiently monitored, and without further evidence considered not relevant for ecosystems by the German environmental agency [178].

5.2.3 Analysis of pesticides in fungi mycelium

Most published analytical methods concerning xenobiotics in fungi are about pesticides in edible fungi [179]. Mycelium was seldomly analyzed for contaminants. Extraction methods depend on physical and chemical properties of the analytes. Polar compounds like flame retardants were extracted from fungi mycelium with methanol [180]. In contrast DDT, a non-polar pesticide, and its metabolites were extracted with hexane from mycelium and medium and analyzed by GC-MS [181]. More polar pesticides like phenylurea herbicides and their metabolites were extracted from the medium with acetate/hexane and subsequently analyzed by HPLC-UV [182]. In most toxicity studies metabolite and analyte concentrations were determined in the medium, rather than in mycelium. For example, degradation of the pesticides terbuthylazine, difenoconazole and pendimethalin by fungi grown in liquid culture were assessed by analysis of medium [183]. Metolachlor analysis to assess degradation and metabolism of the pesticide in acclimated field soil was tested by analysis of medium, extracted with hexane and ethyl acetate [184]. Degradation of fluorenes by fungi grown in liquid culture was investigated and concentrations in fungi and media analyzed by liquid-liquid extraction with ethyl acetate and HPLC-UV detection [185].

In pesticide analysis of fruits and vegetables QuEChERS (Quick Easy Cheap Effective Rugged and Safe) extraction, developed by Anasstasiades is widely used [2], and has been applied to edible fungi [179, 186]. QuEChERS extract buffers are required ion is a liquid-liquid extraction with water and acetonitrile. In case of some ionic pesticides buffers are required for the pH depending QuEChERS extraction to extract at certain pH ranges and enhance recovery of acidic labile or ionic pesticides. For propiconazole it was shown, that addition of buffer or pH changes are not necessary [27]. The method has, to the best of my knowledge, not yet been applied to extract fungi mycelium.

5.2.4 Nanoparticles

Investigated York-shell nanoparticles were synthesized in the group of Prof. Anwander at the Institute of Inorganic Chemistry at the University of Tübingen, according to published protocolls [187-188]. The thioether-bridged mesoporous nanoparticles with organo-silica framework (York-shell nanoparticles) were used in toxicity studies of propiconazole with different fungi in liquid culture and agar plate experiments. In all experiments described in this study, the applied York-shell nanoparticle concentrations to reduce the bioavailable fraction of propiconazole in medium were calculated using data from adsorption isotherms. Growth inhibition effects were investigated for propiconazole and nanoparticles solely and reduced bioavailable propiconazole concentrations due to sorption of propiconazole to nanoparticles with their combination.

5.3 EXPERIMENTAL

5.3.1 Reagents, chemicals and consumables

HPLC solvents methanol hypergrade LC-MS (chromasolv), water hypergrade LC-MS (chromasolv), acetonitrile LC-MS grade (Sigma Aldrich) and formic acid (98%, eluent

additive for LC-MS) were supplied by Sigma-Aldrich (Steinheim, Germany). Ethyl acetate analytical reagent grade was purchased by Fisher Scientific (Loughborough, UK).

Propiconazol was purchased by Dr. Ehrenstorfer GmbH (Augsburg, Germany), propiconazole-d_3, ergosterol, picolinic acid, pyridine, triethylamine, 2-methyl-6-nitrobenzoic acid anhydride, 4-dimethylaminopyridine, sodium chloride, magnesium sulfate and potassium hydroxide were purchased from Sigma Aldrich (Steinheim, Germany). PSA bulk sorbent and C18 bulk sorbent were purchased by Agilent Technologies (Waldbronn, Germany).

3 mm PTFE syringe filter (0.45 µm) were supplied by Macherey-Nagel (Düren Germany).

5.3.2 Agar plate experiment with nanoparticles

Experiments with fungi grown on 25 mL agar plates were conducted by Elisabeth Früh, Institute of Evolution and Ecology at the University of Tübingen, Germany, and mycelium samples and agar plates provided for analysis. Experiments were performed at an exposure concentration of 100 µg/L propiconazole and York-shell nanoparticles in concentrations of A) 0.017, 0.098 and 0.577 g/L resulting in 80, 50 and 20 µg/L propiconazole concentration in the medium or B) 0.03, 0.129 and 0.517 g/L resulting in 85, 50 and 15 µg/L propiconazole concentration in the medium. Agar plates contained 1.8 % agar and 0.5 % glucose. Agar plates and mycelium were stored at -20 °C until analysis.

5.3.3 Liquid culture experiments with nanoparticles

Experiments with fungi grown in 20 mL liquid culture medium were conducted by Elisabeth Früh and dried mycelium samples and medium provided for analysis. 1.5 mL liquid medium was extracted by Leyla Guluzada, Center for Applied Geosciences at the University of Tübingen, Germany, and extracts provided for analysis. In 20 mL liquid culture medium *A. muscaria* was exposed to 5 mg/L propiconazole and 0.03, 0.129 and 0.517 g/L nanoparticles resulting in nominal 4, 2.5 and 1 mg/L propiconazole*. L. bicolor* was exposed to 0.1 mg/L in liquid culture and *C. geophilum* to 2 mg/L. Mycelium samples were dried at 60 °C after seven days of exposure and dried samples stored at -20 °C until analysis.

5.3.4 Quantification of propiconazole in fungi mycelium grown on agar plates

20 – 50 mg frozen fungi mycelium originating from agar plate experiments were homogenized with mortar and pestle in liquid nitrogen. The pulverized sample was transferred to Eppendorf tubes and deuterated internal standard was added. Different extraction protocols were tested.

1) 1 mL ethyl acetate was added to the sample and the suspension was subsequently shaken for 2 min. After 1 h incubation at room temperature the sample was centrifuged at 10 000 rpm for 2 min and the supernatant transferred to a vial. The solvent was evaporated under nitrogen stream at room temperature and the residue was redissolved in methanol and deuterated internal standard was added to reach a final concentration of 8 µg/L. After filtration with a 45 µm PTFE filter the sample was analyzed by HPLC-MS.

2) 1.5 mL methanol were added and the sample vortexed for 2 min. After 1 h incubation at room temperature the sample was centrifuged at 10 000 rpm for 2 min and split in two. a) 0.25 mL were transferred to a vial and evaporated to dryness. The residue was resolved in 490 µL methanol and 10 µL internal standard (c = 100 µg/L), filtered through a 0.45 µm PTFE filter and analyzed by HPLC-MS. b) 1 mL sample was transferred to a vial and 120 mg

KOH were added. The sample was then heated to 60 °C for 1 h. 500 µL hexane were added and the sample was extracted twice with additional 500 µL hexane. Combined organic phases were evaporated to dryness under a nitrogen stream and the residue was redissolved in 590 µL methanol and 10 µL internal standard (c = 100 µg/L). After filtration with 0.45 µm PTFE filters the sample was analyzed by HPLC-MS.

3) 0.75 mL water and 0.75 mL acetonitrile were added for QuEChERS extraction. Subsequently, the sample was shaken with a vortex device for 1 min. Afterwards, 90 mg anhydrous $MgSO_4$ and 40 mg sodium chloride were added and the sample was vortexed for 1 min. After centrifugation at 10 000 rpm for 3 min the organic layer was either analyzed as raw extract or cleaned by a dispersive solid phase extraction (dSPE) step:

a) Analysis of raw extract: the organic phase was evaporated to dryness and the residue redissolved in 0.46 mL methanol and 40 µL deuterated internal standard (c = 100 µg/L) resulting in a final concentration of 8 µg/L, and analyzed by HPLC-MS after filtration.

b) dSPE cleanup: For dSPE cleanup the organic phase was transferred to another Eppendorf tube containing the dSPE sorbent. For cleanup 15 mg PSA, 15 mg C18 or 15 mg of each mixed with 120 mg anhydrous $MgSO_4$ were used. The tube was vortexed for 1 min. After centrifugation at 10 000 rpm for 3 min the organic layer was evaporated to dryness, the residue redissolved in 0.46 mL methanol and 40 µL deuterated internal standard (c = 100 µg/L) was added resulting in a final concentration of 8 µg/L. The sample was analyzed by HPLC-MS after filtration with 0.45 µm PTFE filter.

5.3.5 Quantification of propiconazole in fungi mycelium grown in liquid culture

20 mg fungi mycelium grown in liquid culture were dried at 60 °C until dryness and homogenized with mortar and pestle by addition of 10 mg sodium chloride. The pulverized sample was transferred to Eppendorf tubes and 40 µL deuterated internal standard (c = 100 µg/L) was added. 0.75 mL water and 0.75 mL acetonitrile were added and the sample was subsequently vortexed for 1 min. Afterwards 90 mg anhydrous $MgSO_4$ and 20 mg sodium chloride were added and the sample was vortexed for 1 min. After centrifugation at 10 000 rpm for 3 min the organic layer was either analyzed as raw extract or cleaned by a dSPE step:

a) Analysis of raw extract: the organic phase was evaporated to dryness and redissolved in 0.5 mL methanol and after filtration samples were diluted 1/10 with methanol and analyzed by HPLC-MS.

b) dSPE cleanup: For dSPE cleanup the organic phase was transferred to another Eppendorf tube containing 15 mg PSA, 15 mg C18 or 15 mg of each in their mixture together with 120 mg anhydrous $MgSO_4$. The tube was vortexed for 1 min, and centrifuged at 10 000 rpm for 3 min. The organic layer was separated and evaporated to dryness. Samples were redissolved in 0.5 mL methanol, filtered with PTFE syringe filter, diluted with methanol 1/10 and analyzed by HPLC-MS.

5.3.6 Quantification of propiconazole in agar plates

Agar plates were dried at 60 °C in the dark for 24 h and homogenized with mortar and pestle. 40 mg pulverized agar were transferred to Eppendorf tubes and extracted. For method development different extraction solvents were used: a) 1.5 mL acetonitrile, b) 0.75 mL acetonitrile and 0.75 mL water with 20 mg sodium chloride, c) 1.5 mL methanol, d) 1.5 mL ethyl acetate, and e) 0.75 mL ethyl acetate and 0.75 mL water. The organic layer

was transferred and the solvent evaporated. 225 µL methanol and 25 µL internal standard solution in methanol (c = 100 µg/L) were added resulting in a final concentration of 10 µg/L deuterated propiconazole-d_3. Samples were filtered with 0.45 µm PTFE filters and analyzed by HPLC-MS.

The final extraction was carried out with 80 mg dried agar and 1.5 mL ethyl acetate. After 4 h incubation at room temperature samples were centrifuged at 10 000 rpm for 3 min and evaporated to dryness. Residues were reconstituted in 450 µL methanol and 50 µL deuterated propiconazole (c = 100 µg/L) were added resulting in a concentration of 10 µg/L of the internal standard in the final extract. Samples were filtered through 0.45 µm PTFE filters and analyzed by HPLC-MS.

5.3.7 Quantification of propiconazole and transformation products in liquid culture medium

1.5 mL liquid medium samples were extracted with ethyl acetate (3 x 1.5 mL). Organic phases were combined, the solvent removed and the residue redissolved in 1 mL methanol. 10 µL of the extract were injected and analyzed by HPLC-MS.

5.3.8 Instrumental methods

For LC-MS analysis of propiconazole and metabolites, a 1260 Infinity LC system coupled to a 6550 iFunnel QTOF LC/MS system (Agilent Technologies, Waldbronn, Germany) was used. Aliquots of 10 µL sample were injected onto a Zorbax Eclipse Plus C18 column (2.1 x 150 mm, 3.5-Micron, narrow bore, Agilent Technologies, Waldbronn, Germany) at 40 °C. A jet stream electrospray ionization (ESI) source was operated in the positive ionization mode with a nebulizer pressure of 35 psig, drying gas temperature of 160 °C, a flow rate of 16 L/min and a fragmentor voltage of 360 V. In the positive ionization mode capillary voltage was set to -4000 V, skimmer voltage to 65 V and a nozzle voltage to -500 V. The mass range was 100 – 1200 m/z with a data acquisition rate of 1 spectrum/s. The sheath gas temperature was set to 325 °C with a flow rate of 11 L/min. For internal calibration purine and HP0921 (Agilent Technologies, Waldbronn, Germany, m/z = 121.0508, 922.0097) were used. A gradient elution at a flow rate of 0.3 mL/min using water and methanol, both containing 0.1 % formic acid, was used. The initial content of 95 % water was decreased after 1 min to 5 % water over 7 min and after another 7 min at 5 % increased to 95 % water over 0.5 min. Data analysis was performed with MassHunter Workstation software Version B.06.00 (Agilent Technologies, Waldbronn, Germany).

The mass range for MS/MS spectra was 100 – 1700 m/z and isolated masses were m/z = 256.0039, 258.0195, 358.072, 342.0771 and 345.0964 at an isolation range of 4 m/z and a retention time window of 2 min. Collision energy was set to 24 V and the acquisition time for MS/MS spectra was 200 ms/spectrum and the mass range 50 – 400 m/z.

5.3.9 Photochemical transformation

Propiconazole standard in acetonitrile (c = 10 mM) was diluted with water to 10 µM and a volume of 10 mL was irradiated with 220 - 250 nm at 200 watt for 60 min with a SUV DC Lumatec (Munich, Germany) in glass Petri dishes. 20 µL samples were taken every minute for 10 min and afterwards every 10 min until 1 h irradiation was reached. Samples were stored at -20 °C till analysis by CE-MS, see Section 5.3.12.

5.3.10 Ergosterol derivatization

Ergosterol standard in methanol used for calibration or the hexane phase of samples were evaporated to dryness and 170 µL derivatization solution were added. The ergosterol derivatization was carried out according to literature [189]. The derivatization solution contained 100 mg 2-methyl-6-nitro-benzoicanhydride (1.1 eq, 0.29 mmol), 80 mg picolinic acid (2.5 eq, 0.65 mmol), 30 mg 4-dimethylaminopyridine (1 eq, 0.25 mmol), 1.5 mL pyridine and 200 µL trimethylamine. Extract and solution were incubated at 80 °C for 60 min. After addition of 1 mL hexane the sample was vortexed for 20 sec and centrifuged at 10 000 rpm min for 3 min. The supernatant was transferred to a vial and the solvent removed to dryness. The residue was redissolved in 250 µL acetonitrile, filtered with PTFE syringe filters and analyzed by HPLC-MS.

5.3.11 Desorption of propiconazole from nanoparticles

1.3 mg York-shell nanoparticles were spiked with 10 µL propiconazole in methanol (c = 5 mg/L). The solvent was completely evaporated under a stream of nitrogen. Spiked nanoparticles were extracted with a) 10 mL ethyl acetate, b) 10 mL acetonitrile, c) 5 mL acetonitrile and 5 mL water or d) 5 mL ethyl acetate and 5 mL water. Samples were centrifuged at 10 000 rpm for 3 min. From each sample 2 mL of the organic phase were transferred to an LC vial and evaporated to dryness. The supernatant was resolved in 0.5 mL methanol and filtered with a PTFE 0.45 µm filter and analyzed by HPLC-MS.

5.3.12 Analysis of photo-transformation products

Samples were stored at -20 °C for 8 weeks until they were analyzed. Analysis was performed using a CE 7100 (Agilent Technologies, Waldbronn, Germany) coupled to a 6550 iFunnel mass spectrometer QTOF (Agilent Technologies, Waldbronn, Germany) as an autosampler and delivery system. Undiluted samples in water/acetonitrile (1/100) were injected hydrodynamically at 100 mbar for 15 sec and flushed to the MS with water at 1 bar for 30 sec using a bare fused silica capillary (50 µm diameter, 64.5 cm length). The mass spectrometer was run by an ESI source in the positive ionization mode at 3500 V. Gas temperature and flow rate were set to 150 °C and 11 L/min and nebulizer pressure 5 psig. Data were analyzed by MassHunter Qualitative software (Agilent Technologies, Waldbronn, Germany). Sum formula were calculated from exact masses by the software.

5.3.13 Statistics

Measured values were tested for normal distribution by Shapiro-Wilk-test with Origin 9.1.0 (OriginLab, Northampton, USA) at a 0.05 level. If normal distribution was proven, significant differences of variances were tested with one-way ANOVA with the software Origin 9.1.0 (OriginLab, Northampton, USA) at a level of 0.05. Non-normal distributed samples were tested with Kruskal-Wallis-test for differences with the software R (3.3.1, R Foundation for Statistical Computing). For linear regression in calibration experiments the software Origin 9.1.0 (OriginLab, Northampton, USA) was used.

5.4 RESULTS AND DISCUSSION

Blank matrices, corresponding to uncontaminated samples, were used for optimization steps including cleanup, detection and quantification levels of the method.

5.4.1 Method development

Separation and detection of propiconazole

Separation was performed with an eluent made of methanol/water and a C18 column using gradient elution. Retention time for propiconazole was 13.2 min. Quantification was based on the deuterated internal standard propiconazole-d_3. The fragment ion with the highest signal intensity was the triazole fragment Figure 27. As the isotopic labeling was at the aromatic ring, the fragment ion was the same for propiconazole and the internal standard propiconazole-d_3 (compare Figure 27). Therefore, quantification was carried out in the MS mode without fragmentation.

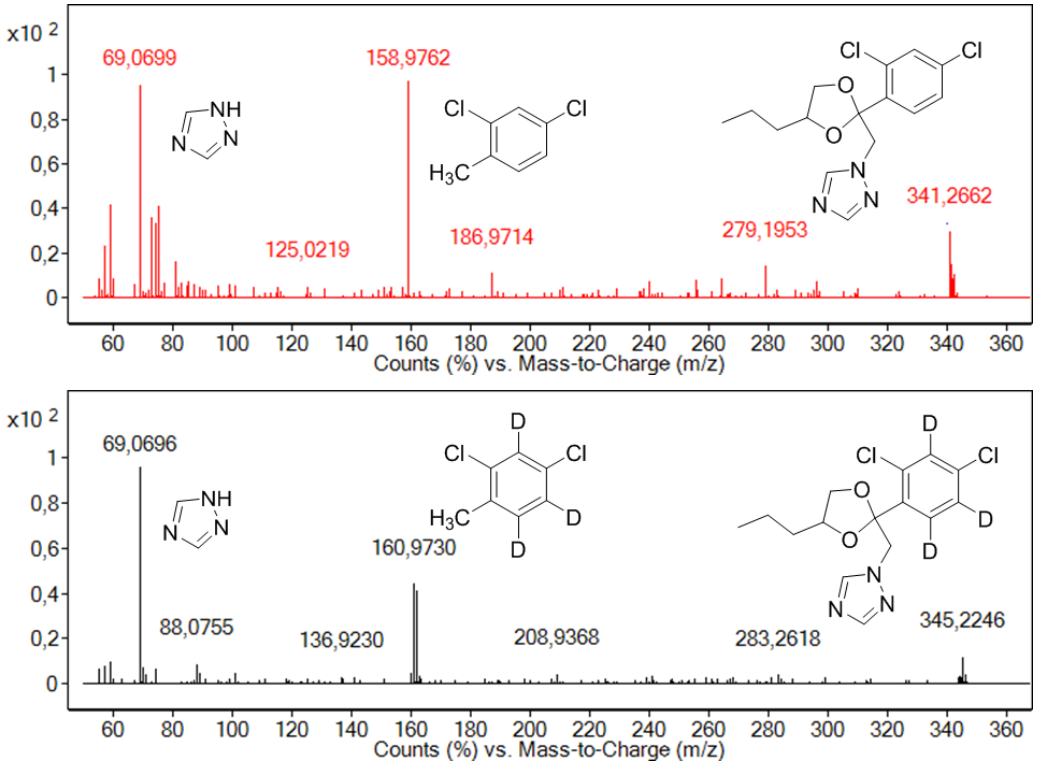


Figure 27: MS/MS spectra of propiconazole (red) (m/z $[M+H]^+$: 342.0771) and isotopically labeled internal standard propiconazole-d_3 (black) (m/z $[M+H]^+$: 345.0964) and the two most abundant fragment ions the azole 1H-1,2,4-triazole (m/z $[C_2H_4N_3]^+$: 69.0697) and the aromatic fragment 2,4-dichloro-1-methylbenzene-3,5,6-d_3 m/z $[C_6H_5Cl]^+$: 158.9759 (m/z $[C_6H_2D_3Cl]^+$: 160.9755) at the retention time 13.2 min, LOQ = 0.2 µg/L and LOD = 0.1 µg/L, CE 24 V and a fragmentation voltage of 360 V.

Measured MS/MS fragment ions of propiconazole corroborate literature data [190]. The limit of detection (LOD) was below 0.1 µg/L for both compounds, determined by the concentration producing a signal three times larger than background noise (S/N = 3). The limit of quantification (LOQ) was 0.2 µg/L based on a signal to noise ratio of ten (S/N = 10). In comparison with literature the LOD determined in this study was higher than reported for wastewater samples with an LOD of 15 ng/L [191].

Quantification of propiconazole in mycelium grown on agar plate experiments

Extraction method development

For mycelium grown on agar plates, different extraction methods were compared. To analyze signal suppression by interfering matrix components and the extraction efficiency of different solvents four extraction procedures, listed in Table 8, were compared based on relative signal intensities calculated as the percentage of peak areas of propiconazole and

spiked samples vs. standards at the spiked concentrations in methanol as reference. QuEChERS extraction (water + acetonitrile) was performed for raw extracts and with dSPE cleanup as commonly applied in food analysis [179]. Ethyl acetate and methanol were used for liquid extraction. To simultaneously determine ergosterol and propiconazole content samples were extracted a) with methanol or b) methanol followed by subsequent acidic hydrolysis following published procedures [192]. Every extraction method was performed with one sample spiked before extraction, to determine the recovery and with one sample spiked after extraction and before analysis to determine signal suppression by interfering matrix components.

Table 8: Extraction procedures applied for fungi mycelium grown on agar plate to assess propiconazole content in wet weight mycelium. Signal intensities of samples spiked before (sample + spike) and after extraction (extract + spike) to compare recovery and matrix effect are presented.

extraction solvent	sample	signal area [%]
water + acetonitrile	sample + spike	21
	extract + spike	41
ethyl acetate	sample + spike	11
	extract + spike	18
methanol	sample + spike	9
methanol + acidic hydrolysis	sample + spike	6

Signal areas of propiconazole in samples extracted with water and acetonitrile were 6 to 4-fold higher than in the other extracts. Especially the extraction followed by subsequent acidic hydrolysis revealed signal areas of propiconazole below 10 % compared to reference material in methanol. Extraction with methanol and ethyl acetate showed comparable signal areas of propiconazole, but in both cases approximately 4-fold lower than in the QuEChERS extract. Therefore, liquid-liquid extraction with water and acetonitrile was further optimized.

Optimization of QuEChERS extraction

Signal area of propiconazole was reduced in QuEChERS extract by 59 % and in the ethyl acetate extract by 82 % in extraction experiments, data shown in Table 8. Therefore, the implementation of a cleanup step in QuEChERS extraction was tested to increase signal intensity by removal of interfering matrix components. For cleanup PSA (primary secondary amines) was used, which removes fatty acids, organic acids, saccharides and some pigments from the QuEChERS raw extract by interaction with basic amino-groups [2]. PSA and raw extract analysis was performed at three spiked concentrations of 0.5, 1 and 2 µg/L for two separate replicates. Quantification of propiconazole was achieved by external calibration. For each concentration two samples were spiked before and after the extraction prior to analysis. Results are presented in Figure 28.

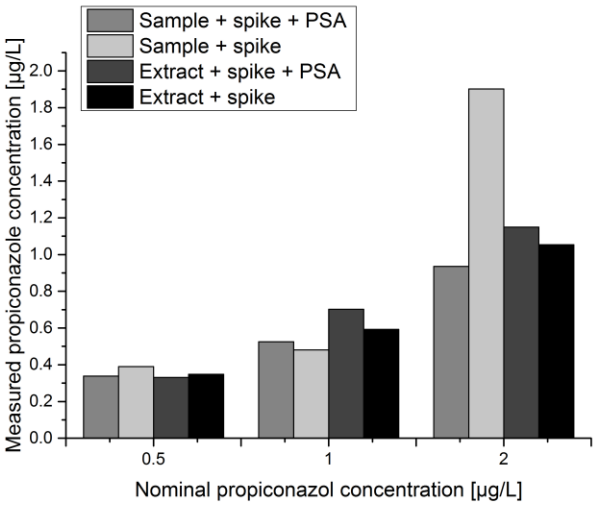


Figure 28: Propiconazole concentration in fungi mycelium samples grown on agar plates, spiked before extraction (sample + spike) (grey and dark grey) and spiked before analysis (extract + spike) (light grey and black), for comparison cleaned extracts (grey and dark grey) and raw extracts (light grey and black) were analyzed at three concentrations 0.5, 1 and 2 µg/L (n = 2).

The comparison of propiconazole signal areas with and without PSA cleanup clearly indicated that PSA treatment was not beneficial.

Table 9: Comparison of dSPE cleaned extract and raw extract of fungi mycelium grown on agar plates. Recovery was calculated from samples spiked before extraction and matrix effect in samples spiked after extraction, the relative recovery is based on the absolute recovery corrected for matrix effects. For details of the calculation see text.

	0.5 [µg/L]	**1 [µg/L]**	**2 [µg/L]**
recovery (+PSA)	58 %	53 %	46 %
recovery (-)	70 %	53 %	91 %
matrix effect (+PSA)	37 %	28 %	32 %
matrix effect (-)	30 %	37 %	41 %
rel. recovery (+PSA)	93 %	74 %	68 %
rel. recovery (-)	100 %	83 %	155 %

Recovery was calculated based on the ratio of the peak area of propiconazole in the sample spiked before extraction to the peak area of a reference solution in methanol of the same concentration. The matrix effect was calculated by the ratio of the peak area of sample spiked after extraction and before analysis (extract + spike) to the peak area of a reference solution at like nominal concentration. To determine losses during extraction without signal suppression relative recovery was calculated as the ratio of peak area of sample spiked before extraction (sample + spike) to the peak area of the sample spiked after extraction (extract + spike). Average signal intensities of 66 ± 6 % were achieved for extracts cleaned with dSPE and 62 ± 7 % in raw extracts (Table 9). As no significant signal enhancing effect was observed and to keep sample preparation as simple as possible further method validation was performed using the raw extracts.

Method validation

Method validation was accomplished using mycelium samples to best reflect possible matrix effects. Calibration was performed with mycelium grown without propiconazole on agar plates. Samples were spiked at five concentrations 0.1, 0.2, 0.5, 1 and 2 µg/L in two independently treated samples (shown in Figure 29).

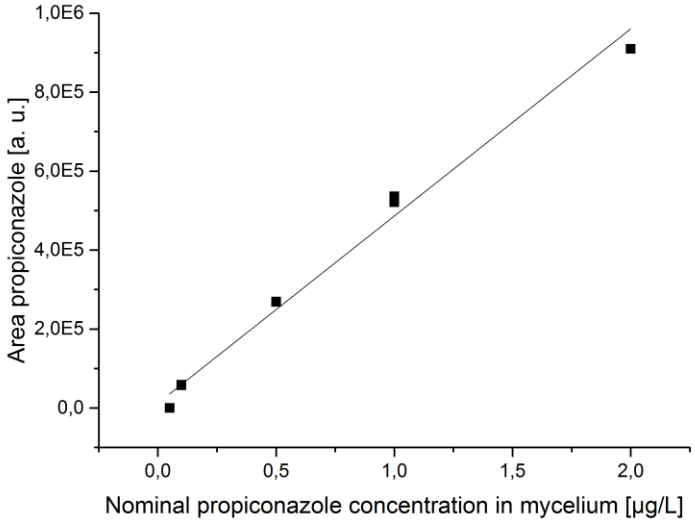


Figure 29: External calibration of propiconazole in mycelium grown on agar plates at five concentrations, (R^2 = 0.99377, n = 2) and LOD = 0.1 µg/L and LOQ = 0.2 µg/L, 20 mg wet weight mycelium were used.

Calibration data for mycelium grown on agar plates revealed an LOQ of 0.2 µg/L and an LOD below 0.1 µg/L for extraction of 20 mg frozen mycelium in 0.5 mL methanol. This corresponds to a method quantification limit of 5 ng propiconazole per g fungi wet weight. The calibration graph was linear over the whole range investigated (R^2 = 0.99377).

Quantification of propiconazole in fungi mycelium grown in liquid culture

Optimization of QuEChERS extraction

Mycelium grown in liquid culture was dried at 60 °C and homogenized with mortar and pestle by addition of 25 mg sodium chloride to avoid electrostatic effects. Consequently, in the following QuEChERS extraction only anhydrous $MgSO_4$ was added in the salting out step. To optimize QuEChERS extraction the implementation of cleanup by dSPE (dispersive solid phase extraction) was investigated by addition of different sorbents. Propiconazole peak areas in the raw extract, in extracts treated with non-endcapped C18, PSA cleaned extracts or, a combination of non-endcapped C18 and PSA, were compared. Non-endcapped C18 is a sorbent attracting nonpolar matrix components with its groups functionalized with octadecylsilane and polar basic compounds with non-endcapped silanol-groups [193]. Recoveries and peak areas were calculated via peak area of propiconazole in the sample and in a methanolic reference solution and are given in Table 10.

Table 10: Method validation parameters for propiconazole in fungi mycelium grown in liquid culture after QuEChERS extraction with different dSPE sorbents. Recovery is based on peak area of propiconazole in samples spiked before extraction and peak area is based on peak area of extracts spiked after extraction (see main text) (n = 2).

	recovery [%]	**peak area [%]**	**rel. recovery [%]**
raw	104	124	84
PSA	107	126	85
C18	133	134	100
PSA+C18	110	101	109

Recovery of analyte in the raw extract was in the range of 104 ± 10 %. As revealed by one-way ANOVA, only the treatment using C18 and PSA + C18 showed a significant difference to the results for the raw extract regarding recovery. Addition of a cleanup step led to recoveries with more than 100 %, especially for C18 cleanup. This fact might be due to signal enhancing effects of matrix components [194]. With the good results for the most simple sample preparation, I continued work and validation using raw extracts.

Method validation

Linear range and detection limits were determined via calibration curves in matrix, for all three fungi species grown in liquid culture (Figure 30). Extraction was performed with the optimized QuEChERS extraction (see Section 5.3.5) with 20 mg mycelium for each sample. Six concentrations in three replicates were used for calibration in the range of 0 - 80 µg/L, results of linear regression are presented in Table 11.

Table 11: Parameter of the linear regression (y = a + bx) used for external calibration for propiconazole in dried mycelium from different fungi species grown in liquid culture and extracted with QuEChERS by analysis of raw extracts for six spiked concentrations in the range 0 – 80 µg/L (n = 3).

	a	b	r^2	LOD	LOQ	recovery
A. muscaria	-390000	276000	0.99926	< 1 µg/L	< 1 µg/L	85 ± 6 %
L. bicolor	-409000	293000	0.99839	< 1 µg/L	< 1 µg/L	93 ± 11 %
C. geophilum	-489000	262000	0.99762	< 1 µg/L	< 1 µg/L	85 ± 12 %

The sensitivity, determined as slopes b of the linear regression, was very similar for all three species, ranging from 276000 to 293000. The largest increase in peak area was detected for *L. bicolor* and the smallest for *C. geophilum*. R^2 values were greater than 0.99 for the concentration range of 0 – 80 µg/L and limits of quantification and detection were below 1 µg/L for the three analyzed species. Differences in the calibration curves can be explained by different type and concentrations of co-extracted matrix components, resulting in different signal suppression during MS analysis. Therefore, individual external calibrations were required for each fungi species.

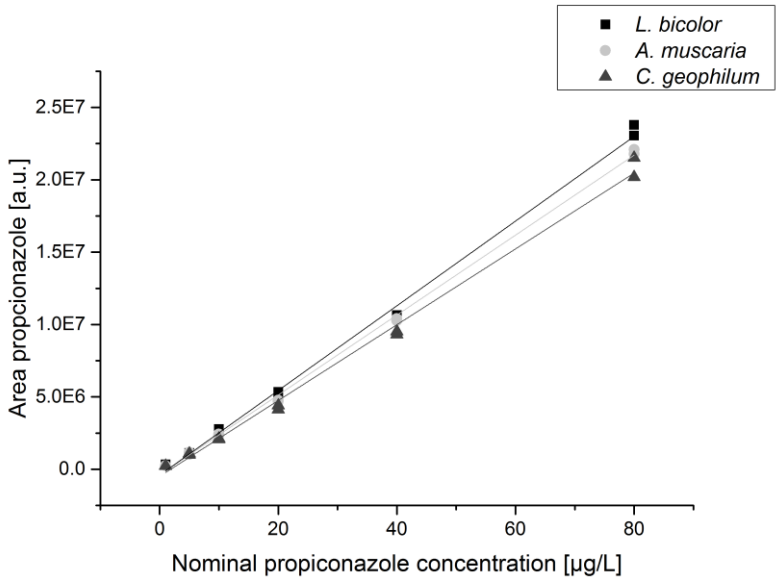


Figure 30: Calibration curves for propiconazole measured in dried fungi mycelium grown in liquid culture for three fungi species.

Recoveries were significantly higher for mycelium grown on agar plates vs. in liquid culture. This is due to higher matrix load in 20 mg dried samples originating from liquid culture experiments compared to 20 mg wet weight mycelium from agar plate experiments. Mycelium samples grown on agar plates were extracted without drying and thus, concentrations are based on wet weight, whereas liquid culture mycelium was dried at 60 °C and internal concentrations are calculated based on dry weight. The high water content of mycelium results in higher concentrations based on dry weight vs. wet weight.

Analysis of ergosterol in mycelium samples

Ergosterol is used in environmental and food chemistry as an indicator for fungal contamination of food or construction materials, for example in barley or malt [195] or processed food like ketchup [196] or treated wood [197]. To assess the inhibitory effect on ergosterol biosynthesis by propiconazole, ergosterol quantification was envisaged. It was hypothesized, that the ergosterol concentration in fungi decreases with increasing internal propiconazole concentration. Thus, quantification of ergosterol could be used as a bioindicator for ergosterol inhibitor burden for example in soil, like it is used as indicator for fungal contamination in building materials [197]. Quantification methods of ergosterol were published for edible fungi [198].

A significant fraction of ergosterol in fungi is bound to the cell wall via ester-bonds. Thus, ergosterol quantification may discriminate three fractions of ergosterol: Free, total free and absolute ergosterol content, which can be distinguished by appropriate extraction procedures. Total free ergosterol is obtained by alkaline hydrolysis with potassium hydroxide in methanol after extraction. Absolute ergosterol is determined after alkaline hydrolysis during extraction and free ergosterol without alkaline hydrolysis. Extraction is carried out with methanol and alkaline hydrolysis with potassium hydroxide. Partitioning of ergosterol into the hexane phase used for analysis is achieved by subsequent liquid-liquid extraction [195]. Ultrasonic extraction is not possible due to decomposition of ergosterol molecules and thus reduced extraction of intact ergosterol [199]. As most steroids, ergosterol has a low ionization potential in ESI-MS and is preferably ionized by APCI (atmospheric pressure chemical ionization) instead of ESI (electrospray ionization) or has to be derivatized as picolinyl ester to be suitable for ESI [200]. This derivatization method has not yet been applied to environmental samples. For quantification in human blood samples [201] ergosterol was derivatized to the picolinyl ester with picolinic acid, pyridine and triethylamine with the use of 2-methyl-6-nitrobenzoic anhydride and 4-dimethylaminopyridine at 80 °C [189]. In this work ergosterol was extracted from fungi mycelium and derivatized to quantify the ergosterol content according to the procedure described in Section 5.3.10. However, the resulting ergosterol-derivatives could not be detected by ESI-source despite its higher polarity than ergosterol itself.

Optimization of extraction conditions for agar plates extraction and liquid culture medium

To assess the exposure concentration during experiments, the propiconazole content was quantified in the exposure media, these were, liquid culture medium and agar plates. Whereas for the liquid culture medium the analysis was straight forward by using liquid-liquid extraction with ethyl acetate, extraction from agar plates was more difficult. First agar plate extraction was performed according to literature for extraction of natural products [202]. Frozen agar plates were thawed and solids separated from water by filtration. The water phases differed in volume for different biological samples from 0.5 to 3 mL for three agar plates. Thus, liquid-liquid extraction with ethyl acetate did not deliver reproducible results. Direct

extraction of agar plates with acetone or ethyl acetate after thawing did not give reproducible results either.

Therefore, an extraction procedure based on dried agar plates instead of frozen agar plates was developed. Agar plates were dried at 60 °C for 24 h and homogenized with mortar and pestle before extraction.

Different solvents were compared for liquid-liquid extraction (for details, see Section 5.3.6): 1) ethyl acetate or ethyl acetate/water as used in liquid culture extraction, 2) methanol and 3) acetonitrile/water according to mycelium extraction. Method development was performed with aliquots of 40 mg agar of one agar plate, containing 100 µg/L propiconazole. Recoveries and extracted concentrations, for two replicates of each procedure are given in Figure 31. For recovery experiments, internal standard was added prior to extraction and signal areas of internal standard in spiked samples were compared to the signal area of reference material in methanol (Figure 31). Recovery thus takes analyte losses during workup and matrix effects in MS analysis into account.

Extraction procedures showed comparable recoveries between 20 and 30 % and propiconazole concentrations between 55 and 65 µg/L for the different treatments. The highest recovery was achieved for ethyl acetate extraction (29 ± 1 %), however, with slightly lower relative propiconazole concentrations (56 ± 0.2 µg/L) compared to extraction with other solvents. The lowest relative standard deviation (RSD) of recovery was achieved for ethyl acetate and ethyl acetate/water extraction (both 1.1 % RSD). Methanol extraction had an RSD of 2.4 % and acetonitrile/water of 1.8 %. The highest variability of 3.9 % for recovery was detected for pure acetonitrile extraction. This procedure showed best extraction performance of 66 µg/L but also the lowest recovery of 15 % in the same extract.

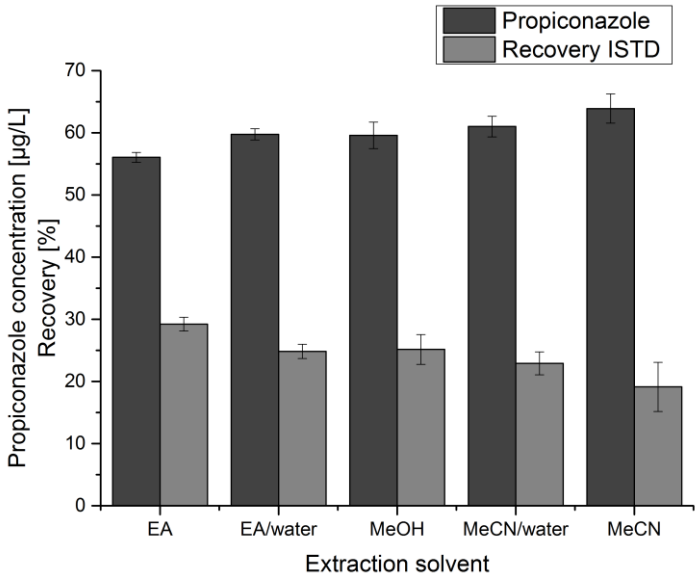


Figure 31: Propiconazole concentration in samples originating from the same agar plate extracted with different solvents. Recovery was calculated based on spiked isotopically labelled propiconazole-d_3 (light grey). The propiconazole concentration corresponds to nominal 100 µg/L. EA: ethyl acetate, MeCN: acetonitrile.

Comparison of extraction with acetonitrile vs. extraction with ethyl acetate revealed better extraction (+ 8 µg/L) but lower recoveries (- 10 %) for acetonitrile as extraction solvent. This indicates better extraction properties of acetonitrile, which might be due to the higher polarity

and higher dipole moment of acetonitrile. The lower recovery might indicate interaction between the solvent and the pulverized agar, which lead to adsorption of internal standard to agar matrix or extraction of MS interfering matrix components to a higher extent.

For further agar extraction ethyl acetate was used as solvent. This is advantageous as both extraction protocols for agar plates and liquid culture medium could be carried out with ethyl acetate. From the exposure experiment with 730 mg dried agar suspended in a final aqueous solution of 25 mL, only 80 mg of dried material were sufficient for quantification also when nanoparticles were added to the plates. Extraction of liquid culture medium was carried out with ethyl acetate (1/1) three times.

Proof for co-extraction of propiconazole from nanoparticles in exposure experiments

Nanoparticles were incubated with 5 µg/L propiconazole in methanol for 2 hours. After evaporation of the methanol nanoparticles were extracted with different solvents to assess the impact of different solvents on propiconazole desorption from nanoparticles. Solely nanoparticle samples were extracted with acetonitrile, ethyl acetate, acetonitrile/water (1/1) or ethyl acetate/water (1/1) (for details, see Section 5.3.11). Results showed reduced recoveries in extractions containing water, but comparable desorption efficiency of acetonitrile and ethyl acetate, as shown in Figure 32. This indicates that differences in propiconazole concentrations detected in fungi mycelium and exposure medium extracts are not due to differences in desorption efficiencies of different solvents used in the different extraction protocols but rather to different matrix effects.

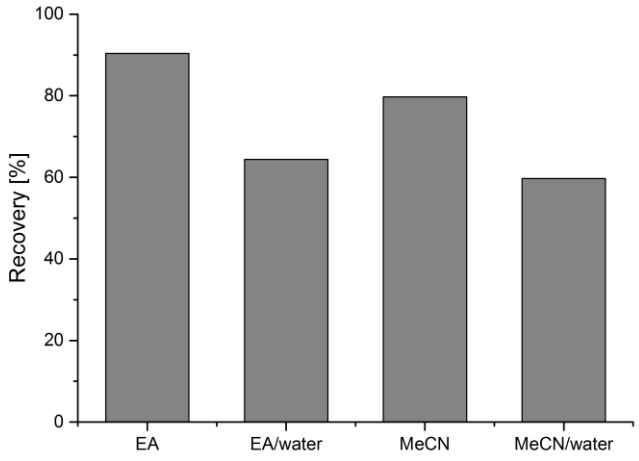


Figure 32: Propiconazole concentration in nanoparticle extracts. Nanoparticles were extracted with different solvents (see Section 5.3.11) and recovery was calculated by comparison of the signal area of propiconazole in extracts to the signal area of propiconazole standard in methanol. EA: ethyl acetate.

5.4.2 Identification of propiconazole transformation products

To assess the fate of propiconazole during the experiments beside sorption and uptake, the parent compound and its biotic and abiotic transformation products were analyzed in mycelium and growth medium.

Detected biotransformation products of propiconazole

Biotransformation products of propiconazole were investigated in transformation studies with rainbow trouts [203] and gammarids [204]. Rösch et al. detected two main metabolites, the first one resulting from hydroxylation and the second one by ether cleavage from propiconazole [204]. Published molecular structures of metabolites and propiconazole [205] are shown in Figure 33, with structural isomers denoted BTP 2.1 - 2.3.

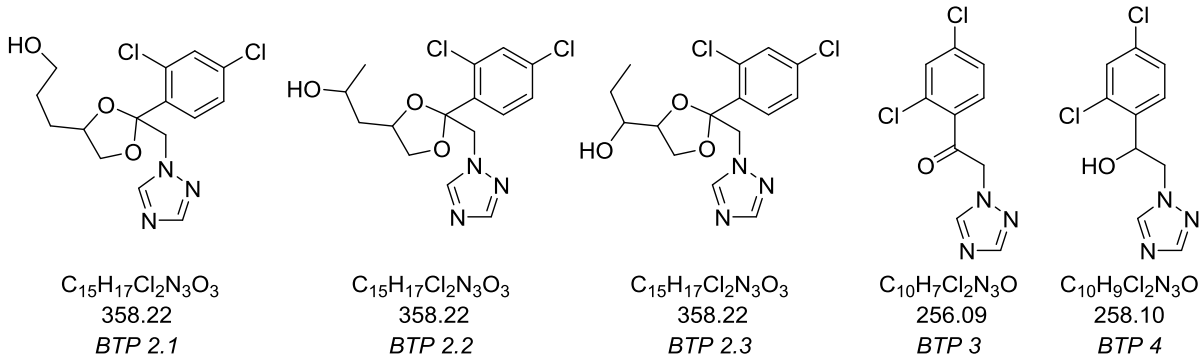


Figure 33: Molecular structures of propiconazole and five biotransformation products (BTP), among them three isomeric forms (BTP 2.1-2.3). Chemical names of the metabolites are 2.1 3-(2-((1H-1,2,4-triazol-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)propane-1-ol, BTP 2.2 1-(2-((1H-1,2,4-triazole-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)propane-2-ol, BTP 2.3 1-(2-((1H-1,2,4-triazole-1-yl)methyl)-2-(2,4-dichlorophenyl)-1,3-dioxolan-4-yl)propane-1-ol, BTP 3 1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethane-1-one and BTP 4 1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethane-1-ol.

The metabolites known from literature were analyzed by HPLC with high-resolution MS and MS/MS. Five metabolites were found consistent with those given in Figure 33, three of them being stereoisomers *BTP 2.1 - 2.3*. An HPLC-MS/MS method was developed to separate and analyze all metabolites in mycelium and medium extracts. For separation, a C18 column and gradient elution with methanol and water was used (see Section 5.3.8). The three stereoisomers had very similar retention times, but were visible as shoulders. The extracted ion chromatograms of five metabolites in fungi extracts are shown in Figure 34.

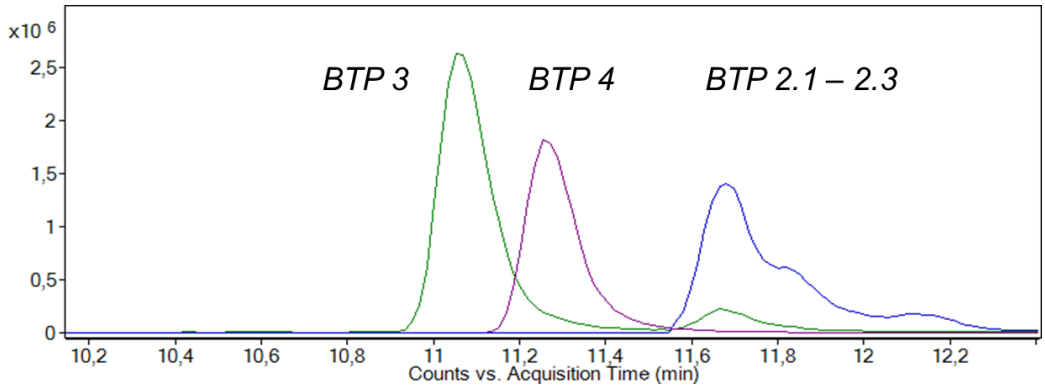


*Figure 34: Extracted ion chromatograms of metabolites in fungi extract, BTP **3** (green), BTP **4** (purple) and the partially separated 3 isomeric metabolites BTP **2.1** - **2.3** (blue).*

All three metabolites were detected in fungi mycelium and medium extracts (see Section 5.5.3 and Section 5.5.4). Compound identification was confirmed by MS/MS analysis and absolute mass as well as retention time, data listed in Table 12.

Table 12: Identification criteria for metabolites of propiconazole, exact masses and retention time as well as major fragment ions obtained at CE 24 V and fragmentation voltage 360 V.

metabolite	$[M+H]^+$	t_R [min]	formula	fragment
BTP 3	256.0039	11.04	$C_{10}H_7Cl_2N_3O$	69.07
BTP 4	258.0195	11.24	$C_{10}H_9Cl_2N_3O$	69.07
BTP 2.1 - 2.3	358.0720	11.6 – 12.6	$C_{15}H_{17}Cl_2N_3O_3$	256.004 + 203.034

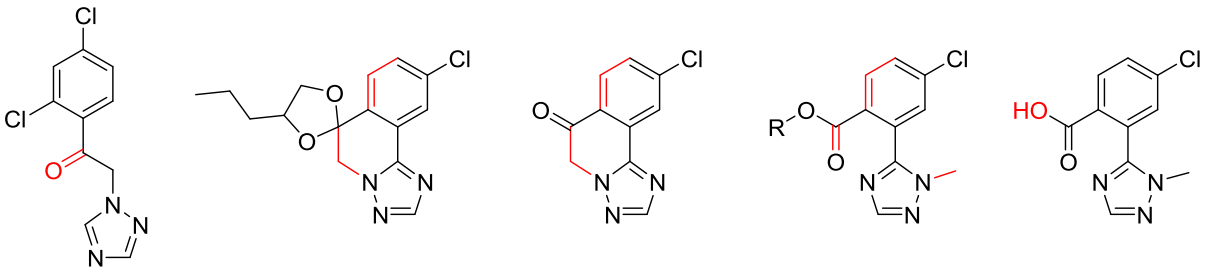
Reference material for metabolites was not commercially available and thus qualitative evaluation via signal intensities was performed for exposure experiments. In the following signal areas are always given relative to the signal area of isotopically labelled propiconazole-d_3.

Photo-transformation experiments of propiconazole

Photo-transformation experiments were carried out for 60 min with irradiation at 220 – 250 nm (see Section 5.3.9). Aliquots of the irradiated propiconazole solution were sampled and injected to the MS via a capillary electrophoresis autosampler and capillary. The products were not purified or isolated, thus, signal areas of transformation products are given in % of propiconazole, assuming that ionization efficiencies are similar to propiconazole. Photo-transformation products were identified according to literature [205-206]. Kinetics of the photo-degradation processes were not considered and would have necessitated longer reaction times.

Five transformation products could be identified by their exact masses (Figure 35), three of them are known from literature (*PTP 2*, *PTP 3*, *PTP 5)* [206]. The most abundant signal was obtained for m/z = 324.11, corresponding to the calculated molecular formula $C_{15}H_{18}ClN_3O_3$, relating to compound PTP 5.

Extracted ion chromatograms (EICs) were plotted for the transformation products in mycelium samples analyzed by HPLC-MS. Only transformation product $C_{10}H_7Cl_2N_3O$, compound PTP 2, was detected in fungi mycelium extracts, this compound is identical to BTP 3 originating also from metabolic transformation processes (see Figure 33). Therefore, it is likely that only biotic processes are relevant during the exposure experiments.



$C_{10}H_7Cl_2N_3O$ *PTP 2*

$C_{15}H_{16}ClN_3O_2$ *PTP 3*

$C_{10}H_6ClN_3O$ *PTP 4*

$C_{15}H_{18}ClN_3O_3$ *PTP 5* R=$(CH_2)_4CH_2OH$

$C_{10}H_8ClN_3O_2$ *PTP 6*

Figure 35: Molecular structure of propiconazole and photo transformation products PTP, changes highlighted in red. PTP 2 1-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-yl)ethane-1-one, PTP 3 9-chloro-4'-propyl-5H-spiro[[1,2,4]triazolo[5,1-a]isoquinoline-6,2'-[1,3]dioxolane], PTP 4 9-chloro-[1,2,4]triazole[5,1-a]isoquinoline-6(5H)-one, PTP 5 4-hydroxybutyl 4-chloro-2-(1-methyl-1H-1,2,4-triazol-5-yl)benzoate and PTP 6 4-chloro-2-(1-methyl-1H-1,2,4-triazole-5-yl)benzoic acid.

The time dependence of the formation of photo-transformation product PTP 5 is shown in Figure 36. With a small induction period visible below 5 min, it is likely that it is a secondary product and a daughter of a short-lived species. The compound is formed by hydrolysis of the dechlorinated cyclic ketal PTP 3 resulting in the side chain connected via ester bonding and methylation of the triazole ring. At the end of the experiment at 60 min, the signal area of compound PTP 5 was in the range of 3 x 10^7 and thus one order of magnitude below the initial propiconazole concentration of a peak area of 3 x 10^8. This concentration makes compound PTP 5 the main transformation product of propiconazole formed by irradiation.

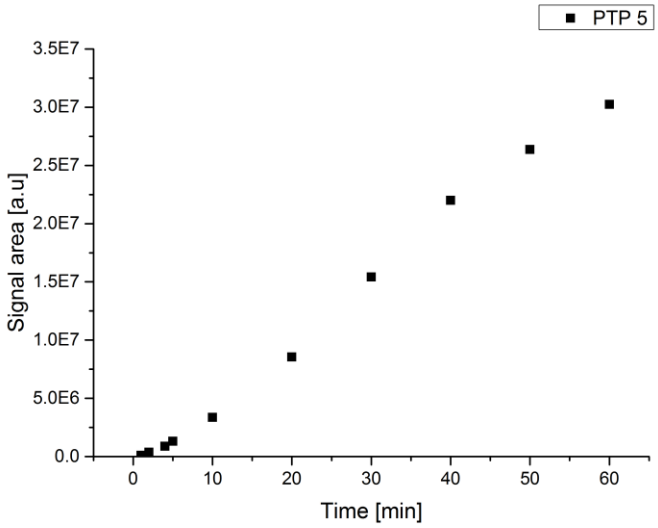


Figure 36: Kinetic of photo-transformation product PTP 5.

The time dependence of the formation of photo-transformation product $C_{15}H_{16}ClN_3O_2$, compound PTP 3, is exponential. After about 35 min or PTP 3 further degrades in a secondary reaction and its concentration diminishes again, most likely because secondary products PTP 4 - 6 are formed (Figure 37). Vialaton et al. predicted HCl elimination followed by photo-cyclization as the first step and the major pathway of propiconazole photo-degradation [206]. Compound PTP 3 as precursor for products PTP 4 to 6 explains the kinetic shown in Figure 37 in grey ▲. Assuming similar ionization efficiencies, the concentrations of PTP 5 and PTP 6 exceed the concentration of PTP 3 at 60 min leading to drastically decreasing concentrations of the precursor compound. Compound PTP 5 is detected after 4 minutes after an induction period with increasing concentrations. PTP 3 is detected from minute 20 on in correlation with the kinetics of PTP 3.

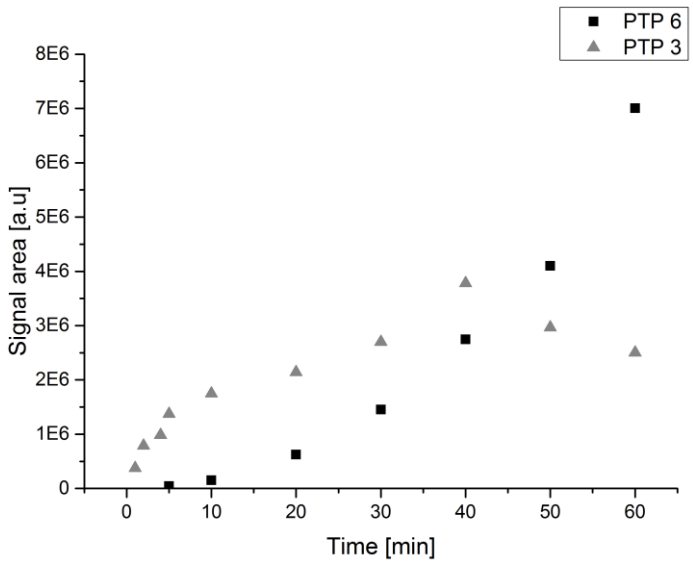


Figure 37: Kinetic of photo-transformation product PTP 3 and PTP 6.

The reaction product PTP 6 is formed from PTP 5 by hydrolysis of the ester bound in the side chain and possesses a benzoic acidic functional group. The exponential kinetic (Figure 37) can be explained, because formation of PTP 6 depends on the concentration of precursor PTP 5. Reaction product PTP 6 reaches a signal area approximately 25 % of the one of precursor PTP 5.

Photo-transformation product $C_{15}H_{18}ClN_3O_3$, compound PTP 2, is formed following a zero order linear kinetic, the secondary product PTP 6, compound $C_{10}H_8ClN_3O_2$, is formed in a first order exponential kinetic. Both photoproducts are detected in increasing concentrations starting already 1 min after starting the irradiation. Vialaton predicted PTP 2 as formed by hydrolysis of the cyclic photo-product PTP 4 [206]. PTP 2 is formed by hydrolysis of the ketal resulting in the metabolite with a keto functional group, the kinetic is shown in Figure 38. This compound is also formed in biotic transformation. Compared to the other photo-transformation products, compound PTP 2 has the lowest signal area, resulting in the lowest concentration of 0.3 % of the initial propiconazole concentration, whereas it is one of the most abundant metabolites in biotic transformation processes [204].

At 20 min the photo-transformation product $C_{10}H_6ClN_3O$ (PTP 4) is formed with linearly increasing concentration (Figure 38). Cleavage of the ketal in PTP 3 results in PTP 4 containing a tricyclic system with a keto functional group at the photo-cyclized ring.

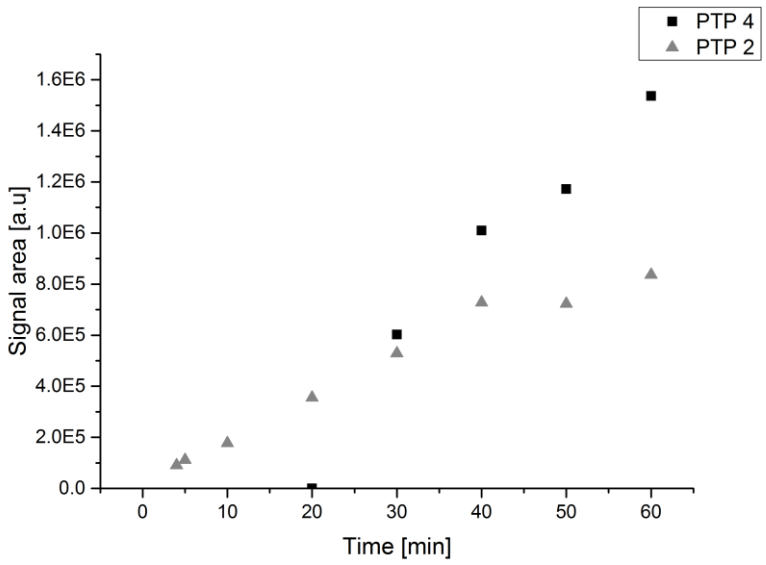


Figure 38: Kinetic of PTP 2 and PTP 4 formed by irradiation within 60 min.

All photo-transformation product kinetics start with a slower initiating phase, indicating that, they are secondary products. Propiconazole was degraded in water at 269 nm by direct photolysis as well as indirectly photodegraded by photo-induced reaction of dissolved organic matter leading e.g. to radicals [206]. Product formation depends on the wavelength. At short wavelength the photo-cyclization (PTP 3) was the major pathway, at longer wavelength like sunlight oxidation processes occurred [206]. The main products found by Vialaton et al. are PTP 5 and PTP 3 like in the experimental results shown here. Vialaton et al. confirmed the transformation products and their stereochemistry by independent synthesis [206].

To exclude abiotic transformation processes as a reason for decreasing propiconazole concentrations, photo-transformation products known from literature were analyzed [205-206]. Both photo- and oxidative transformation processes are known to be relevant for propiconazole [207] [208]. The impact of UV light for photo-degradation of propiconazole was observed to play a minor role under field conditions [207].

Photo-transformation products were neither detected in fungi sample extracts originating from agar plate and liquid culture experiments nor in liquid culture medium and agar plates. As both treatments were carried out without daylight, that seems likely. The only mass, which was detected, correlating to photo-transformation product PTP 5 is known to be also formed in biological processes (BTP 2 - 4) [204].

5.4.3 Evaluation of analytical procedures with regard to literature methods

The developed method enables the analysis of extracts from 20 mg dried mycelium grown in liquid culture and 20 mg fungi mycelium grown on agar plates with the same extraction protocol based on QuEChERS extraction procedure. To my knowledge, this is the first time that this extraction method is applied to fungi mycelium. With the developed method propiconazole and metabolites are detectable in the same extract without further extraction or cleanup steps. The analysis by HPLC-MS allows detection of propiconazole in fungi grown on agar plates at 0.1 µg/L and quantification at 0.2 µg/L corresponding to 25 ng per g wet weight mycelium with recoveries of 66 ± 6 %. For mycelium sample in liquid culture the developed method is applicable to different fungi species tested and comprises a limit of detection and quantification below 1 µg/L in fungi extract from 20 mg dried mycelium.

Recoveries of 104 ± 10 % were achieved based on deuterated internal standard. The developed agar extraction procedure of dried agar plates provides a robust protocol to analyze the propiconazole content of agar plates.

5.5 APPLICATION

The developed method was applied to fungi samples grown on agar plates and in liquid culture exposure experiments. Fungi were exposed to the fungicide propiconazole and to the combination of propiconazole and York-shell nanoparticles to evaluate the effect of nanoparticle sorption on uptake and transformation of the fungicide. Sorption experiments of nanoparticles and propiconazole were conducted and nominal exposure concentrations were calculated based on sorption isotherms. In comparison of both experiments the impact on a) the uptake rate of propiconazole by the fungi, b) the metabolic transformation rate of the fungi and c) the inhibition of growth was investigated.

L. bicolor was used in agar plate experiments. In these experiments the propiconazole content in agar plates and fungi mycelium grown on these plates exposed to the combination of different nanoparticle concentrations and 100 µg/L propiconazole was analyzed.

Experiments in liquid culture were performed with three fungi species and propiconazole concentrations according to the sensitivity of the fungi. *A. muscaria*, *L. bicolor* and *C. geophilum* were exposed to 5 mg/L propiconazole without nanoparticles. In this experiment fungi mycelium and liquid culture medium were analyzed with the developed method.

To assess the impact of nanoparticles in liquid culture *A. muscaria* was exposed to different propiconazole concentrations without nanoparticles and to 5 mg/L propiconazole and different nanoparticle concentrations resulting in the same nominal propiconazole exposure concentrations after sorption equilibrium. In these experiments the medium and dried fungi mycelium were analyzed with the developed method.

5.5.1 Sorption of propiconazole to nanoparticles

Leyla Guluzada and Stefan Haderlein Center of Geosciences, University of Tübingen, conducted batch experiments for sorption of propiconazole to York-shell nanoparticles. Freundlich/Langmuir isotherms were obtained with a K_d value of 7735.562 and in the experiment on agar plates a K_d value of 10164. From these isotherms, bioavailable concentrations of propiconazole were calculated for different amounts of nanoparticles added to the exposure experiments. Bioavailable concentrations were set to 0.085, 0.05 and 0.015 mg/L reduced from 0.1 mg/L in agar plate experiments and to 1, 2.5 and 4 mg/L reduced from 5 mg/L in liquid culture experiments.

5.5.2 Analysis of exposure medium

Control of propiconazole exposure concentrations in agar plates

In agar plate experiments 1.2, 3.7 and 6.4 mg/L nanoparticles were added to a mixture of agar containing 100 µg/L propiconazole. According to the calculated sorption isotherms, the added nanoparticles were expected to sorb propiconazole. In equilibrium bioavailable concentrations were not sorbed. The bioavailable propiconazole concentrations were 0.015, 0.05 and 0.085 µg/L. The highest exposure concentration was without nanoparticles.

Fungicide concentration was measured in agar in order to verify the calculated bioavailable concentrations in the presence of nanoparticles. The propiconazole concentration was analyzed at the end of exposure experiments in 80 mg dried agar with two independently treated samples per agar plate and three agar plates for each tested concentration. Extraction was carried out with ethyl acetate as described in Section 5.3.6. Calculations included corrections via recoveries (see Section 5.3.6).

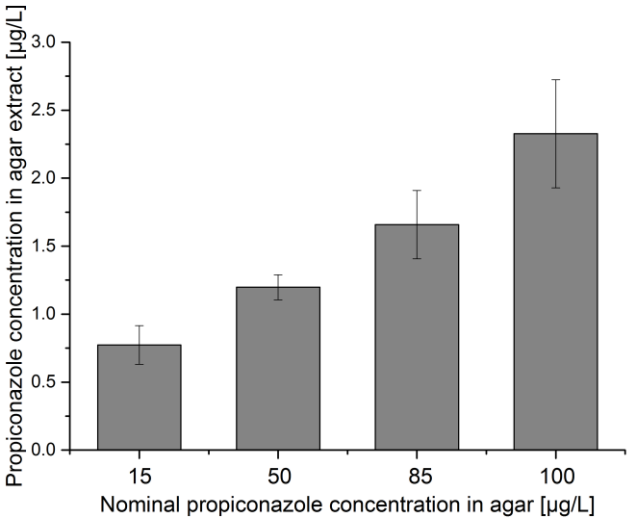


Figure 39: Measured propiconazole concentration in agar extracts at the end of the experiment (n = 6, 3 biological replicates per concentration, and two analytical replicates). Bioavailable propiconazole concentrations were reduced from 100 µg/L to 85, 50 and 15 µg/L by addition of appropriate amounts of nanoparticles, calculated via adsorption isotherms.

For all samples, the measured propiconazole concentration decreased with increasing nanoparticle concentrations (Figure 39). In all experiments 100 µg/L propiconazole and 0, 0.03, 0.129 and 0.517 g/L York-shell nanoparticles were present, leading to a sorption of 15, 50 and 85 %. As expected the available propiconazole concentration was reduced due to sorption to added nanoparticles. Biotic and abiotic transformation products were not detected in agar plate extracts with fungi present.

The amount of available agar differed after drying for the treatments. On average for all 6 treatments, 661 ± 105 mg dried agar were obtained, with differences between the treatments according to added nanoparticle concentrations: in treatments without nanoparticles 720 ± 27 mg were obtained after drying, for samples with 15 % and 50 % sorption nearly the same amount with 680 ± 24 and 684 ± 30 mg was obtained. The lowest amount of agar (549 ± 154 mg) was obtained for the samples with the highest nanoparticle concentration. Addition of nanoparticles might lead to modification of agar but further replicates would be needed for verification of this hypothesis.

Analysis of agar plate extracts prove, that propiconazole concentrations were reduced by sorption to nanoparticles as calculated from adsorption isotherms. In the agar plates neither biotic nor abiotic transformation products were detected at the end of the treatment. Therefore, the exposure concentration during the experiment was assumed to be stable.

Control of propiconazole exposure concentration in liquid culture medium

In the same way as for agar plate cultivation, the exposure concentration of propiconazole was monitored for liquid culture medium. For this, samples of the medium were taken at the

beginning of experiment day (0) and the last day (7) for different nominal propiconazole concentrations of 1 to 5 mg/L. To exclude competing non biological degradation processes with metabolism, samples were analyzed for propiconazole and transformation products (see Section 5.3.5 and Section 5.3.7). In all samples except 2.5 mg/L the concentration was stable in flasks without fungi (Figure 40). In every flask 5 mg/L propiconazole and different nanoparticle concentrations reducing the bioavailable fungicide concentration to 1, 2.5 and 5 mg/L as well as glucose were present. For nominal concentrations of 1 and 5 mg/L no changes in the propiconazole concentration were observed (see Figure 40). In the exposure experiment with 2.5 mg/L a fungal contamination was observed, which can explain formation of the metabolite BTP 4. The measured concentrations were higher than the calculated concentrations in experiments with 2.5 and 4 mg/L. This might be explained by desorption processes of propiconazole from nanoparticles in the presence of medium components.

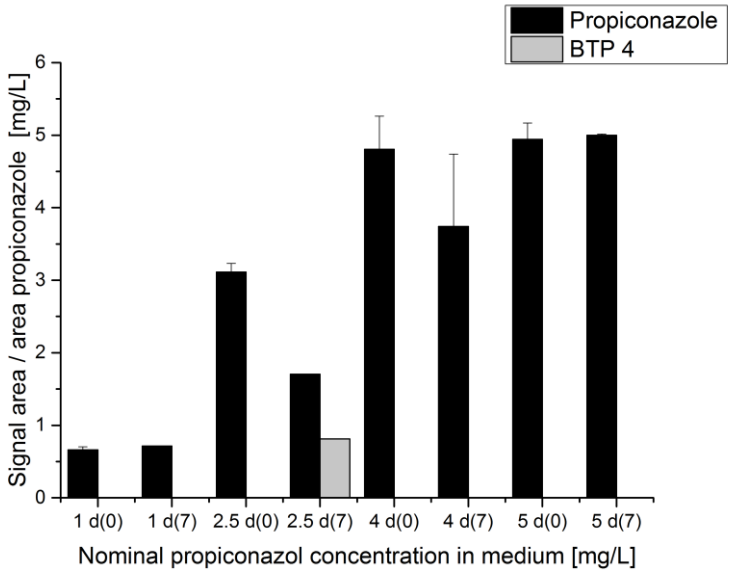


Figure 40: Propiconazole concentration in medium without fungi with reduced fungicide concentrations by sorption to nanoparticles from 5 mg/L to 4, 2.5 and 1 mg/L at the beginning of the experiment day (0) and end of the experiment after one week day (7). Two samples were analyzed per concentration. BTP 4, see Figure 33.

Analysis of liquid medium extracts prove that propiconazole concentrations were reduced by sorption to nanoparticles as calculated from adsorption isotherms. Medium extracts were tested for biotic and abiotic transformation products. In medium extracts from liquid culture experiments only in one contaminated sample biotic transformation products were observed in the other samples neither biotic nor abiotic transformation products were detected. Propiconazole concentrations were in the same range for day (0) and day (7). Therefore, the exposure concentration during the experiment was assumed to be stable.

5.5.3 Uptake and metabolism of propiconazole in *L. bicolor* grown on agar plates in the presence of nanoparticles

Exposure experiment

For growing and uptake experiments with agar plates *Laccaria bicolor* was grown for 2 weeks on agar plates in the presence of nanoparticles and propiconazole (see Section 5.3.4 and Section 5.3.6). Propiconazole concentrations in agar were reduced from 100 µg/L by addition of 0, 0.032, 0.129 and 0.517 g/L York-shell nanoparticles to nominal propiconazole concentrations of 80, 50 and 20 µg/L. The nominal concentrations were calculated based on

adsorption isotherms (see Section 5.5.1). For each concentration three biological replicates were used.

Propiconazole uptake

The propiconazole concentration in *L. bicolor* mycelium after exposure to different bioavailable propiconazole concentrations was quantified using QuEChERS extraction as described in Section 5.3.4. For each exposure experiment (4 concentrations, 3 biological replicates) two independent workups were conducted using 20 mg of homogenized mycelium each. The internal concentration was calculated based on wet weight fungus using internal standard (see Section 5.3.4).

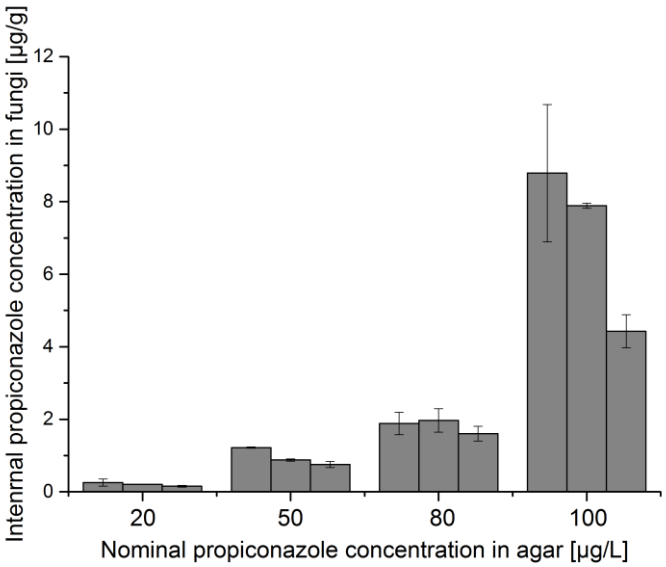


Figure 41: Propiconazole concentration in Laccaria bicolor mycelium extracts grown on agar plates when exposed to propiconazole. Nominal propiconazole concentrations were calculated from sorption isotherms for propiconazole on organo silica nanoparticles. The bioavailable concentrations were reduced from 100 µg/L by addition of nanoparticles to 80, 50 and 20 µg/L. Quantification was based on peak area of propiconazole in ratio to ISTD in QuEChERS extracts. Concentrations are given on a µg/g scale based on the wet weight of the mycelium. For every exposure concentration three biological replicates were conducted, two samples of at least 10 mg were taken per replicate followed by independent workup and analysis.

Figure 41 shows the internal concentration of propiconazole in mycelium plotted against the nominal concentration calculated via adsorption isotherms (see Section 5.5.1). Clearly, the uptake increased with increasing bioavailable exposure concentrations. Low variance was observed for biological replicates with 0.2 ± 0.1, 1 ± 0.2, 2 ± 0.3 and 7 ± 2 µg/g. The results demonstrate that the addition of sorbing nanoparticles reduces the bioavailable concentration of propiconazole and leads to lowered internal concentrations. The non-linear dependence of internal concentration on the nominal concentration, may be explained by propiconazole's effect on fungal growth: Fungal biomass can be used as an indicator for the effects of the fungicide, because propiconazole decreases mycelium growth by inhibition of ergosterol synthesis. Consequently, internal concentrations were plotted against the mass of the fungi from different exposure experiments (Figure 42). The reduced growth at higher bioavailable exposure concentrations indicates the inhibitory effect of the fungicide on ergosterol biosynthesis and thus reduced growth due to a lowered cell wall stability. Treatments can be clearly distinguished one from another.

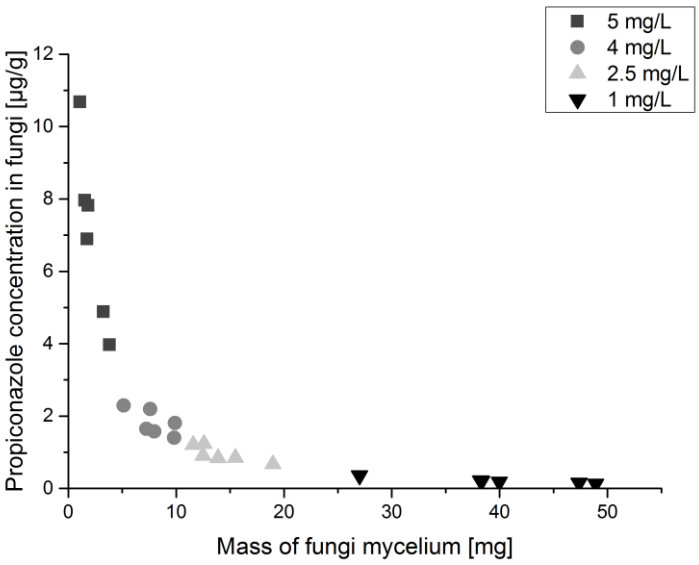


Figure 42: Correlation of mycelium mass and internal concentration of incorporated propiconazole. Fitting with a two-phase exponential association equation model reveals $R^2 = 0.98741$.

Fitting confirms an exponential correlation between growth and propiconazole exposure concentration. This may be explained by a combined effect of higher uptake rates and increased inhibition of growth at higher exposure concentrations.

Propiconazole transformation by *Laccaria bicolor*

To analyze the metabolism of propiconazole all metabolites (BTP 2.1 - 2.3 as combination, because signal were not resolved, BTP 3 and BTP 4) identified in Section 5.4.2 were analyzed in mycelium extracts, according to the procedure described in Section 5.4.2. Due to the lack of commercial availability of metabolite standards, data were evaluated only qualitatively. Results are given in Figure 43.

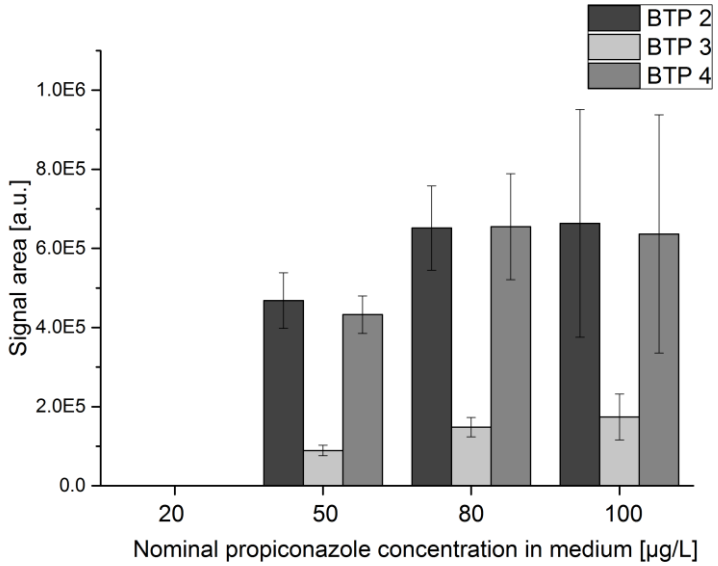


Figure 43: Metabolites in L. bicolor mycelium grown on agar plates exposed to propiconazole at reduced bioavailable concentrations by sorption to nanoparticles (see Section 5.5.1). Signal areas of the metabolites are given. For every exposure concentration three biological replicates with two independent extractions per replicate were analyzed.

In the extract according to the lowest exposure concentration no metabolites were detected, maybe metabolite concentrations were below the limit of detection. The internal concentration of metabolites increases with increasing propiconazole concentration. All signal areas of the metabolites were 10-fold lower compared to propiconazole (Figure 41). Assuming similar ionization efficiencies, a low degree of metabolism was present. Of all metabolites detected in mycelium extracts, BTP 3 revealed the lowest signal intensity of about one third of the one of BTP 2 and 4, regardless of the nominal exposure concentration of propiconazole. Peak intensities of BTP 2 and 4 were in the same order of magnitude.

Metabolites concentrations in mycelium show a metabolic activity of the fungus towards propiconazole and the capability to metabolize the fungicide at exposure concentrations up to 100 µg/L in agar plates. The detection of the metabolites in signal areas 10-fold lower than the one of propiconazole (Figure 41 and Figure 43) and high amounts of propiconazole remaining in the agar (Figure 39) indicates a slow metabolism or reduced availability due to sorption to agar or diffusion limitation. For the different exposure concentrations different mycelium amounts were available for analysis: 2 ± 2, 4 ± 2, 20 ± 14 and 30 ± 14 mg for 100, 80, 50 and 20 µg/L, respectively. Taking the amount of extracted sample into account the detected concentration of metabolites increases with increasing available propiconazole concentrations. This shows, that sorption to nanoparticles prevents propiconazole from degradation. The lower propiconazole content at lower exposure concentrations indicates a reduced uptake of the fungicide correlating with lower metabolite abundancies. To answer the question, if sorption to nanoparticles leads to a stable exposure concentration due to desorption processes from the nanoparticles, more experiments are required.

5.5.4 Liquid culture experiments

Fungi in liquid culture experiments were grown in 20 mL medium with 500 mg fungi mycelium as starting material for 1 week. In these exposure experiments three fungi species *L. bicolor*, *A. muscaria* and *C. geophilum* were exposed to propiconazole at 5 mg/L. *geophilum* to 2 mg/L. According to their sensitivity towards the fungicide, exposure concentrations were chosen to induce a growth inhibition of 70 %. Samples were provided by Elisabeth Früh. Five biological replicates per exposure concentration were analyzed in two independent extractions, according to the developed procedure, described in Section 5.3.5.

Propiconazole uptake by different fungi species without nanoparticles

Quantification or propiconazole in mycelium

Mycelium samples were dried and two independent extractions carried out per biological replicate with the optimized QuEChERS extraction procedure as described in Section 5.3.5. To determine the propiconazole content in mycelia 7 - 42 mg were taken per biological sample. In addition, the liquid medium was analyzed as described in Section 5.5.2. Results are given in Figure 44 with propiconazole concentration in µg/g dried mycelium. Clearly, the detected internal concentration differs significantly between the three species (Figure 44).

The highest internal content of 785 ± 47 µg/g was detected in *C. geophilum*. In *A. muscaria* the content was only 33 ± 21 µg/g and thus lower by a factor of 24. Propiconazole content was intermediate (284 ± 80 µg/g) in *L. bicolor*, but here, the highest standard deviations were observed. The fungi with the highest internal concentration in the *A. muscaria* experiment was excluded from statistical analysis: Without this outlier the measured values in all three species are normally distributed and with significant differences ($F = 1.6E-7$, $F = 2.5E-12$, $p > 0.05$).

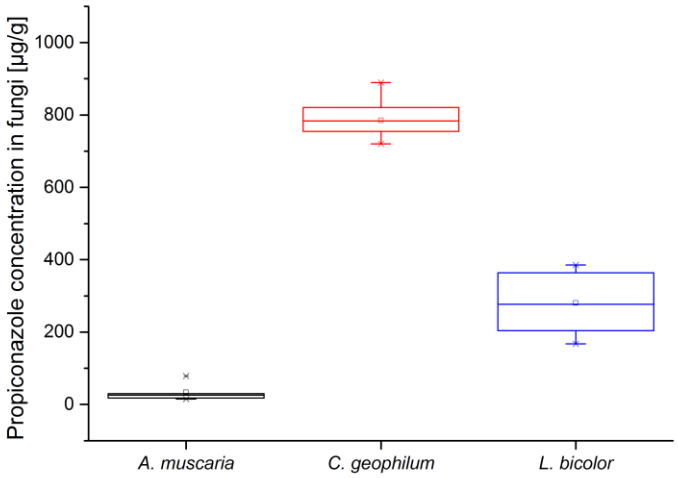


Figure 44: Comparison of the internal content of propiconazole in three fungi species grown in liquid culture, exposed to 5 mg/L propiconazole for 7 days. Five biological replicates per fungi species each divided into two analytical replicates were analyzed. Samples were extracted by QuEChERS extraction. Quantification was based on external calibration, see Section 5.4.1.

The mycelium mass analyzed was 42 ± 3 mg for *C. geophilum*, 12 ± 2 mg *L. bicolor* and 7 ± 2 mg for *A. muscaria*. Results reveal, that the internal concentration is inversely correlated to biomass. This indicates different metabolic activities of the different species. Except for the medium for *L. bicolor*, at the end of the experiment no propiconazole was observed in the medium. This indicates that propiconazole was fully metabolized by the fungi. These finding corroborate literature observations. In experiments on agar plates *A. muscaria* and *C. geophilum* were able to tolerate up to 0.1 ppm propiconazole and one strain of *C. geophilum* was even stimulated in growth [161]. In the study presented here, *C. geophilum* showed the highest increase in biomass combined with the highest concentration of incorporated fungicide. For *L. bicolor* and *A. muscaria* distinct differences in glucose transport were observed [209]. Glucose was taken up at maximal speed from the beginning of the experiment by *L. bicolor* whereas *A. muscaria* exceeded this uptake rate by a factor of seven at the end of experiment [209 163 210]. The higher metabolic activity of *A. muscaria* could explain the lower incorporated fungicide concentration. If propiconazole is metabolized faster in *A. muscaria* than in the other species, this would lead to a lower internal concentration although the uptake rate is higher.

These results clearly show strong inter-species differences for the tested fungi. Thus, the transfer of results from one species to another and the assessment of a general impact on fungi is difficult, as results of this study indicate that metabolism in different fungi species tends to differ to a great extent. Results provide evidence for bioaccumulation of propiconazole in some of the treated fungi species.

Propiconazole transformation in liquid culture medium by different fungi species

Metabolites were not detectable in mycelium extracts. Still they might be present but at lower concentrations than the limit of detection, or released to growing medium.

The exchange between fungi and liquid medium is fast for nutrient provision. In addition, there are many extracellular enzymes secreted by the fungi. Therefore, the medium was analyzed for differences in metabolic activity between the fungi species to explain the different propiconazole concentrations after 7 days of exposure. Three biological replicates

were analyzed for each fungus. The analytical methodology is described in Section 5.3.8. Metabolites BTP 2 - 4 were identified by HPLC-MS and their signal area is given in Figure 45 together with the signal area of propiconazole, where detected. In samples from *L. bicolor* all analytes were detected. In *C. geophilum*, BTP 2 - 4 but not propiconazole were detected and samples of *A. muscaria* did not reveal any of the compounds.

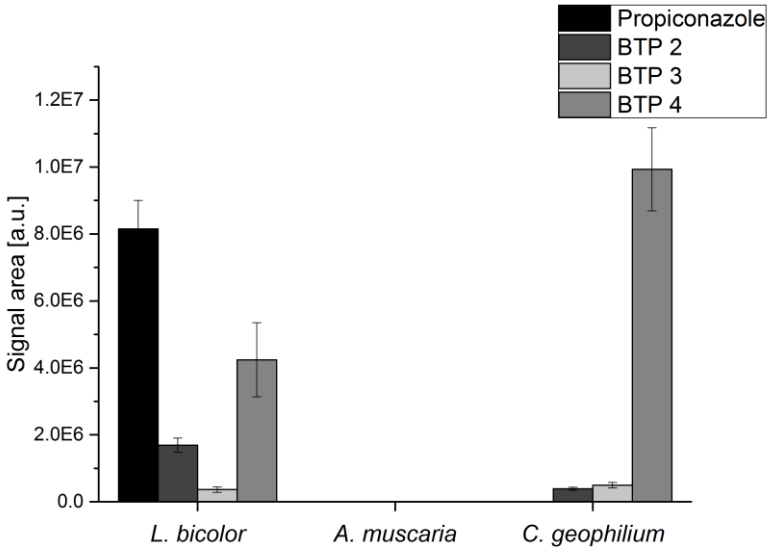


Figure 45: Propiconazole and metabolite concentrations in liquid culture medium after 7 days of exposure to different fungi species. Peak area of metabolites and propiconazole are given. Three biological replicates were analyzed for every fungi species.

Detected metabolite concentrations clearly differ between species. In case of *A. muscaria* levels below the limit of detection were observed for all metabolites and thus significantly lower than in *L. bicolor* and *C. geophilum* ($p > 0.05$). Obviously, *A. muscaria* is capable of metabolizing propiconazole faster than the other two fungi species (excluding abiotic transformation, see Section 5.5.2). In *C. geophilum* BTP 4 was the most abundant metabolite and propiconazole were not detectable after 7 days. Propiconazole was only detected in the experiments with *L. bicolor* at the end of exposure time. Here, all metabolites showed similar peak areas and thus presumably similar concentrations. BTP 3 had the lowest concentration, which is in accordance with the findings from growing studies on agar plates, where it is the least abundant metabolite (see Section 5.5.3 and Figure 43). The results indicate that all investigated fungi species are able to metabolize propiconazole at concentrations of 5, 2 and 0.1 mg/L in liquid culture for *A. muscaria*, *C. geophilum* and *L. bicolor*, respectively.

In case of *A. muscaria* and *C. geophilum* at the end of the exposure experiment propiconazole was not detectable in the medium, although these experiments were conducted at higher exposure concentrations. *C. geophilium* showed the highest increase in biomass with 42 mg present at the end of the experiment and the highest propiconazole content in mycelium at a medium exposure concentration of 2 mg/L. *A. muscaria* had the lowest increase by 7 mg at the end of the experiment and the lowest propiconazole content in mycelium at the highest exposure concentration of 5 mg/L. These findings show, that the incorporated propiconazole concentration was not reflected by inhibition of growth between the different species. *A. muscaria* seems to transform propiconazole, as only low concentrations were incorporated and no propiconazole was present in the medium, but the

growth rate is reduced, whereas *C. geophilum* seems to incorporate propiconazole and transform it to mainly one metabolite, the growth rate was reduced to a lower extent.

To summarize the different patterns of propiconazole and metabolite composition and concentrations observed in the extracts indicate differences in metabolic activity among the species and growing conditions studied. Differences in metabolic activity may at least partially explain the observed propiconazole concentrations resulting in different bioaccumulation patterns.

Comparing agar plate and liquid culture experiments the propiconazole content in mycelium grown on agar plate was 30 fold lower at the same exposure concentration of 100 µg/L for *L. bicolor* samples. In case of agar plate experiment the internal concentration is wet weight whereas in liquid culture mycelium it is based on dry weight. Data of the range of mass reduction during drying were not available. With assuming a reduction of mass by approximately 90 %, the accumulation potential of the fungus would be in the same range for both growing conditions. In agar plate experiments metabolites were detected in mycelium in 10-fold lower concentrations compared to the detected propiconazole concentrations. In liquid culture experiments metabolites were not detected in mycelium extracts and at 10-fold lower concentrations in medium than the remaining propiconazole concentration. This indicates a faster metabolic activity in liquid culture than in agar plate experiments. The higher transformation rate in liquid culture medium may be explained by a higher metabolic activity of the fungus in liquid culture, where nutrients and propiconazole are more easily accessible than in agar plates. The higher amount of mycelium available at the end of the exposure time supports this hypothesis.

In some cases metabolites are more active than the parent compound. To investigate the toxicity of propiconazole metabolites experiments with isolated metabolites are required. But the higher growing rate in liquid culture medium experiments in the presence of metabolites indicates a reduced toxicity of the metabolites compared to the parent compound.

Changes in propiconazole uptake by Amanita muscaria grown in liquid culture by the addition of sorbing nanoparticles

The impact of nanoparticles on toxicity of propiconazole towards fungi was investigated in experiments with *A. muscaria* in liquid culture using five biological replicates per exposure concentration. For reference, a first exposure experiment used concentrations of 1, 2.5, 4 and 5 mg/L propiconazole without nanoparticles to assess the impact of the fungicide (see Figure 46). In a second step, nanoparticles were added to the liquid medium containing 5 mg/L propiconazole in to order sorb propiconazole to a certain extent. The nanoparticle content was chosen from isotherm data to give rise to a nominal concentration in the medium of 2.5 mg/L. Exposure experiments were conducted for seven days. Biological replicates were divided in duplicates of each 3 - 11 mg. They were processed with QuEChERS extraction procedure according to Section 5.3.5 and analyzed for propiconazole as described in Section 5.3.8.

Uptake of propiconazole by mycelium in the presence of nanoparticles

The incorporated concentrations in this experiment were in the same order of magnitude like the concentration detected in *A. muscaria* mycelium from the previous experiment where fungi were exposed to 5 mg/L propiconazole (see Section 5.5.3 and Figure 44) corroborating the robustness of the methodology used. An increasing internal concentration with increasing exposure concentration of propiconazole was detected (Figure 46). The internal

concentration was significantly different for samples with nominal concentrations 1 vs. 4 mg/L (p = 0.00038) and 1 vs. 5 mg/L (p = 5.9E-5), and for 2.5 vs. 5 mg/L (p = 0.00011).

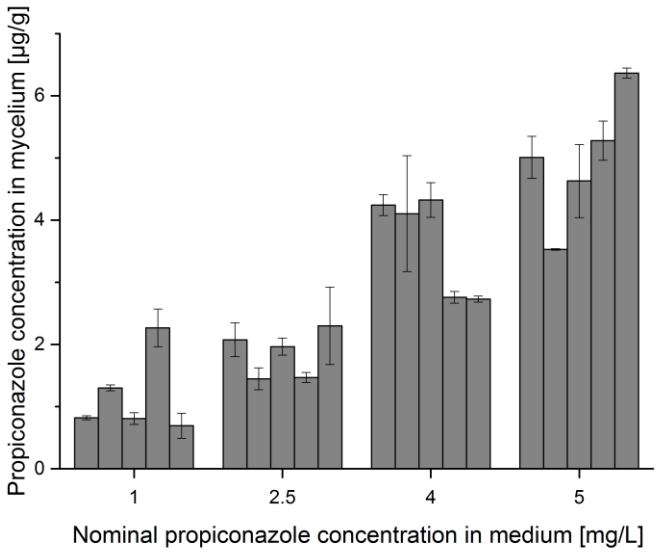


Figure 46: Propiconazole concentration in A. muscaria grown in liquid culture exposed to different fungicide concentrations. For each exposure concentration five biological replicates were analyzed in two independently extracted aliquots. Average values for the two extracts are given. Quantification is based on peak area calculated based on peak area of internal standard.

One set of samples was exposed to 2.5 mg/L propiconazole directly and the other one in an experiment, where the original propiconazole concentration of 5 mg/L was reduced by addition of nanoparticles sorbing propiconazole to yield a nominal bioavailable concentration of 2.5 mg/L as calculated by sorption isotherms. The difference in propiconazole concentration in mycelium from these two exposure experiments were highly significant with (p = 2.75E-9) (see Figure 47).

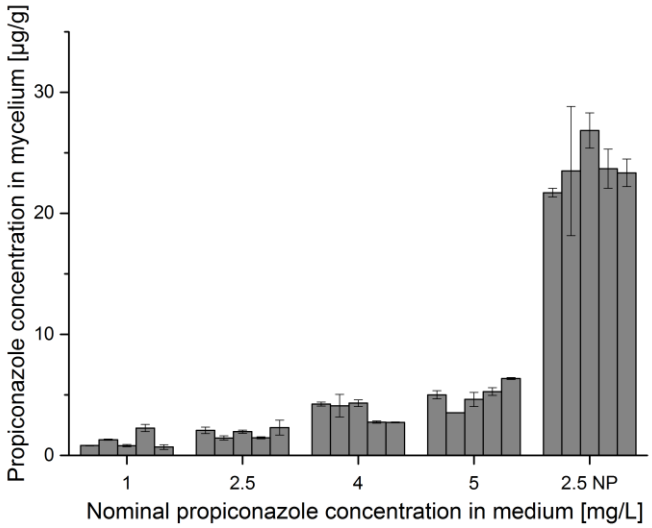


Figure 47: Propiconazole concentration in QuEChERS extracts from A. muscaria mycelium exposed to 1, 2.5, 4 and 5 mg/L propiconazole as well as exposed to a medium with 5 mg/L propiconazole and nanoparticles reducing the bioavailable concentration by sorption to 2.5 mg/L (calculated by sorption isotherms). For each exposure concentration 5 biological replicates were analyzed each in two independently extracted aliquots (n = 2). Quantification is based on deuterated internal standard.

The higher concentration in the fungi exposed to the combination of nanoparticles and propiconazole gives rise to the suspicion of a pool of propiconazole being sorbed to the nanoparticles. This pool is not bioavailable and protected from being metabolized by the fungus (compare Section 5.5.4). Therefore, it would still be detectable at the end of the experiment. This hypothesis is further supported by the difference in the mass of mycelium (dry weight) present after 7 days of exposure shown in Table 13.

Table 13: Available fungi mycelium mass (dry weight) grown after 7 days at different exposure concentrations of propiconazole with and without addition of nanoparticles (NP).

exposure concentration [mg/L]	1	2.5	4	5	5 + NP (2.5 nominal)
mycelium mass [mg]	13 ± 5	14 ± 1	11 ± 4	9 ± 2	18 ± 1

With increasing propiconazole concentration, growth is inhibited, which is reflected in the mycelium mass present at the end of the experiment decreasing from 13 to 9 mg. In the exposure experiment with nanoparticles, a medium nominal concentration of 2.5 mg/L was defined. In this experiment, the highest mycelium mass was observed. Different explanations can be given: It might indicate that nanoparticles (absolute amount in the medium was 6 mg) sorb to mycelium surface and are included in QuEChERS extraction and remain in QuEChERS extracts. 0.129 g/L nanoparticles were added in samples 2.5 NP with 20 mL medium resulting in 6 mg absolute nanoparticles and a bioavailable concentration of 2.5 mg/L propiconazole. A co-extraction of propiconazole from nanoparticles is unlikely, because desorption experiments gave no hint to different solvation properties of acetonitrile and ethyl acetate (see Section 5.3.11 and Figure 32). Experiments with nanoparticles and without propiconazole did not affect the increase of biomass.

When nanoparticles were added to the liquid culture medium containing 5 mg/L fungicide, the internal concentration was up to five times higher compared to all further experiments without nanoparticles. To better evaluate the effect of nanoparticles, exposure experiments with different propiconazole concentrations are compared to exposure experiments conducted at 5 mg/L propiconazole and different nanoparticle concentrations reducing the bioavailable propiconazole concentration to the same nominal concentrations used in the experiment without nanoparticles.

Internal propiconazole concentration in Amanita muscaria exposed in the presence of different amounts of nanoparticles

Exposure experiment

A. muscaria was exposed to different propiconazole concentrations as in the previous experiment. In this experiment the same nominal concentrations were reached by addition of nanoparticles reducing the bioavailable concentration due to sorption of propiconazole to nanoparticles. The nominal (bioavailable) propiconazole concentration was reduced from 5 mg/L propiconazole by addition of different concentrations of York-shell nanoparticles, calculated via adsorption isotherms. Fungi were treated as in the previous experiments and exposed for one week in liquid culture medium. Dried mycelium (at least 10 mg) from five biological replicates was extracted in independently treated duplicates and analyzed with the optimized HPLC-MS method described in Section 5.3.5. Nanoparticle concentrations were 0.03 g/L resulting in 4 mg/L bioavailable propiconazole, 0.129 g/L resulting in 2.5 mg/L propiconazole and 0.517 g/L nanoparticles resulting in 1 mg/L nominal propiconazole as exposure concentration.

Uptake of propiconazole at reduced bioavailable concentrations

As shown in Figure 48, the highest propiconazole concentrations were detected in mycelium exposed to nominal propiconazole concentrations of 1 and 2.5 mg/L with 7 ± 1 and 9 ± 3 µg/g. The five biological replicates exposed to 4 mg/L propiconazole showed a high variation of 2 ± 1 µg/g for three fungi and 9 ± 2 µg/L for two fungi. The lowest propiconazole content was detected in fungi exposed to the highest concentration of 5 mg/L without addition of nanoparticles with a content in mycelium of 1 ± 0.5 µg/g. The analysis of mycelium showed an increasing propiconazole concentration with higher nanoparticle concentrations, correlating with lower nominal propiconazole concentrations. The detected concentration in the sample exposed to 5 mg/L was five times lower compared to the sample without nanoparticles and the concentration detected in the sample exposed to 2.5 mg/L was three times lower. The analyzed concentrations are in the same order of magnitude and standard deviation of concentrations within one experiment was lower especially for samples exposed at the same concentration. This shows the robustness of the method but differences between experiments. These differences may be due to different laboratory conditions during exposure time.

The high variability in samples exposed to 4 mg/L bioavailable propiconazole agrees with a high variability of propiconazole concentration detected in reference medium samples at this concentration (Figure 40), the reasons for this are unknown.

The results reveal strong differences of the propiconazole concentration in mycelium exposed when nanoparticles were added compared to exposure experiments with only propiconazole (Figure 46). Fungi grown in the highest exposure concentration of propiconazole-only extracts showed the lowest internal concentration, whereas at high nanoparticle concentrations and thus at the second lowest bioavailable concentrations the highest internal concentrations were measured (Figure 48).

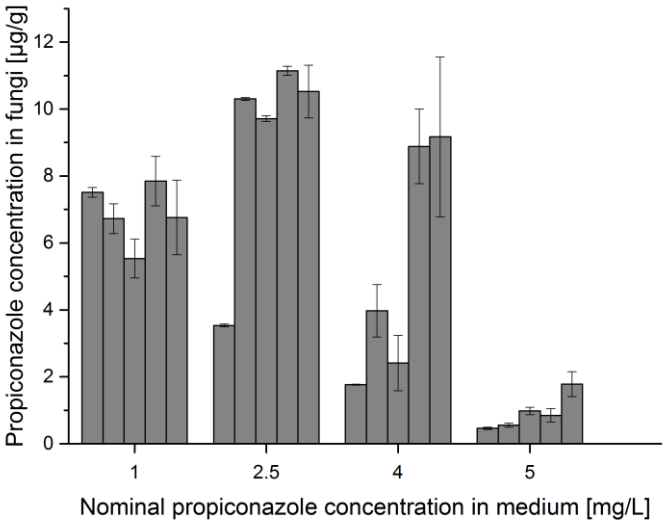


Figure 48: A. muscaria mycelium grown in liquid culture exposed to 5 mg/L propiconazole and different concentrations of nanoparticles reducing the bioavailable concentration to nominal concentration of 1, 2.5 and 4 mg/L propiconazole. For each exposure concentration 5 biological replicates were analyzed each in two independently extracted aliquots. Quantification is based on deuterated internal standard. To reduce the bioavailable propiconazole concentration 0.032, 0.129 and 0.517 g/L nanoparticles were present in 20 mL medium.

Higher detected propiconazole concentrations in experiments including nanoparticles might be due to reduced degradation by the fungi of sorbed and thus non-bioavailable propiconazole as discussed in the previous section. The real propiconazole concentration may be lower than expected from the experimental design. During the extraction procedure propiconazole may desorb from nanoparticles themselves. Real propiconazole concentrations may differ from nominal ones by bio transformation, see Section 5.5.4. These may sorb to fungi mycelium by higher affinity to organic material than to water. Consequently, propiconazole concentration may not fully reflect internal concentrations.

In previous experiments with different fungi species (Figure 44 and Section 5.5.4) metabolic activity was observed by detection of metabolites in the medium (Figure 45) and in fungi mycelium grown on agar plates (Figure 43). Another indication for metabolic activity of the fungus is the decreasing concentration of propiconazole during exposure time. At the end of the experiment no propiconazole was detected in the exposure medium (Figure 45). If *A. muscaria* is able to quickly metabolize propiconazole, sorption of the fungicide to nanoparticles would inhibit this degradation processes and only bioavailable fungicide is degraded. As organic nanoparticles might sorb to organic material like mycelium rather than being dissolved in medium, during the extraction procedure propiconazole would partially desorb from nanoparticles and be detected in addition to the internal concentration in fungi. To prove this hypothesis mycelium and medium were analyzed for metabolite concentrations.

Identification of metabolites in mycelium

Chromatograms were re-evaluated for metabolites. The mycelium extracts contained the metabolites BTP 2 - 4, see (Figure 49). Metabolites were quantified relative to propiconazole-d_3 signal area. The most abundant metabolite in all samples was BTP 4, detected in higher concentrations than propiconazole, followed by BTP 2 in about 10-fold lower amount assuming similar ionization efficiencies. All samples reveal very similar ratios in metabolic composition. Surprisingly, all analytes showed decreasing concentrations at higher nominal initial propiconazole concentrations. In the previous experiment with different fungi species (Section 5.5.4) metabolites were not detected nether in the *A. muscaria* mycelium (Figure 44) nor in medium (Figure 45), this indicates fast complete degradation of propiconazole. In the experiment with nanoparticles propiconazole and metabolites were detected at the end of the exposure time in medium and mycelium. This shows an effect of nanoparticle sorption to the degradation of propiconazole by the fungus.

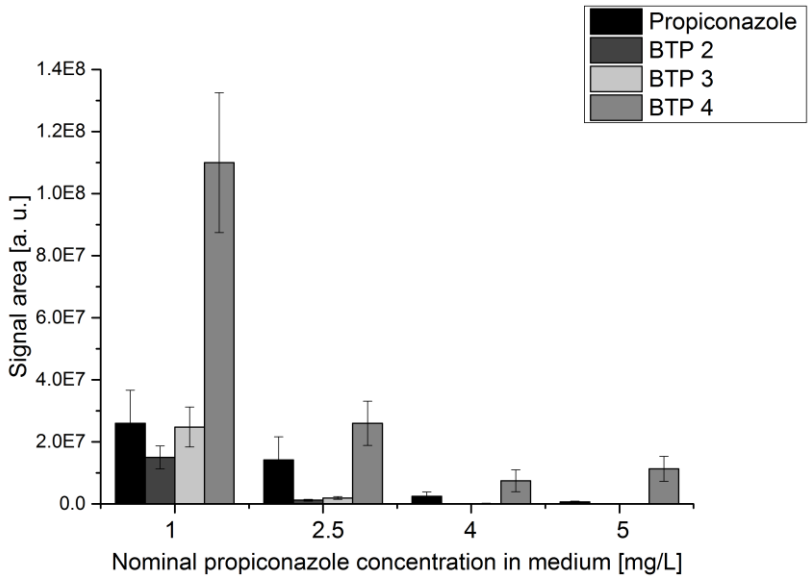


Figure 49: Peak area of propiconazole and of three metabolites in A. muscaria mycelium grown in liquid culture for seven days exposed to propiconazole in the presence of nanoparticles reducing the bioavailable concentration from 5 mg/L by sorption processes. Samples were extracted with ethyl acetate and analyzed by HPLC-MS.

The metabolite composition shown in in Figure 49 was different from the one observed in exposure experiments with *L. bicolor*, grown on agar plates (Figure 43), where BTP 4 signal area was in the same order of magnitude but BTP 3 signal area was one third of its intensity. In contrast, in *A. muscaria* the BTP 4 was the most abundant metabolite with 10-fold higher intensities than the other metabolites. The concentration of BTP 2 was close to the limit of detection in samples exposed at high concentrations. This indicates differences in metabolic activity between fungi species.

Concentration of propiconazole and metabolites in liquid culture medium

Liquid culture medium was analyzed for metabolite and propiconazole content, given as signal area. After 7 days propiconazole was not detectable at the highest fungicide concentration without nanoparticles (5 mg/L) and with increasing concentrations in samples with nominal 4 and 2.5 mg/L propiconazole (Figure 51). This increasing propiconazole concentration correlates with an increasing nanoparticle content present in the samples. At the lowest nominal exposure concentration, a low concentration of propiconazole was detected. In reference medium the concentration was stable over the exposure period, thus abiotic transformation can be excluded (Figure 40).

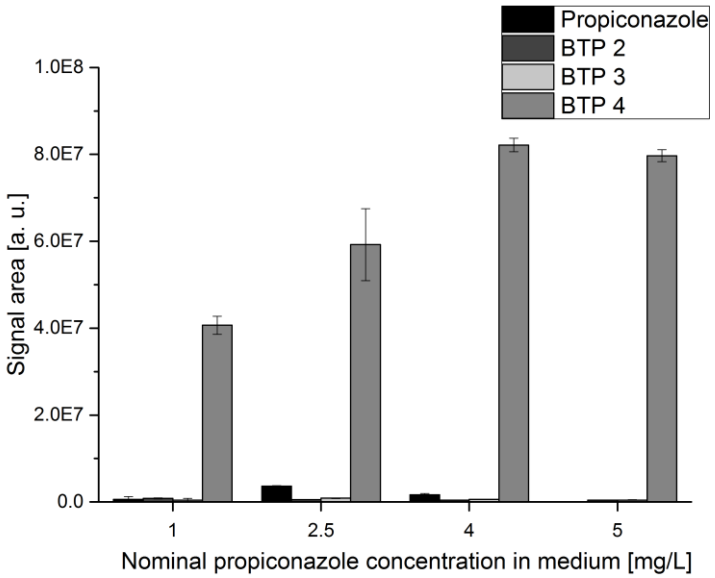


Figure 50: Metabolite concentrations in medium from A. muscaria exposed in liquid culture to propiconazole for 7 days with nanoparticle concentrations reducing the nominal propiconazole concentration. Two samples were analyzed per exposure concentration.

In the medium BTP 4 was the most abundant metabolite (Figure 50), in accordance with the metabolite composition detected in mycelium extracts (Figure 49). In case of BTP 4 the concentration in medium was increasing with increasing exposure concentration.

Metabolite composition between analysis of medium and mycelium differed. In both sample types the metabolite BTP 4 was the most abundant but with different intensities. Metabolites BTP 2 and 3 were at the limit of detection in mycelium extracts but at quantifiable concentrations in medium extracts. The differences in metabolite concentrations and composition show, that for a comprehensive assessment medium and mycelium have to be analyzed, although analysis of medium is less time consuming. The different metabolite compositions may be explained by extracelluar enzymes excreted form the fungus into the medium, as observed in soil [166-167].

In this example two different effects occur. Higher exposure concentrations result in increased propiconazole uptake and higher nanoparticle concentrations lead to a reduced metabolic transformation rate and reduced metabolite concentrations. The combination of both effects results in a maximal propiconazole concentration for a medium nominal propiconazole exposure concentration.

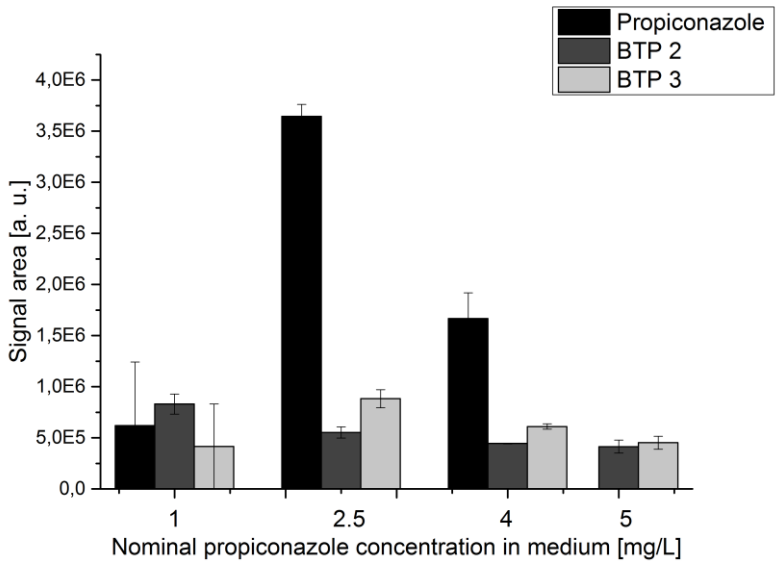


Figure 51: Propiconazole and metabolite concentrations without BTP 4 concentrations in liquid culture, same data as Figure 50.

In contrast to metabolite ratios in mycelium, in the medium BTP 2 with its three isomeric forms of the mono oxidized propiconazole and BTP 3, bearing a keto functional group were detected equally concentrated and independent from the exposure concentration. The fact, that metabolites were detected in mycelium and liquid culture medium samples confirms the hypothesis of mineralization of bioavailable propiconazole by *A. muscaria*.

In medium analysis equal metabolite concentrations were detected independently from the exposure concentration. Furthermore in mycelium extracts higher concentrations were detected at lower nominal concentrations. This finding may be explained by equilibrium exposure conditions. The propiconazole concentration during the experiment was not monitored but sorbed propiconazole could desorb from the nanoparticles at decreasing propiconazole concentrations resulting in a long term exposure. This would lead to relatively higher metabolite concentrations at lower exposure concentrations, if propiconazole is constantly desorbed after bioavailable propiconazole is metabolized. At higher exposure concentrations the metabolism would be faster as propiconazole is available from the beginning of the experiment on. The potential toxicity of the formed metabolites was not investigated in this study. To assess the toxicity, experiments with isolated metabolites are required. The observation, that metabolites were detected in higher concentrations than the parent compound, makes this question an interesting topic.

5.5.5 Mass balance

To assess the sorption, uptake and transformation of propiconazole and thus discuss also its environmental fate, mass balance was calculated for one agar plate experiment and for one liquid culture experiment both with nanoparticles present.

Agar plate experiment with *L. bicolor*

In the agar plate experiment with *L. bicolor* 25 – 52% of the initial propiconazole concentration was detected in the agar plates (Figure 39). Only a minor part was taken up by the fungus (1 -12 %) (Figure 41). The major part remained in the agar as propiconazole. Metabolites were not detected in agar plates and only in concentrations lower than the one

of propiconazole in mycelium extracts. Therefore, in case of agar plate experiments the metabolism was negligible.

Liquid culture experiment with *A. muscaria*

In the liquid culture experiment with *A. muscaria* the mass balance was below 5 % compared to the initial concentration in medium. For calculation, the concentration of propiconazole and its metabolites in mycelium and liquid culture medium were taken into account. In the medium propiconazole was not detectable at the end of the experiment (Figure 50). The complete amount of residual propiconazole was present in mycelium. Due to strong biodegradation only minor concentrations of propiconazole were detected in the mycelium of *A. muscaria* (Figure 46). Assuming the metabolite ionization efficiency being equal to propiconazole (enabling a quantification based on the labeled internal standard) metabolites in liquid culture experiments would contribute with additionally 1 - 14 % in case of liquid culture experiments. Degradation is thus the most important way for decreasing propiconazole concentrations. Whether complete mineralization or formation of further metabolites, not taken into consideration, occurred has to be investigated in further experiments. Other explanations could be, that metabolites sorb to the nanoparticles or surfaces of equipment and extraction of metabolites was not in complete.

Discussion of mass balance

In this study the most important path for decreasing propiconazole concentrations in liquid culture experiments with *A. muscaria* was mineralization. Assuming that growth in liquid culture promotes higher metabolic rates by easier access to nutrients than in agar plate experiments, uptake and degradation was faster in these experiments compared to agar plate experiments. This hypothesis would agree with the finding of different uptake rates of monosaccharides by different fungi species and the different uptake rates of propiconazole for different species tested [163]. In contrast to literature, in this study metabolites contributed to the overall exposure especially in liquid culture experiments. In the EU assessment report for propiconazole metabolites are counted as not relevant, as they were below 10 % of parent compound concentrations [168], like in case of agar plate experiments. However, in experiments with *Gammarus pulex* metabolite concentrations were only three times lower than the one of propiconazole parent compound [204]. This is in agreement with results of liquid culture experiments presented here. In this study, especially for liquid culture experiments metabolite concentrations exceeded the propiconazole concentration in mycelium as well as in liquid culture. The high metabolite concentrations in the medium indicate that metabolism, which is a major excretion part of xenobiotics for organisms, works in case of propiconazole and fungi in liquid culture or propiconazole is metabolized in the medium by excreted enzymatic degradation of the fungus.

Addition of nanoparticles resulted in higher detected propiconazole concentrations in liquid culture experiments, whereas in agar plate experiments the propiconazole content decreased with higher nanoparticle concentrations. This shows that in case of agar plate experiments sorption to nanoparticles reduced the bioavailable concentration of propiconazole whereas in liquid culture bioavailable propiconazole was metabolized and sorbed propiconazole was available during the exposure experiment most likely due to equilibration processes. In this case nanoparticles reduced the transformation of propiconazole and this might lead to a long term exposure, if propiconazole is desorbed in equilibrium conditions.

5.5.6 Bioconcentration factor

One parameter to assess the bioaccumulation potential of compounds is the bioconcentration factor (BCF). It is calculated by the ratio of propiconazole concentration in fungi mycelium compared to the concentration in medium. The bioaccumulation factor also takes metabolic activity into account.

$$BCF = \frac{c\,(fungi)}{c\,(medium)}$$

BCF can only be calculated for the equilibrium state. A measured BCF greater than 100 is used as indication for a potential bioaccumulating property of a substance [211]. In the experiments reported in this thesis only the internal concentration at one point, the end of experiment, was determined. However, experimental BCFs were calculated from laboratory experiments without nanoparticles for liquid culture and with nanoparticles in case of agar plate experiments. Values (Table 14) are calculated as average of all incorporated concentrations in relation to bioavailable exposure concentration.

Table 14: Calculated bioaccumulation factors for different fungi species exposed to propiconazole at different growing conditions.

fungi	BCF	condition
A. muscaria	5 ± 2 L/kg	liquid culture
C. geophilum	157 L/kg	liquid culture
L. bicolor	56 L/kg	liquid culture
L. bicolor	4600 ± 1600 L/kg	agar plate

In literature, not much data is available on propiconazole uptake in organisms. The results of this study for fungi, thus have to be compared to uptake by other organism. In bluegill fish, the BCF was 180 L/kg [168]. This value is in the range of the calculated BCF for *C. geophilum*. Comparison of calculated values is complicated, because the variability between fungi species as well as between different growing conditions is high (e.g. a factor of 80 comparing agar plate vs. liquid culture conditions). This differences for both treatments in case of *L. bicolor* can be explained by different metabolic activity due to availability of nutrients but it has to be considered that in liquid culture experiments dried mycelium was analyzed and in agar plate experiments wet weight mycelium.

5.6 CONCLUSION

The presented data show differences in uptake rate and metabolic activity between fungi species and growing conditions and the presence of nanoparticles. For all tested species the ability to metabolize propiconazole was observed. Based on QuEChERS extraction a miniaturized extraction procedure was developed for quantification of propiconazole in fungi grown in different exposure conditions. For the first time propiconazole and three metabolites were analyzed in different fungi species and fungi grown on agar plates and in liquid culture. The method using 0.75 mL water and 0.75 mL acetonitrile allowed the analysis of propiconazole and its three main metabolites. In agar plate the limit of detection was 0.1 µg/L and the limit of quantification 0.2 µg/L corresponding to 5 ng propiconazole per g wet weight fungi mycelium. In extracts of dried mycelium samples a limit of quantification of 1 µg/L corresponding to 25 ng/g was achieved. Quantification based on deuterated internal standard revealed recoveries of 100 ± 7 % for agar plate experiments and 85 ± 6 % to 93 ±

7 % for liquid culture experiments of three different fungi species. The developed method was well suited to determine the propiconazole content in agar plates. A strong correlation between growth inhibition and the internal concentration was found for fungi mycelium grown on agar plates. Results of liquid culture experiments indicate the ability of investigated fungi to quickly metabolize propiconazole. Differences between the fungi species and growing conditions with respect to uptake and metabolism were observed. The results clearly indicate a bioaccumulation potential of propiconazole, as it was detected in every fungi mycelium analyzed at bioconcentration factors of 5 to 157 L/kg. Experiments with the combination of propiconazole and nanoparticles revealed different effects of sorption. In case of agar plate experiments the bioavailable concentration was reduced and lower propiconazole content in mycelium was detected. In liquid culture experiments sorbed propiconazole was not metabolized but slowly released to the medium when sorption equilibrium changed and thus higher propiconazole concentrations were detected at the end of the exposure time. The environmental fate of propiconazole may be strongly different depending upon whether it is sorbed to particles or bioavailable.

6 ANALYSIS OF WASTEWATER AND SURFACE WATER SAMPLES FRACTIONATED BY DIFFERENT FREE FLOW ELECTROPHORETIC TECHNIQUES

6.1 ABSTRACT

In this project, free flow electrophoresis was used to preconcentrate and fractionate ionic compounds, mostly organic micropollutants in surface water and wastewater samples. Continuous separation by FFE provides the possibility to remove interfering matrix components and simultaneously concentrate compounds. Fractions were analyzed by HPLC-ESI-QTOF-MS via screening for 92 micropollutants. Two fractionation techniques were used for separation: free flow isotachophoresis and free flow interval zone electrophoresis in pH mode. Ionogenic compounds from water samples were separated according to their electrophoretic mobility or isoelectric point in up to 96 fractions. Absorbance at 260 nm indicated enrichment in the fractions compared to the raw water samples. Fractions with high UV activity were analyzed with the optimized HPLC-MS method in full scan mode. The results show the ability of FFE to separate and enrich wastewater contaminants.

6.2 INTRODUCTION

6.2.1 Wastewater analysis

Multi-component detection techniques by HPLC-MS represent a powerful tool for tackling analytical challenges of trace analysis in surface waters. The increasing number of chemicals on the market and their metabolites give rise to concerns about pollution of surface and drinking water. Chemicals might enter surface waters via numerous ways. Pharmaceuticals and personal care products are released into surface waters via wastewater treatment plant effluents if they are not sufficiently removed. Pesticides applied in agriculture enter surface waters via diffuse entry pathways. The EU water framework commits the Member States to monitor micropollutants in surface waters. For prioritized compounds limits were defined [212-213]. Concerning the complexity of the samples (number and physicochemical characteristics of compounds) and the low compound concentrations in water samples new separation and preconcentration techniques are required. The number of emerging pollutants not covered by environmental legislation brought water analysis, particularly non-target screening and analytical methods comprising several hundred compounds in one run, into the focus of research. Beside the high coverage of different sample components, advantages of multi-component methods include faster analysis and higher cost-efficiency compared to target analysis of single substances. In addition, retrospective analysis may become possible. For environmental analysis of intermediate polar compound in surface water samples, HPLC-MS is used as separation and detection method. An overview of screening methods for the analysis of micropollutants in environmental samples was published by Krauss et al. [214] and a review on non-target water analysis by Schymanski et al. [215]. Three different screening approaches are distinguished by

the authors target analysis based on reference material, screening for suspected analytes without reference standard and screening for unknowns [214].

High-resolution mass spectrometry (HRMS) by Orbitrap or QTOF in full scan mode is gaining more and more popularity due to the advantage, that - theoretically - an unlimited number of compounds is detectable [23]. The use of a triple quadrupole MS for detection limits the number of compounds by the MS/MS capabilities of the instrument [216]. In screening methods, identification of compounds detected in samples is often performed with compound databases comprising retention time, accurate mass and insource fragmentation [194, 217]. In this study, a multi-component method based on HPLC-QTOF-MS target analysis was developed and identification based on retention time and exact mass of reference material.

Low concentrations of emerging pollutants present in surface waters as well as interfering matrix components in MS analysis are one of the greatest challenges in water analysis. Detection of contaminants in drinking water in concentrations below 0.1 µg/L, which is the allowed concentration limit for pollutants in the European Union, requires preconcentration of organic trace pollutants like pharmaceuticals or personal care products, even when very sensitive quantification methods are used [23]. In multi-component analysis of water and wastewater samples, offline or online solid phase extraction (SPE) is often applied for preconcentration of trace contaminants [191, 194, 217-218]. In SPE, a large sample volume is filtered through the SPE cartridge and pollutants are retained by the sorbent material, whereas interfering compounds like salts pass through. In the elution step pollutants are desorbed by a smaller volume of a suitable organic solvent, resulting in preconcentrated samples and often a partial removal of unwanted matrix components. The limit of detection in water samples enriched by SPE and analyzed by TOF-MS is usually in the ng/L range for wastewater chemicals [194]. Interfering matrix components can both enhance and reduce signal intensities of analytes during the ionization step in MS analysis [194]. In one case during an analysis of 400 compounds in wastewater samples, 43 % of the compounds were affected by matrix effects by more than 25 % [194]. Two methods analyzing 539 and 72 compounds in the µg/L range in drinking water observed signal suppression higher than 20 % for 60 % or 8 % of all compounds [23, 218]. These data demonstrate the importance of sample pretreatment to eliminate unwanted matrix components and increase pollutant concentrations for their trace analysis in water. In this project, free flow electrophoresis was applied to surface and wastewater samples to investigate the potential to fractionate and simultaneously preconcentrate ionizable or ionic compounds and remove interfering matrix components.

A general challenge in wastewater analysis is the detection of ionic and highly polar compounds, even when using hydrophilic interaction chromatography (HILIC) and reversed phase liquid chromatography (RPLC) [24]. This often includes metabolites or transformation products as these are mostly more polar than their parent compounds [1]. In RPLC polar compounds are not retarded by the column material and therefore, elute in the dead volume without separation. HILIC methods were developed, though they have the great disadvantage that the aqueous sample has to be diluted to about 80 % acetonitrile before injection. FFE fractionation rises the possibility to obtain fractions compatible with appropriate further separation techniques such as liquid chromatography or capillary electrophoresis. Moreover, the combination of electromigrative and chromatographic separation techniques increases selectivity and peak capacity and come along with a more

effective elimination of interfering matrix components. Quantification reliability is enhanced by the reduction of interfering matrix components or coeluting/comigrating analytes.

6.2.2 Electromigrative separation techniques

In this study, preparative FFE was used to reduce the complexity of surface water samples by fractionation for simplified subsequent chromatographic separation steps by HPLC. Raw water and fractionated samples were analyzed by HPLC-MS to evaluate the complexity of substance mixtures and to preconcentrate pollutants to reach the desired detection limits. Two different techniques were evaluated for applicability: free flow isotachophoresis (FFITP) and free flow interval zone electrophoresis (FFIZE-pH). In the fractionation step, ionic interfering compounds were expected to be separated from the ionic compounds of interest and thus fractions were made accessible for following LC-MS analysis. The focus was on compounds with acidic functional groups to include phenols, often encountered upon microbial transformation.

Ion migration in the electric field

In electromigrative separation techniques, ionic or ionizable species in solution are accelerated by an applied electric field. An ionic compound in solution will move in the direction parallel to the applied electric field and experiences friction upon migration. The velocity (v_i) of the ion is based on the electrophoretic mobility (μ_i) and the electric field strength (E) [219].

$$v_i = \mu_i E$$

The electrophoretic mobility of an ion (μ_i) is given by the charge of the ion (z_i), the elementary charge e_0 (1.6022×10^{-19} C), the viscosity of the solution (η) and the hydrodynamic radius of the ion (r_i) by

$$\mu_i = z_i e_0 / 6 \pi \eta r_i$$

assuming a spherical shape. Every substance has a unique charge-to-size-ratio and therefore, a particular electrophoretic mobility [219]. The differences in electrophoretic mobility are the principles of separation by capillary electrophoresis. Capillary electrophoresis has successfully been applied as analytical separation technique in pesticide and pharmaceutical analysis especially for separation of polar analytes with acidic or basic functional groups [220-224].

Free flow electrophoresis

FFE is a continuous preparative isolation and purification technique based on electromigrative processes [25]. It provides the possibility to fractionate a sample in continuous flow mode compared to batch procedures as for example solid phase extraction, liquid chromatography or gel permeation chromatography [219] which allows processing large sample volumes. The instrumental setup is schematically shown in Figure 52. Separation medium flow and electric field are perpendicular to each other with the sample being continuously injected into the carrier flow as a narrow band [25]. The electrodes are spatially separated from the cell by a membrane to reduce flow perturbation, gas bubbles or electrochemical reactions [225]. Ionic compounds are deflected along their way down the separation chamber and sample into channels according to their effective electrophoretic mobility [225]. The charged analytes migrate perpendicular to the direction of hydrodynamic

buffer flow [226]. The original narrow sample zone thus spreads according to the differences in the effective electrophoretic mobilities of analytes. The separated sample streams are then continuously collected in fraction outlets [25].

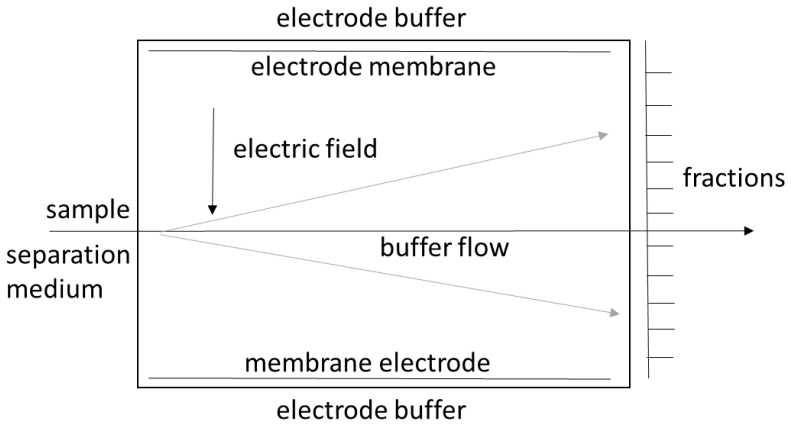


Figure 52: Schematic experimental setup of a free flow electrophoretic cell. [25]

Free flow electrophoresis is frequently used to separate biomolecules on a preparative scale. An advantage is that mild conditions of separation buffers can be used [227]. This enables separation without denaturation of biomolecules which keep their native functions [225]. FFE has been successfully applied to separate enzymes [227], microorganisms [228], amino acids [219], proteins [229] and viruses [230]. FFIEF exhibits the possibility to fractionate proteins with differences in the isoelectric point (pI) by less than 0.5 pH units [226, 231]. In this project, the applicability of FFE to preconcentrate and fractionate small organic ionizable pollutants was investigated. Common sample volumes are 10 mL for one run [225]. The idea was therefore, to fractionate water samples by FFE.

Modes of FFE

Four modes of FFE are commonly used: ZE (zone electrophoresis), IEF (isoelectric focusing), ITP (isotachophoresis) and SFE (step-field electrophoresis) [232]. These four modes are determined by the particular buffer composition used as separation medium [219].

Free flow isotachophoresis (FFITP)

In ITP three zones are present: a leading zone containing an electrolyte with a coion of high effective electrophoretic mobility, an analyte zone containing the sample and a terminating zone comprising a coion of low effective electrophoretic mobility [219]. The ions arrange according to their ionic mobilities by the Kohlrausch regulation function in steady-state zones which are in direct contact [25, 219]. These zones experience self-sharpening [25]. For proteins enrichment factors of 10 or even higher were observed [25]. Fractionation can be facilitated when spacers migrating between the zones of interest are added. For example, Good's buffers can be applied, resulting in diluted mixed zones of analyte and spacer [25].

Free flow isoelectric focusing (FFIEF)

Amphoteric compounds like amino acids have a characteristic pH point, where the overall charge is neutral. This point is called the isoelectric point (pI). In a cell with a pH gradient, the amphoteric compound will stop migrating when the surrounding pH equals to the isoelectric point and the net charge is zero [219]. This effect is used in isoelectric focusing (IEF). In this method again, the sample flow is perpendicular to the applied electric field but

compounds are separated and focused according to their isoelectric point [25, 225]. By application of an electric field, a pH gradient along the chamber is induced due to electrolysis of water or by an appropriate choice of anolyte and catholyte at the electrodes and ampholyte buffer for separation [219, 225]. Compounds migrate until they reach the pH when they are neutral, for ampholytes the pH would equal their pI, for basic or acidic molecules, full protonation or deprotonation would be reached [219]. Amphoteric compounds are refocused by a focusing effect, as compounds may diffuse into regions of higher/lower pH where again a net charge is induced and forces the analyte to migrate back into the zone [225].

Free flow interval zone electrophoresis (FFIZE)

In zone electrophoresis (ZE) fractionation is carried out at a uniform pH and conductivity across the chamber and separation is based on the electrophoretic mobility of analytes [219]. With the interval mode, a higher resolution can be reached. The sample is then injected batch-wise instead of continuously.

Free flow interval zone electrophoresis in pH mode (FFIZE-pH-mode)

In this free flow mode several pH steps are introduced to form band sharpening during the interval zone electrophoresis. The method is similar to FFIEF, however, only few discrete pH steps are introduced with a small number of ampholytes in the separation medium. This technique was developed by FFE-Services and was applied to water samples analyzed in this study.

Migration zone broadening

Different phenomena cause broadening in FFE [233]. Signal-broadening is caused by hydrodynamic, electrodynamic and electrohydrodynamic processes and diffusion. Another limiting factor is the width of the initial injection zone [25]. In the cell, a laminar flow velocity profile for buffer and sample solution evolves. This leads to different migration times of charged compounds in the cell. Compounds in the center of the cell are transported faster than compounds near the cell wall. This effect, named electrohydrodynamic distortion, causes band broadening [25]. Joule heating, the heat produced by friction forces from electric current inside the buffer system is another effect that causes band broadening by thermoconvection [234]. Application of higher voltages improves separation but causes Joule heating [225, 232]. The produced heat is dissipated via the cell walls and therefore a temperature gradient occurs within the cell. In two-side cooling cells, the gradient is towards both cell walls with a maximum in temperature in the middle [25]. A change of 1 °C causes an increase in electrophoretic mobility by about 2 % due to viscosity changes [25]. Application of media with high conductivity reduces zone broadening by electromigration dispersion and thus allows a higher sample throughput [226]. A last aspect to be considered is adsorption to cell walls.

6.2.3 Project

In this project, instrumental development was combined with environmental application. An HPLC-QTOF-MS method was developed for determination of 92 pollutants in water samples after preconcentration by FFE. The developed method was applied to surface water samples. The workflow of this project, combining sampling, fractionation and preconcentration and subsequent analysis by HPLC-MS is shown in Figure 53.

Figure 53: Workflow of waste and surface water sampling, fractionation and preconcentration by different FFE techniques (FFITP and FFIZE-pH) and analysis of fractions and raw water samples by HPLC-MS.

To fractionate and preconcentrate analytes in surface and wastewater samples, two FFE techniques were investigated: FFITP and FFIZE-pH. In the fractionation step a reduction of the sample complexity was expected to allow better quantification possibilities due to fewer coeluting compounds and less quenching effects in the HPLC-MS step. The combination of both techniques leads to the high separation capacity of ionic compounds by electromigrative separation with the high sensitivity and further selectivity of HPLC-MS analysis.

6.3 EXPERIMENTAL

6.3.1 FFE-Experiments

Fractionation experiments were conducted by FFE Services München, Germany in Octopus universal apparatus (Dr. Weber GmbH, Kirchheim, Germany). Raw and fractionated water samples were provided by FFE Services.

FFITP

An Isar surface water sample was fractionated in a separation module of 500 x 100 x 0.4 mm with 96 fractionation outlets. The cell was built with spacers of 0.4 mm. Filter paper of 0.6 mm with a 200 µm membrane in a cell volume of 22.4 mL were used. For anode electrode solution (leader) 150 mM HCl, 300 mM NH_3 and for cathode solution (terminator) 100 mM KOH and 40 mM $Ba(OH)_2$ were used. Fractionation was carried out at 10 °C with Orafol foil. The sample was injected with 168 mL/h medium and 800 µL/h sample at Fraction 58. The pH gradient forming in the ITP zones in the cell was pH 9.4 - 12.9. Separation was carried out in the cell for 7 min at 850 V and 700 V in Experiment 1 and 2. Volumes of 2 mL per fraction were sampled from the continuous free flow system and stored at -20 °C until analysis. Samples were filtered with PTFE syringe filter (0.45 µm, 3 mm supplied by Macherey-Nagel (Düren, Germany)) before analysis by HPLC-MS.

FFIZE-pH

A wastewater sample was fractionated in HAc/NH_3 buffer and a pH gradient of pH 5 - 9. Cell dimensions were 500 x 100 x 0.2 mm and cell temperature was 8 °C. For anode solvent 150 mM HAc, 300 mM NH_3, 9.2/13660 was used and for cathode solvent 150 mM HAc, 300 mM NH_3. Further parameters: LF = 5870 µS membrane PP60, cell-foil-type Ora, spacer 0.2 mm and filter paper 0.3 mm, cell volume 11.2 mL and a 0.44 mm diameter tube was used for sampling and 75 mL/h buffer. The water sample was injected at fraction 71 - 85. Optical density (OD) was measured at 260 nm. Samples were filtered with PTFE syringe filter 0.45 µm, 3 mm supplied by Macherey-Nagel (Düren, Germany) before analysis by HPLC-MS.

6.3.2 Data analysis

For method development a standard mixture containing 270 compounds, provided by Zweckverband Landeswasserversorgung Langenau, Germany, was used. Samples were analyzed by HPLC-QTOF-MS (Agilent Technologies, Waldbronn, Germany) and data analysis performed by MassHunter software. Results were compared to results from Langenau.

For LC-MS/MS analysis, a 1260 Infinity LC system coupled to a 6550 iFunnel QTOF LC/MS system (Agilent Technologies, Waldbronn, Germany) was used. Aliquots of 10 µL sample were injected onto a Zorbax Eclipse Plus C18 column (2.1 x 150 mm, 3.5-Micron, narrow bore, Agilent Technologies, Waldbronn, Germany). A jet stream electrospray ionization (ESI) source was operated with a nebulizer pressure of 35 psig, drying gas temperature of 160 °C, a flow rate of 16 L/min and a fragmentor voltage of 360 V. In the positive ionization mode capillary voltage was set to -4000 V, skimmer voltage to 65 V and a nozzle voltage to -500 V. The mass range was 80 - 1200 m/z with a data acquisition rate of 1 spectrum/s. The sheath gas temperature was set to 325 °C with a flow rate of 11 L/min. For internal calibration purine and HP0921 (Agilent Technologies, Waldbronn, Germany, m/z = 121.0508, 922.0097) were used. A gradient elution at a flow rate of 0.3 mL/min using water and acetonitrile, both eluents containing 0.1 % formic acid, was used. The initial content of 98 % water was decreased after 2 min to 80 % water over 2 min and to 2 % over 14.5 min and after another 11 min at 2 % increased to 98 % water over 0.5 min and 98 % water applied for 10 min.

Data analysis was performed with MassHunter Workstation software Version B.06.00 (Agilent Technologies, Waldbronn, Germany). Retention time window of ± 1 min and m/z extraction window of ± 100 ppm were criteria for identification based on reference compounds in methanol. pI and pKa values were calculated with ChemAxon software (Cambridge, USA). Further physicochemical parameters of analytes were taken from the databases DrugBank (University of Alberta, Canada), PubChem (National Center for Biotechnology Information, USA) and PPDB (University of Hertfordshire, UK).

6.4 RESULTS AND DISCUSSION

6.4.1 Development of target analysis method by HPLC-ESI-QTOF-MS

For separation of a reference material mixture containing 92 wastewater chemicals, a C18 column was used with gradient elution by acetonitrile and water. C18 columns are commonly used in water analysis for separation by liquid chromatography due to their ability to separate pollutants in a broad polarity range from medium polar to nonpolar [194, 216-217]. Detection was carried out with ESI-QTOF-MS in the positive ionization mode. The separation and detection method enables the analysis of 92 pollutants relevant in wastewater analysis in 20 min, the extracted ion chromatograms of all compounds are shown in Figure 54.

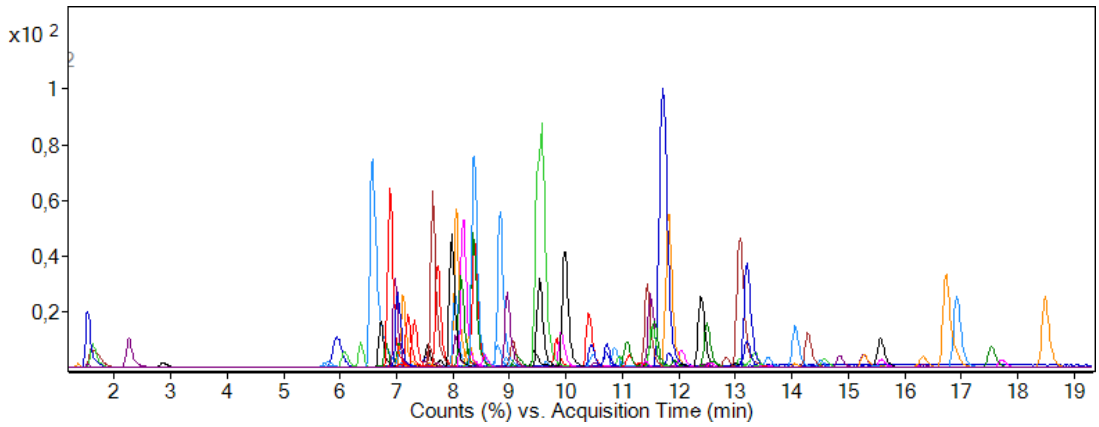


Figure 54: Extracted ion chromatograms of 92 compounds in the reference mixture at a concentration of 5 µg/L analyzed by HPLC-QTOF-MS in the positive ionization mode within 20 min. Further parameters see text.

For quantification of analytes in water samples the reference mixture was analyzed in three concentration steps: 5, 10 and 20 µg/L. Confirmation of compounds was carried out by two identification criteria: retention time and exact mass. The retention time window was set to 1 min and for exact mass a range of 100 ppm was used. The developed method comprises substances of different compound categories, including pharmaceuticals, lifestyle products and pesticides. The developed method was applied to raw water and fractionated wastewater samples.

Table 15: Identification criteria for pollutants in water separated by HPLC and detected with ESI-QTOF-MS.

compound	$[M+H]^+$	t_R [min]	compound	$[M+H]^+$	t_R [min]
1,2,3-Benzotriazole	120.0556	8.1	**Metamitron**	203.0927	8.8
2-Aminobenzothiazole	151.0325	7.2	**Metazachlor**	278.1055	13.2
2-Hydroxy-benzothiazole	152.0165	10.1	**Metformin**	130.1087	2
8-Hydroxychinoline	146.0600	6.7	**Methabenz-thiazuron**	222.0696	11.8
Acebutolol	337.2122	8	**Metolachlor**	284.1412	15.6
Acridin	180.0808	7.811	**Metolacarb**	166.0863	10.9
Amantadine	152.1434	7.6	**Metoprolol**	268.1907	7.6
Amisulpride	370.1795	7.7	**Metoprolol acid**	268.1543	7.2
Atenolol	267.1703	6.8	**Metoxuron**	229.0738	10.5
Atrazine	216.1011	12.3	**Monocrotophos**	224.0682	7.7
Azoxystrobin	404.1241	14.5	**Monuron**	199.0633	10.9
Betaxolol	308.2220	9.5	**N,N-Diethyl-toluamide (DEET)**	192.1383	12.4
Bisoprolol	326.2326	8.9	**N-Acetyl-4-aminoantipyrind**	246.1237	7.6
Bupirimate	317.1642	13.2	**Nadolol**	310.2013	7.3
Caffeine	195.0877	7.6	**Naphazoline**	211.1230	8.1
Candesartan	441.167	11.8	**Nicotine**	163.1230	2.4
Carbamazepine-10,11-epoxid	253.0972	10.5	**N-Methyl-2-pyrrolidone (NMP)**	100.0757	6.3
Carbamazepine	237.1022	11.1	**Norflurazon**	304.0459	12.8
Chloridazon	222.0429	9.14	**N-Oxide-Tramadol**	280.1907	8.4
Chlorotoluron	213.0789	9.9	**Penconazole**	284.0716	15.2
Cyprodinil	226.1339	13.2	**Phenanthridinon**	196.0757	11.4

compound	$[M+H]^+$	t_R [min]	compound	$[M+H]^+$	t_R [min]
Diazinon	305.1083	16.8	**Phenazone**	189.1022	8.4
Difenoconazole	406.0720	16.3	**Phenylalanine**	166.0863	6.2
Dihydrocodeine	302.1751	7.1	**Phosphamidon**	300.0762	10.4
Disulfoton-sulfoxide	291.0307	12.0	**Picoxystrobin**	368.1104	16.7
Diuron	233.0243	12.5	**Piperophos**	354.1321	17.4
Epoxiconazole	330.0804	14.5	**Pirimicarb**	239.1503	8.4
Ethoprophos	243.0637	14.8	**Pirimiphos-ethyl**	334.1349	18.5
Fenamiphos-sulfone	336.1029	11.4	**Pirimiphos-methyl**	306.1036	16.7
Fenpropidin	274.2529	11.3	**Pregabalin**	160.1332	7.0
Fenpropimorph	304.2635	11.4	**Prochloraz**	376.0381	13.5
Fenuron	165.1022	9.0	**Prometon**	226.1662	9.5
Flecainide	415.1451	9.9	**Propyphenazone**	231.1492	11.7
Flurtamone	334.1049	14.0	**Quinoxyfen**	308.0040	17.6
Gabapentine	172.1332	7.1	**Ritalinic acid**	220.1332	7.7
Gabapentine-Lactam	154.1226	9.8	**Sitagliptin**	408.1254	8.6
Imazalil	297.0556	10.3	**Spiroxamine**	298.2741	11.4
Imazapyr	262.1186	8.3	**Sulpiride**	342.1482	6.9
Imazaquin	312.1343	11.0	**Sulpiride N-Oxide**	358.1431	7.1
Iomeprol	777.8614	6.7	**Tebutam**	234.1852	15.5
Lamotrigine	256.0151	7.4	**Telmisartan**	515.2442	10.6
Levocarnitine	162.1125	1.5	**Terbutryn**	242.1434	11.7
Mecarbam	330.0593	13.9	**Tramadol**	264.1958	8.2
Melamin	127.0727	1.7	**Tyrosine**	182.0811	5.0
Mephosfolan	270.0382	10.7	**Valsartan**	436.2343	13.4
Mepronil	270.1489	15.2	**Xanthon**	197.0597	14.2

6.4.2 Application

For identification of pollutants, the target method comprising 92 substances was applied, witg identification criteria listed in Section 6.4.1. In water samples analyzed by HPLC-MS, substances were identified by comparison of retention time and exact masses of compounds in the water samples to the ones of reference material analyzed with the same method. 25 known wastewater pollutants, structures are given in Figure 55, and two metabolites were identified in water samples.

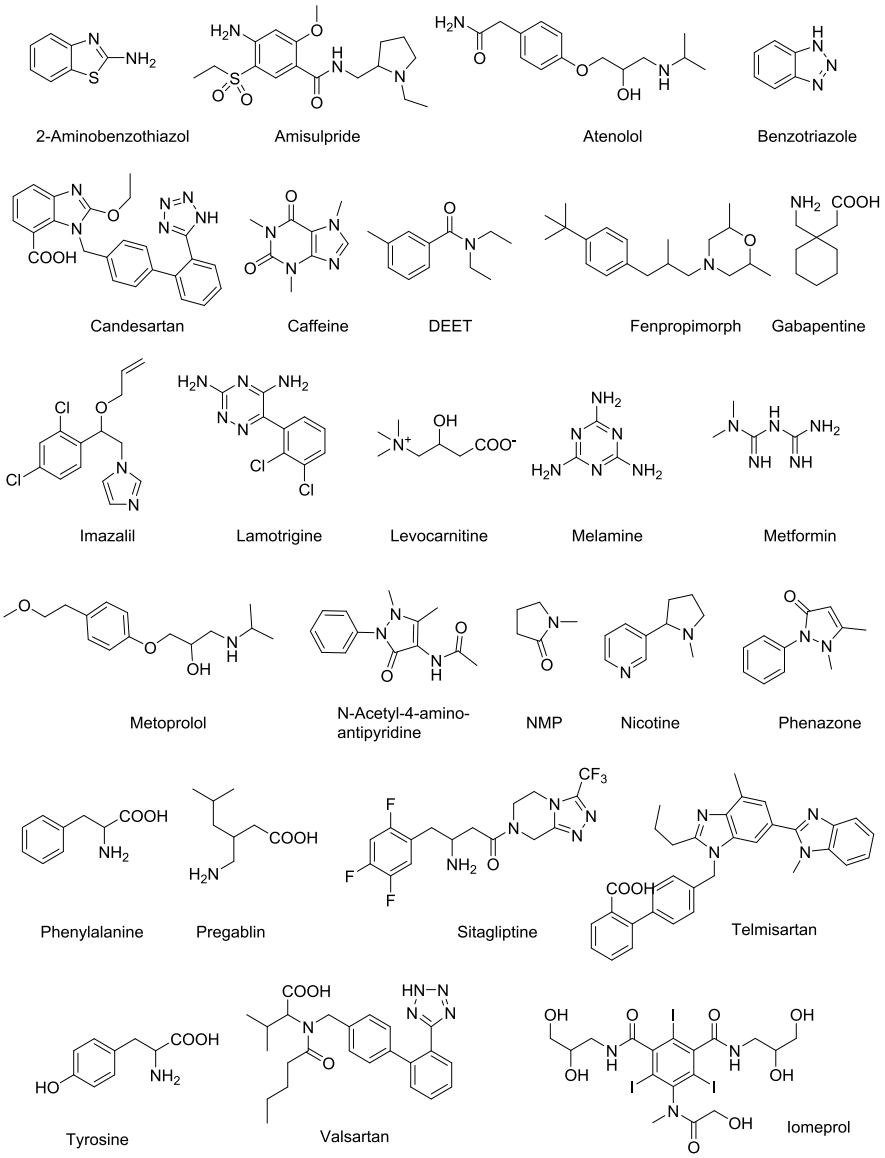


Figure 55: Compounds detected in waste water samples fractionated by different FFE techniques and identified with the developed target method.

Retention time on C18 columns correlates with the polarity of analytes. Therefore, it can be used as an indicator for polarity. Compounds with lower polarity should be more strongly retained by the column material. These compounds eluted later in the chromatogram, at a higher content of organic solvent as eluent, resulting in longer retention times. For confirmation of the retention time as polarity criteria, retention times and octanol-water coefficient (log P_{ow}) values of all detected compounds are plotted in Figure 56.

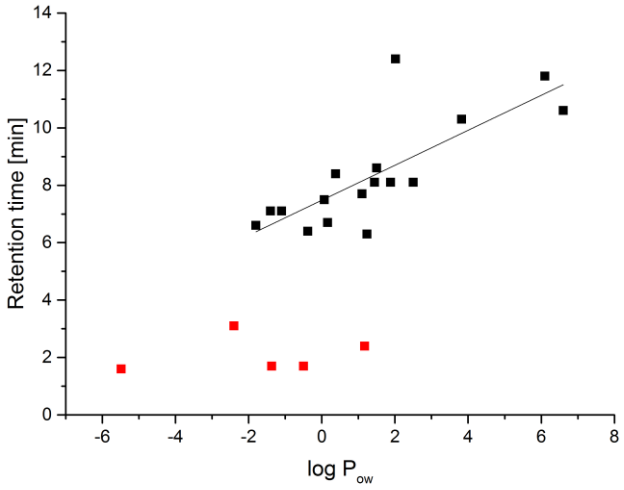


Figure 56: Correlation between retention time and log P_{ow} for all compounds detected in waste water samples R^2 = 0.771 for all compound except the ones with t_R < 3 min.

Three compounds show a different behavior (log P_{ow}, t_R): nicotine (1.17 and 2.4 min), metformin (-0.5 and 1.7 min) and melamine (-1.4 and 1.7 min). All these compounds are detected with a retention time below 2 min in the so called “dead volume” of the chromatographic method. This means these compounds are not retained by the column material. Therefore, retention time is not a criterion correlating with the log P_{ow}. Polar compounds are usually detected in the first minutes of RPLC separation due to low or missing interaction with the column material.

Free flow isotachophoresis (FFITP) I

Non-target analysis

To test the preparative preconcentration capacity of free flow electrophoresis for micropollutants, two Isar surface water samples were fractionated in ITP mode. A selection of fractions to be analyzed later was made based on absorbance at 260 nm. Only fractions with high UV activity, indicating high concentrations of pollutants, were analyzed by HPLC-MS. In this fractionation experiment an anionic ITP system with Cl^- and OH^- at pH 9 - 13 as leader and terminator was used. These conditions result in one of the broadest mobility windows possible to give broadest coverage of micropollutants. In isotachophoresis the analytes arrange themselves according to their order of ionic mobility in steady-state zones between the leader and the terminator.

For an overview of the preconcentration and fractionation process, base peak chromatograms (BPCs) were extracted from the analyzed fractions and the raw water sample analyzed by HPLC-MS in the positive ionization mode: results are shown in Figure 57. For extraction mass range was set from 80 to 1200 ± 0.01 m/z. These chromatograms clearly show changes in the number of signals and their retention times for different fractions, which are not present in the raw water sample.

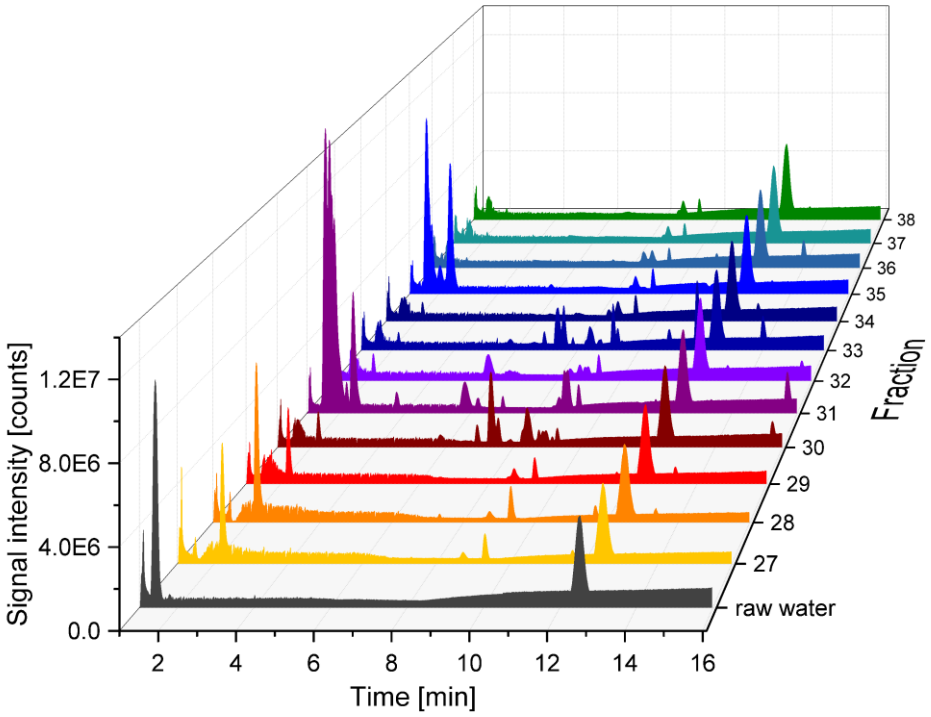


Figure 57: Base peak chromatogram of raw water sample (grey) and fractions (colored), extracted in the mass range of 80 - 1200 ± 0.01 m/z and detected in the positive ionization mode. HPLC-MS conditions as described in Section 6.3.1. The water sample was injected at fraction 58 and an anionic ITP system covering the pH range from pH 9 at fraction 1 to pH 13 at fraction 96 was used.

Exact masses and retention times of selected signals with elevated intensity, in at least one fraction compared to the raw water sample, are listed in Table 16. Chemical formula prediction by MassHunter software with a difference smaller than 5 ppm did not reveal any reasonable compounds. A signal at m/z = 214.919 was only detected in the raw water sample.

Table 16: Mass and retention time of selected compounds apparent in the base peak chromatograms, analyzed in the positive ionization mode with higher signal intensities in fractions' chromatograms compared to the raw water sample. The fraction with the highest signal intensity of an analyte detected in several subsequent fractions are highlighted as max.

fraction	m/z	t_R [min]	fraction with highest signal intensity
raw water	214.919	1.4	
every	250.178	12.5	
27 - 32	102.128	2.2	28
27 - 38	185.115	9.3	
31 - 33	338.217	6.9	
30 + 33	320.207	7.3	
30 + 33	412.209	8.4	
30 - 32	330.265	8.9	31
30 - 32	335.219	15.7	31
33	343.173	9.2	
33	435.176	11.0	
33	325.1620	11.9	
33 + 36	417.165	14.0	

The compound at 250.178 m/z was detected in every sample including the raw water sample with the same intensity, this might indicate a contamination of sampling vials. A compound at

102.128 m/z was detected in fractions 27 – 32 with the highest intensity in fraction 28. Similarly, fractions 31- 33 revealed a signal at 388.217 m/z and fractions 30 - 32 compounds with 330.265 m/z and 335.219 m/z with highest intensities in fraction 31. In fraction 33 four signals were detected, 343.173, 435.176, 325.162 and 417.165 m/z, which were not detected in any other fraction. A signal at m/z 417.165 m/z was detected in fractions 33 and 36. This clearly indicates a fractionation and preconcentration effect. The signal at 185.115 m/z was detected in every fraction except the raw water sample. This might be explained by a process contamination during fractionation or transformation due to electrophoretic conditions. A double signal at 320.207 m/z and a signal at 412.209 m/z were detected in fraction 30 and 33, but not in the fractions in between. Therefore, this signals might stem from two different compounds coeluting in LC but with different electrophoretic mobilities.

The compounds detected in consecutive fractions with the highest concentration in the middle fraction indicate a concentration and fractionation of these substances by FFITP (338.2 m/z in fraction 31 - 33, 102.1 m/z in fraction 27 - 32 and 330.2 m/z and 335.2 m/z in fraction 30 - 32). Further work is necessary to broaden the analysis including compound identification.

Target analysis

Samples were analyzed with the developed HPLC-MS method described above and compounds were quantified by calibration over 3 concentration steps as described in Section 6.3.2, when their signal to noise ratio was five times greater than the background noise (S/N > 5). The limit of detection was set at S/N = 3. Results of the analysis indicate a transient ITP of analytes in the water sample, shown in Figure 58.

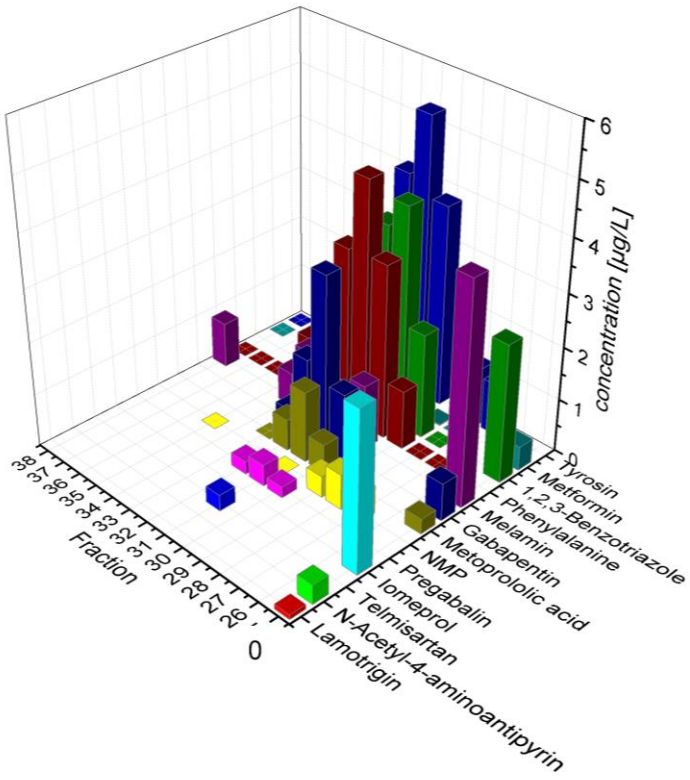


Figure 58: Results of analysis of water fractionation by ITP with the developed target HPLC-MS method. The raw water sample is labeled as 0 and shwon for comparison. Fractions 27 – 40 from FFITP were analyzed by HPLC-MS. Compounds below the limit of quantification but above the limit of detection are shown as squares.

All compounds were detected in the 15 analyzed fractions with the highest UV activity. Some of them were not expected to be anionic at the high pH value chosen. This may indicate diffusion processes, carryover effects or cotransport by anionic matrix components, e.g. humic acids. Iomeprol, N-acetyl-aminoantipyridin and lamotrigine were only detected in raw water sample but not in fractionated samples which is likely, given that these compounds are neutral at the given pH value.

Phenylalanine was detected in all samples but only in samples with high pollutant concentrations 29 - 34 in quantifiable concentrations. The fact that phenylalanine was detected in every sample might be due to contamination during workup or storage or carryover effects. Gabapentin, 1,2,3-benzotriazole, melamine and metoprolol-acid were detected in raw water sample and fractions 29/30 – 33/34. Gabapentine, 1,2,3-benzotriazole and metoprolol acid were detected in higher concentrations in various fractions than in raw water sample. Substances were detected in concentrations up to 470 % higher than in the raw water sample e.g. gabapentin and metoprolol acid in fraction 31 or up to 160 % for benzotriazole in fraction 30. Metoprolol acid was detected in fraction 30 at a concentration 30 % higher than in raw water sample. Melamine and metformin were detected in raw water sample and at least one fraction but at reduced concentrations. These compounds are neutral or cationic at the chosen pH and a cotransport is likely. It is interesting to note that the metabolite of metaprolol, metaprolol-acid, was detected but not the parent compound. Pregablin, NMP, telmisartan and tyrosine were detected in 1 - 7 fractions with highest concentrations in fraction 30 - 32 in case of pregablin, telmisartan and tyrosine and in case of NMP in fractions 27 - 29. These four compounds were not detected in raw water sample. This might indicate a preconcentration.

The pK_a and log P_{ow} values of compounds detected in wastewater samples and fractions are listed in Table 17. In case of metoprolol acid and N-acetyl-4-aminoantipyridine, both metabolites, chemical properties were not available. Fractions of occurrence did not correlate to the listed physicochemical parameters of the analytes.

Table 17: Compounds identified in raw waste water sample and samples fractionated by FFITP, application and physicochemical parameters.

compound	pollutant family	pKa^1	pKa^2	log P_{ow}	t_R [min]	fraction
1,2,3-Benzotriazol	chelating agent	8.37		1.44	8.1	0, 28 - 34
Gabapentin	pharmaceutical	3.68	10.7	-1.1	7.1	0, 30 - 33
Iomeprol	pharmaceutical	2.53	5.65	-1.8	6.6	0
Lamotrigin	pharmaceutical	8.53	9.21	2.5	8.1	0
Melamin	monomer			-1.37	1.7	0, 30 - 34, 38
Metformin	pharmaceutical	12.4		-0.5	1.7	0, 29
NMP	bulk chemical			-0.38	6.4	27 - 29
Telmisartan	pharmaceutical	3.65	6.13	6.6	10.6	31
Pregabalin	pharmaceutical	4.2	10.6	-1.4	7.1	30 - 32
Phenylalanine	amino acid	2.47	9.45	1.24	6.3	29 - 34
Tyrosine	amino acid	2	9.21	-2.4	3.1	27 - 33

In this fractionation experiment the terminator was not detected in any fraction, indicating that the ITP stacking process was not completed by the time of entering the fractionation vials. The flow time was only 7 min, which was too short. Further optimization is necessary. However, already the preliminary results described here, indicate the potential of the methodology for preconcentration of ionic analytes.

FF-ITP II

A second surface water sample was fractionated by anionic FFITP in a similar fractionation system. In this experiment, higher voltage was applied to optimize the separation efficiency. The base peak chromatograms are shown in Figure 59. They clearly show higher abundances of several signals comparing raw water and different fractions as also observed in the previous experiment.

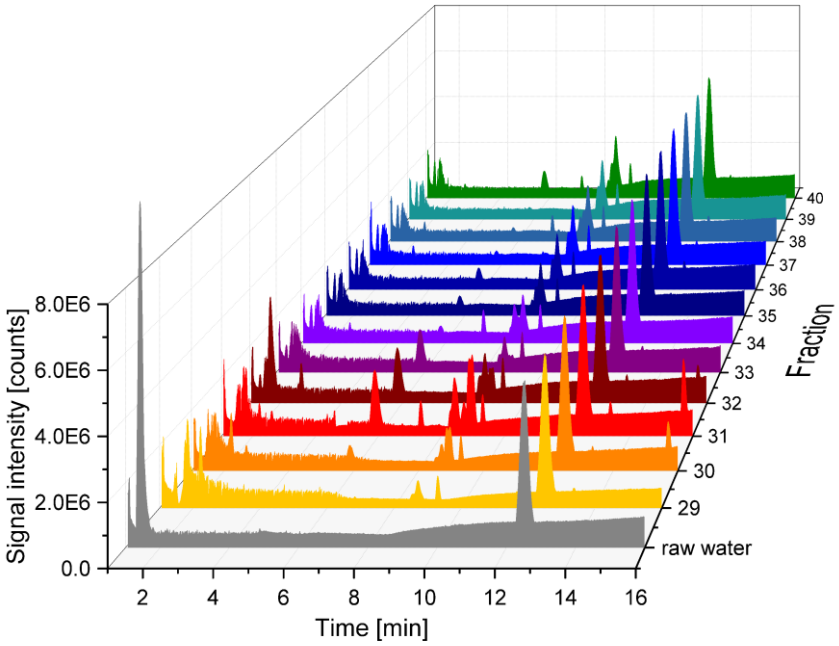


Figure 59: Base peak chromatograms in the range of 80 - 1200 m/z detected in the positive ioniziaton mode, raw water sample and fractions. Further conditions, see Section 6.3.2.

Compared to the previous experiment fewer signals were detected in baseline peak chromatogram analysis in this experiment. Retention time, exact mass and fraction of occurrence of selected masses are listed in Table 15. The raw water BPC showed similar signals at the same retention times, as in the previous experiment (Table 16).

Table 18: Exact mass and retention time of selected compounds detected in base peak chromatograms of a water sample fractionated by FFITP.

fraction	m/z	t_R [min]
raw water	214.919	1.4
raw water	250.178	12.5
28 - 40	185.115	9.3
31 + 38	320.207	7.3
31	412.209	8.4
30 - 31	330.265	8.9
30 - 31	335.219	15.7
31	334.212	5.9

The compound with 185.115 m/z was detected in every fraction except in the raw water sample as in the previous experiment. The signal at 320.207 m/z was detected in fractions 31 and 38. The compounds 330.265 m/z and 335.219 m/z were detected in fractions 30 – 31 with the highest concentration in fraction 31. Two compounds 412.209 m/z and 334.212 m/z were only detected in fraction 31. Chemical formula calculation by MassHunter software with

a difference smaller than 5 ppm did not reveal any reasonable compounds and signal properties could not be matched to target analytes.

Target analysis by HPLC-MS

Fractions with high UV activity were analyzed with the developed target method of 92 wastewater chemicals by HPLC-MS in the positive ionization mode. Six compounds were detected, three of them in raw water sample and fractions. Tyrosine and phenylalanine were detected in every sample analyzed and may be an impurity from previous experiments. Chemical structures of the four compounds fenpropimorph, benzotriazole, melamine and metformin detected in fractions are given Figure 60.

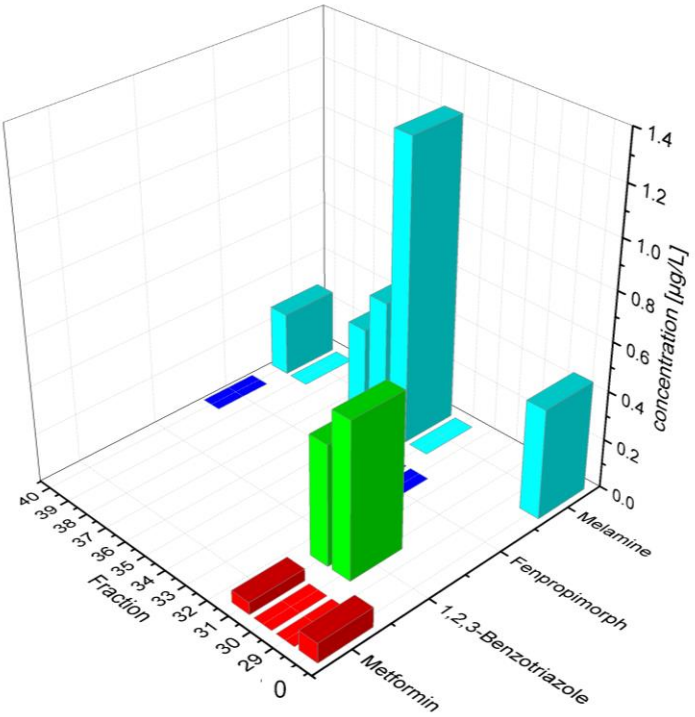


Figure 60: Analytes detected and quantified in a water sample fractionated by FFITP. The raw water sample is labeled as 0. Further parameters are given in Section 6.3.1. Tyrosine and phenylalanine were detected in every sample in 10 to 100-fold higher concentrations than the other analytes and are not shown in the diagram.

Metformin was detected in the raw water sample and in reduced concentrations in three subsequent fractions 29 - 31. Melamine was detected in the raw water and in fractions 33 - 36 and fractions 39 - 40. Most concentrations were at the LOD and thus melamine might be present in all samples in very low concentrations, except fraction 34 with a 270 % higher concentration than in the raw water sample. Fenpropimorph was detected in three fractions below LOQ and benzotriazole was detected in two subsequent samples but not in the raw water sample.

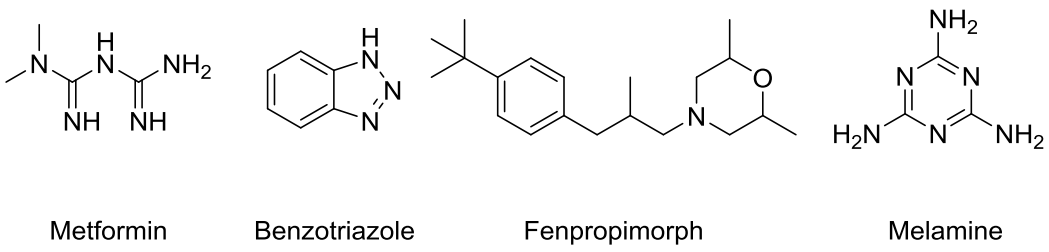


Figure 61: Chemical structures of compounds detected in fractions in FFITP experiment.

The expected enhancement of fractionation efficiency via a higher field strength with sharper fraction boundaries was not achieved. Even with the longest possible separation time the ITP stacking was not complete as described above. The separation is limited in ITP systems to few fractions according to the applied leader and terminator in the range of potential 5 - 10 fractions. However, with the large number of compounds in the sample, more time for an efficient stacking and formation of ITP zones would be required, which is not possible with the available instrumentation.

Free flow interval zone electrophoresis with pH steps (FFIZE-pH)

To investigate the concentration ability of FFIZE to focus and preconcentrate micropollutants, a wastewater sample was fractionated by intervall zone electrophoresis into 96 fractions. In this experiment, a pH gradient of pH 5 - 9 was applied in five steps (pH 5, 6, 7, 8 and 9) each step covering 10 - 15 fractions. The pH profile is given in Figure 62. Analytes were expected to migrate along the pH gradient until they reach a pH step, where they have no effective electrophoretic mobility due to being neutral or reaching their pI. Several analytes can then be expected in each well defined fraction. The advantage compared to ITP experiments lies in the lower time limitation as the migration of analytes does not depend on the number of charged components in the sample.

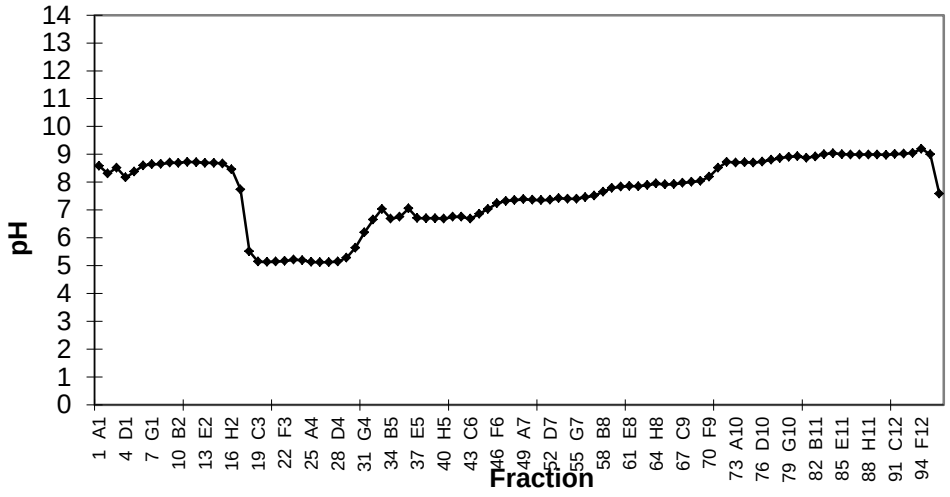


Figure 62: pH profile along the 96 fractions of F-IZE-pH experiment with a waste water sample. Figure provided by FFE Service GmbH.

HPLC-MS analysis was performed only for fractions showing the highest UV absorbance at 260 nm including fractions 11, 12, 24 - 26, 31, 41, 42, 51 - 55, 57, 60, 69 and 75. Therefore, the pH range from 5.0 to 8.9 was covered and detected compounds had pK_a values in the range of 1 to 14.

The base peak chromatograms of the raw water sample and analyzed fractions are shown in Figure 63. Comparison of BPCs revealed increased signal intensities of various unknown compounds in different fractions. Exact masses and retention times of selected compounds are given in Table 19. Detected features did not correspond to any of the 92 compounds in the standard mixture used for method development. Thus, the target method has to be extended, possibly after identifying the signals summarized in Table 19 from nontarget screening, for further investigations.

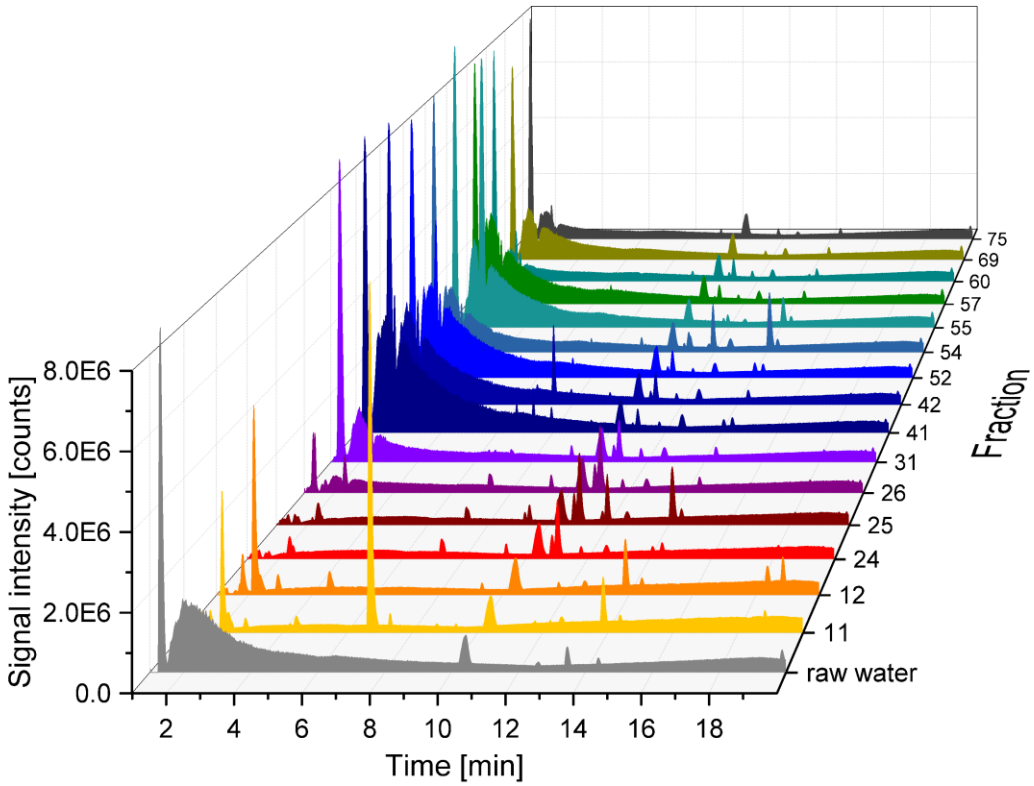


Figure 63: Base peak chromatogram of FFIZE separated samples, BPCs in the range of 80 - 1200 ± 0.01 m/z analyzed by HPLC-QTOF-MS in the positive ionization mode. A pH gradient of pH 5 at fraction 19 - 30 to pH 9 at fraction 83 - 94 was applied and the sample injected at fraction 71 - 85. Further parameters, see Section 6.3.1.

The signal at 102.130 m/z (Figure 63) was detected with increasing signal intensity from fractions 11 - 12 but not in the raw water sample, presumably due to preconcentration. The compound with 217.105 m/z was the most abundant signal among all analyzed fractions and was only detected in fraction 11 and not detected in raw water sample. This indicates a strong concentration of the compound in this fraction with a stacking at a pH step boundary. With an intermediate retention time of 6.7 min in HPLC, the compound can be assumed to become protonated at pH 5 in FFIZE but is uncharged in the HPLC eluent. Its molar mass points to a small organic molecule. The signal at 245.078 m/z was detected in fractions 11 and 12 with equal intensity. In fraction 12, two more compounds were detected at high HPLC retention times of 18.4 and 18.8 min with 345.237 and 321.235 m/z. These were the highest retention times detected for compounds in base peak chromatograms. The high retention times and occurrence in a high pH fraction of FFIZE corresponding to a basic pH value of 9 might be explained by molecules which are uncharged under the acidic conditions of HPLC separation including 0.01 % formic acid. In fractions 24 – 69 a double peak was detected at 357.114 m/z with an intensity maximum in fraction 26. In fraction 25 two signals were detected, which were not present in fractions 24 and 26, at 325.162 and 417.164 m/z. This indicates a sharp focusing of these compounds, possibly at a pH step boundary. In fractions 41 and 42 a new signal at 114.092 m/z was detected with increasing intensity. At HPLC retention times of 1.5 – 4 min, in the base peak chromatograms a higher noise was detected compared to the base peak chromatograms of previous fractions. The reason for this is remains unclear. In fractions 52 – 57, two signals with the highest intensity at fraction 54 were detected at 417.164 and 325.161 m/z.

Table 19: Mass and retention time of selected compounds with higher concentrations in fractions compared to raw water sample. Signals from Figure 63.

fraction	m/z	t_R [min]	max
11 - 12	102.130	2.1	12
11	217.105	6.7	
11 - 12	245.078	13.8	
12	345.237	18.4	
12	321.235	18.8	
24 - 69	357.114	10.9	26
25	325.162	12.0	
25	417.164	14.2	
41 - 42	114.092	7.3	42
52 - 57	417.164	14.1	54
52 - 57	325.161	11.9	54

Comparison of the base peak chromatograms reveals new signals and signals with higher intensities than present in the raw water sample. Identification by any of the previously mentioned criteria was impossible, see Section 6.4.1.

Target screening

The same procedure used to analyze FFITP fractions was applied to FFIZE-pH fractions. Quantification was based on external calibration with reference material in methanol. Results of the analysis are shown in Figure 64. 24 compounds were detected in the samples.

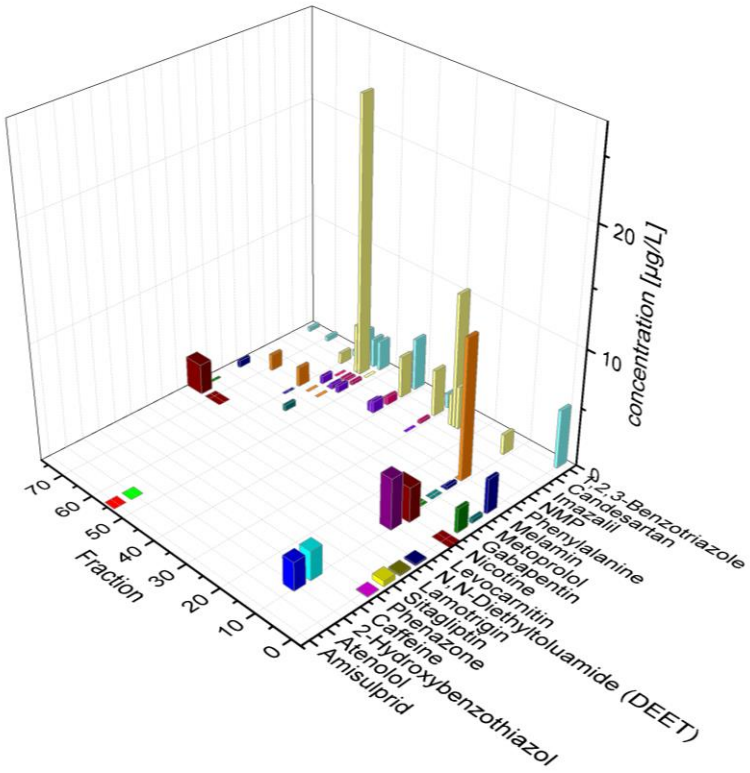


Figure 64: Concentration of compounds detected in raw water and fractions separated by FFIZE-pH. Analytes below the LOQ but above the LOD are indicated by squares.

Seven compounds were detected in the raw water sample and 13 compounds in the analyzed fractions. The detected compounds are listed accompanied by their retention time, fraction of occurrence and classification according to use in Table 20. 50 % of the compounds (9 out of 18) were only detected in one fraction. Compounds detected in the raw water sample were elevated in fractions. 4 out of 8 compounds were only detected in the raw water sample. Gabapentin and DEET are neutral at the chosen pH range and are thus not expected to be affected by electrophoresis. On the other hand, 10 out of 18 compounds were detected in fractions but not in the raw water sample. This could be due to efficient migration and concentration processes. Three compounds imazalil, NMP and phenylalanine were detected in 6 respectively 4 successive fractions. Melamine and nicotine were detected in fraction 11 and 75, both fractions with pH 8.9. Some of these compounds, if identified correctly, need a cotransport phenomenon to be transported in the electric field.

Table 20: Fraction of occurrence, application and retention time of compounds detected in the water sample fractionated by IZE-pH and analyzed by the developed HPLC-MS method.

compound	pollutant class	t_R [min]	fraction
1,2,3-Benzotriazol	chelating agent	8.1	0, 31 - 75
2-Hydroxy-benzotriazol	metabolite	10.1	11
Amisulpride	pharmaceutical	7.7	55
Atenolol	pharmaceutical	6.7	55
Candesartan	pharmaceutical	11.8	11 - 60
Caffeine	waste water	7.5	11
DEET	insecticide	12.4	0
Gabapentin	pharmaceutical	7.1	0
Imazalil	fungicide	10.3	31 - 57
Lamotrigine	pharmaceutical	8.1	0
Levocarnitine	metabolite	1.6	11
Melamine	monomer	1.7	11, 75, 0
Metoprolol	pharmaceutical	8.1	0, 55
Nicotine	waste water	2.4	11, 75
NMP	bulk chemical	6.4	31 - 57
Phenylalanine	amino acid	6.3	11, 12, 57 - 69
Sitagliptine	pharmaceutical	8.6	0
Phenazone	pharmaceutical	8.4	0

Overall, no clear pattern for a fractionation process was observed. Physicochemical properties of detected compounds are listed in Table 21. Among these compounds, 7 substances contain an acidic functional group, which are candesartan, the amino acid phenylalanine, gabapentin, pregabalin, telmisartan, atenolol and levocarnitine. Benzotriazole was detected in fractions 31 - 75 and in the raw water sample like in the previous two experiments. As benzotriazole is uncharged in the applied pH range no concentration at a certain pH was expected. Its transport may be mediated by humic acids. Phenylalanine was also detected in every FFE separated fraction. Therefore, these compounds are expected to represent impurities. DEET, gabapentin, sitagliptine, lamotrigine and phenazone are neutral or cationic at the chosen pH and are hence detected only in the raw water sample.

The detection of neutral or positively charged compounds in several fractions indicates transport phenomena by matrix components. To investigate these phenomena, in further experiments a closer analysis of the raw water sample is required. Candesartan was detected in fraction 11 - 60 and it is a negatively charged compound in the applied pH range.

Table 21: Physicochemical properties of compounds detected in water samples. pKa^1 strongest acidic pK_a value and pKa^2 strongest basic pKa value.

Compound	pKa^1	pKa^2	pI	log P_{ow}
1,2,3-Benzotriazole	8.37	0.6	4.60	1.44
2-Hydroxy-benzotriazole	5.38	-1.73	-	
Amisulpride	14.03	7.05	4.31	1.1
Atenolol	14.08	9.67	11.87	0.16
Candesartan	3.51	1.5	2.5	6.1
Caffeine		-1.16	-	0.07
DEET		-0.95	-	2.02
Gabapentin	4.63	9.91	7.42	-1.1
Imazalil	6.53		-	3.82
Lamotrigine	14.98	5.89	10.93	2.5
Levocarnitine	4.2		-	-5.48
Melamine		9.56	12.62	-1.37
Metoprolol	9.67	14.09	11.88	1.76
Nicotine		8.58	-	1.17
NMP		-1.7	-	-0.38
Phenylalanine	2.47	9.45	5.96	1.24
Sitagliptine		8.78	-	1.5
Phenazone	1.4		-	1.22

The correlation of retention time as an indicator of polarity to the fraction number is presented in Figure 65. A correlation of retention time and fractions was clearly not given. This demonstrates the orthogonality of the two separation mechanism, which may advantageous to enhance selectivity in the offline 2D configuration.

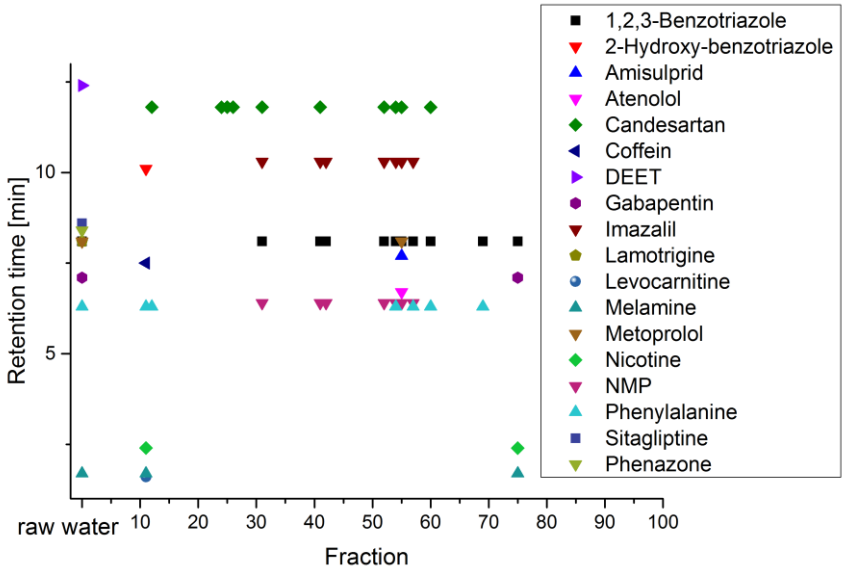


Figure 65: Correlation between retention time and fraction of compounds detected in raw water and FFIZE-pH fractions.

In FFIZE-pH, sample components are separated according to their electrophoretic mobility and their point of no charge, marked by the pI. Amphoteric compounds migrate until they reach a pH regime equaling their pI. Only two detected compounds are amphoteric with a pI in the analyzed range and these are gabapentin, which was only detected in the raw water sample, and phenylalanine, which was a contaminant in these measurements. Therefore,

further experiments conducted in the pH range suitable for the compounds of interest are required to answer the question of the ability of FFIZE-pH for preparative separation of wastewater contaminants. Many open questions can be addressed using standards with broad distribution of pK_a values and electrophoretic mobilities in further experiments.

6.5 CONCLUSION AND OUTLOOK

An HPLC-MS multi-component method was developed for identification of wastewater pollutants by retention time and exact mass comprising 92 substances in the low µg/L range. This method was applied to surface water samples to assess the ability of free flow electromigrative techniques to fractionate and concentrate small molecules. The detection of some compounds like pregabalin in some consecutive fractions indicates the ability of micropollutant fractionation. The detection of pregabalin in fractions but not in the raw water sample points to the concentration ability of the fractionation technique. Both hypotheses should be further investigated in a simpler model experiment containing a small number of compounds with known physicochemical properties and defined concentrations. The preliminary results shown in this study indicate the applicability of FFIZE-pH and FFITP to fractionate smaller molecules similar to bio-macromolecules usually fractionated by these techniques. Results indicate FFE to be a promising strategy for fractionation and preconcentration for ionic compounds. Different dwell times and buffer conditions, as well as electric field strength, are parameters to optimize the method. Another opportunity to increase the separation efficiency could be to combine different electrophoretic separation techniques and thus pre-separate water samples in a first step and fractionate analytes covering a smaller pK_a range by ITP. This project combines the advantages of ion separation by electrophoresis with classical and orthogonal chromatographic separation to give a higher coverage of pollutants and their transformation products.

7 INVESTIGATION OF THE INFLUENCE OF ELECTRODE GEOMETRY, TEMPERATURE AND CONVECTION ON THE TRANSFER OF IONIC ANALYTES BY ELECTROMIGRATIVE ENRICHMENT PROCESSES

7.1 ABSTRACT

Analysis and especially enrichment of ionic compounds in wastewater analysis is challenging. In this study, enrichment experiments were performed aiming at preconcentrating ionic and ionizable analytes from water samples into small volumes with subsequent analysis by HPLC-MS. To achieve this an experimental setup was optimized to transfer charged analytes from a starting volume of 30 mL to a sampling volume of 300 µL via electromigration by application of voltage. Transferring solely charged compounds eliminates neutral (including sample solvent) and oppositely charged matrix components, which remain in the starting vessel during the enrichment procedure. This method only requires simple electrolytes as buffers which makes it environmentally friendly. First proof of principle experiments were performed to evaluate the applicability of electromigration through capillaries for large volume preconcentration. According to the pK_a regime of the analytes of interest an appropriate pH value was adjusted by the buffer and the influence of electrode configuration, temperature, convection and capillary surface on analyte migration behavior was investigated. Four compounds (metformin, terbutryn, clarithromycin and naphazoline) being cationic at pH 2 were proven to be enriched in the electric field at pH 2 within 2 hours. The optimized conditions were elevated temperature of 50 °C, a straight electrode and no convection in the starting vessel. Enrichment factors of 1.3 in case of terbutryn, 1.6 for naphazoline and 3.6 in case of metformin were achieved. However, after 22 hours of voltage applications, in total less than 63% of the analytes were recovered. To assess if electrochemical degradation was the reason for the reduced recoveries of analytes, electrochemical transformation experiments were conducted in an electrochemical cell.

7.2 INTRODUCTION

7.2.1 Preconcentration

Trace pollutant concentrations in environmental samples make preconcentration of analytes mandatory. Common concentration techniques like liquid-liquid extractions are suitable for nonpolar compounds [235], but the consumption of organic solvent can be high. Solid phase extraction requires less organic solvents to elute the analytes of interest, but they still require organic solvents. In contrast, the method for ionic compounds tested here, would be environmentally friendly as only aqueous buffers are required as solvent. Therefore, herein the first attempts are reported to use electromigrative techniques for preparative preconcentration of ionizable compounds in water samples. Related strategies were reported in literature: electromembrane extraction is a preconcentration technique used for ionic analytes, which was applied to concentrate fungicides from water samples [221]. This technique is based on migration of analytes from an aqueous donor to an acceptor solution accelerated by an electrical potential difference. The solutions are separated by an immiscible artificial liquid membrane [236]. Different analytical preconcentration techniques in electrophoresis, among them field-amplified sample stacking, transient ITP or membrane

filtration were reviewed by Breadmore [237]. Electrokinetic injection followed by isotachophoresis is used for inline preconcentration in capillary electrophoresis. This strategy has successfully been applied to concentrate wastewater chemicals in water samples in capillaries [238]. However, these attempts are limited by band broadening at too high injection volumes.

In classical electrokinetic sample injection, analyte molecules migrate into the capillary by the action of the effective electric field applied in the sample vial. For short injections, this process is limited by the effective electric field rather than diffusion. Simulations showed, that diffusion plays a minor role compared to electrode geometry and thus the effective electric field in the vial [26]. The distance between electrode and capillary inlet determines the volume covered by the effective electric field. The greater the distance, the larger the volume sampled by the effective electric field, and thus the amount of analyte injected by electrophoretic migration [26]. These effects were proven for metal ions [239]. Breadmore reported an unlimited-volume stacking by application of a stationary isotachophoresis for 60 min at -20 kV [240]. In this process, analytes are continuously injected at one end and accumulate in the capillary volume between the ITP boundaries allowing a potentially unlimited injection.

In this study electrokinetic sample injection was used to transfer ionic analytes and simultaneously concentrate them by volume differences between the starting and the sampling vessel. To investigate the impact of electrode geometry on preparative preconcentration via permanent electrokinetic injection, two electrode configurations were used in this study. In addition, two other important conditions playing a role for the efficiency of electrokinetic injection were investigated: temperature and convection. Heating was shown to enhance the amount of analyte introduced into the capillary whereas stirring reduced it [26]. Replacing the sampling vial by one with a larger volume was reported to improve the amount of analyte loading by covering a larger volume by the effective electric field for electrokinetic injection [239].

7.2.2 Experimental setup

In the experimental setup a volume of 30 mL filled with starting solution was used and a sampling vessel with only 300 µL buffer. In theory, a complete electromigrative analyte transfer would lead to an enrichment by a factor of 100. Electrolytes were chosen in a way that the buffering coion (coion to analytes) was OH^- or H^+ for the enrichment of anions or cations. Both solutions were adjusted to the appropriate pH value and connected via a 50 - 65 cm capillary filled with buffer. In the sampling vessel a straight electrode was installed whereas in the starting vessel different electrode configurations were applied to investigate the influence of electrode geometry on the transfer process. The starting vessel was equipped with a magnetic-heating-stirrer enabling the investigation of the contribution of convection and temperature to the enrichment process. Schematically the instrumental setup is presented Figure 66.

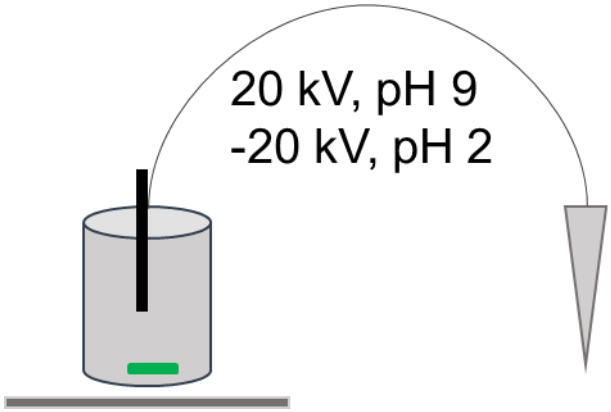


Figure 66: Instrumental setup for the enrichment experiment with starting vessel containing 30 mL with changeable electrode and a sampling vessel of 300 µL both connected via a capillary. For the enrichment of cationic or anionic analytes 20 or -20 kV were applied at pH 2 or pH 9, respectively. The starting vessel was equipped with a magnetic-heating-stirrer to assess the influence of temperature and convection on the injection process.

The influence of electrode configuration was investigated by using different geometries of the Pt wire electrodes in the starting vessel. A straight and a twisted electrode were used, both configurations are shown in Figure 67. A parallel configuration of capillary and electrode was used in case of the straight electrode, and in case of the twisted configuration the capillary was surrounded by the electrode. As reported in literature only analytes present in the effective electric field may be introduced into the capillary [26]. In both modes the distance between capillary ending and electrode ending was approximately 0.5 cm, with the capillary being slightly longer than the electrode. Experiments were conducted with a special focus on influence of electrode configuration, temperature and convection on the effective electric field.

Figure 67: Straight and twisted electrode geometries to broaden the effective electric field in the sampling vessel. The capillary was in parallel to the straight Pt-electrode or in the center of the twisted Pt-electrode in the 30 mL starting vessel.

7.2.3 Experimental design

19 compounds, used in wastewater monitoring as lead substances to evaluate wastewater treatment efficiency [241] were chosen to characterize a preconcentration method based on the electrophoretic mobility of ionic/ionizable compounds. The mixture applied in preconcentration experiments contained anionic, cationic and neutral substances at the pH chosen for buffering. The experiments were conducted at pH 2, with metformin, terbutryn,

naphazoline and clarithromycin being cationic, and at pH 9 with diclofenac, ibuprofen and 2-methyl-4-chlorophenoxyacetic acid (MCPA) being negatively charged. The neutral compounds being uncharged at pH 2 were carbamazepine, irbesartan, benzotriazole, sulfamethoxazole, chloridazon, 2,4-dichlorophenoxyacetic acid (2,4-D), iohexol, epoxiconazole, thiacloprid, propiconazole, imidacloprid and clothianidin. The chemical structures of all analyzed compounds are given in Figure 68.

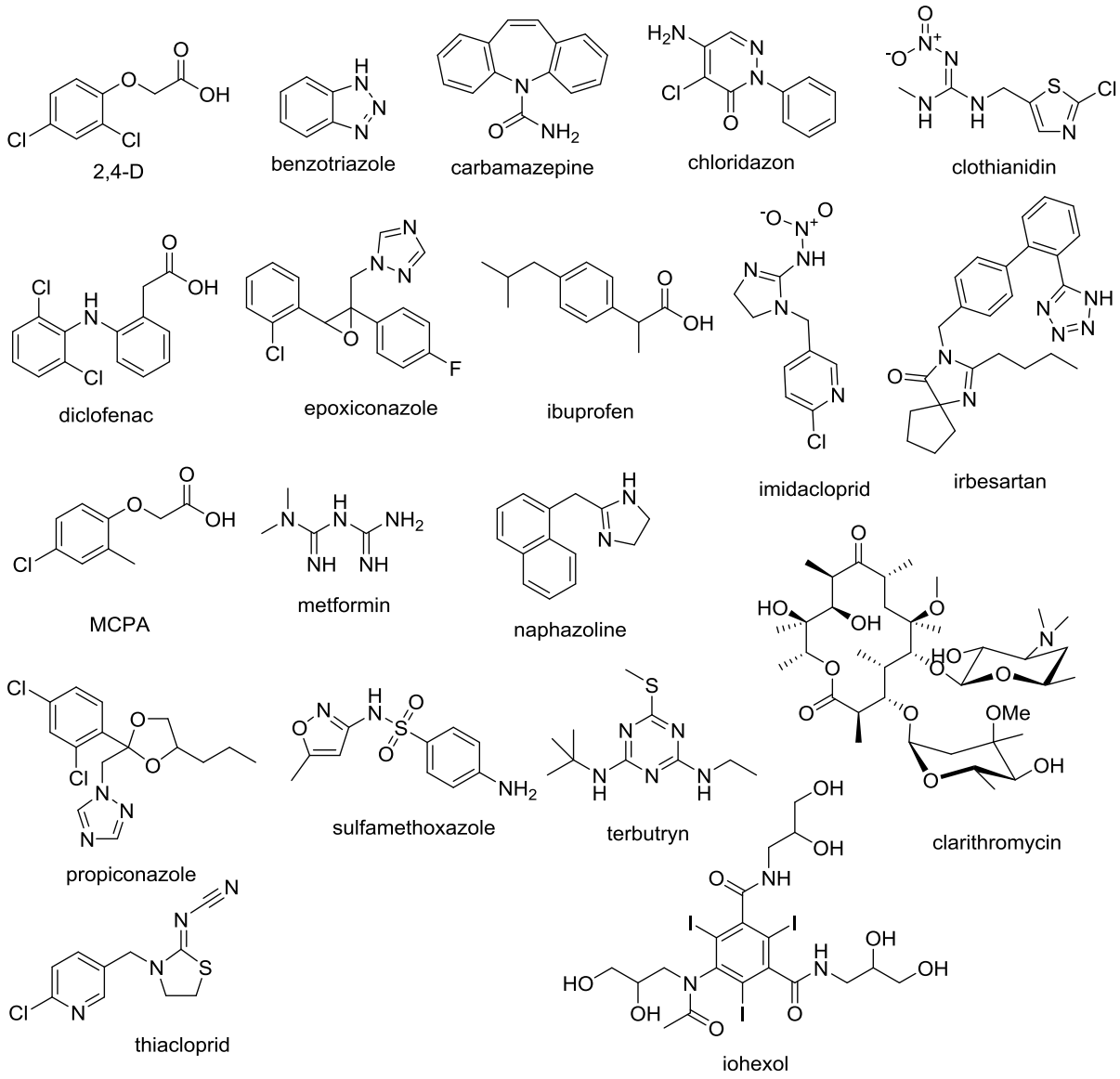


Figure 68: Molecular structures of compounds used in enrichment and electrochemical transformation experiments.

At pH 2 (no electroosmotic flow (EOF) is present in bare fused capillaries) the four cationic compounds and at pH 9 the five anionic compounds were expected to migrate to the sampling vessel, whereas the neutral and oppositely charged compounds were supposed to remain in the starting vessel. At pH 9, the EOF had to be eliminated (see below). The concentrating effect was aimed to be reached by using a larger starting volume and a smaller sampling volume (300 µL vs. 3 mL, thus an ideally 100-fold enrichment). For a quantitative transfer of ionic analytes a voltage of + or -20 kV was applied for 2 - 22 h. Analyte concentrations were monitored in the starting solution, both vessels and the capillary

at different times. The inner volume of the capillary was sampled by rinsing with buffer into an empty sampling vial until 300 µL were collected. For EOF elimination the anionic concentration experiments at pH 9 were conducted with an AAEE coated capillary, developed in our working group [242]. Analysis was conducted by HPLC-MS (see Section 7.3.4). To my knowledge, this experimental strategy is used for the first time for preconcentration of ionizable analytes. The pharmaceuticals clarithromycin, metformin and naphazoline were successfully separated by CE [224, 243-244]. But, to my knowledge, a CE method for the pesticide terbutryn, which is treated as priority substance in the European Union [245], has not yet been reported.

7.2.4 Electrochemical cell

To consider electrochemical degradation or hydrolysis, processes likely to occur in these experiments, electrochemical transformation studies were conducted with an electrochemical cell (EC). Electrochemical cells allow the analytical or preparative synthesis of electrochemical transformation products, which might be generated in wastewater treatment by different processes in oxidative or reductive mode. EC is used as screening method for identification of possible transformation products [246]. For example, metabolites of metoprolol and diclofenac prepared in electrochemical cells, could be detected in treated wastewater samples [247]. An advantage of electrochemical transformation studies is the possibility to directly couple EC with HRMS, and thus analyze transformation products without the need for sample workup to remove interfering matrix components. In contrast, in cell culture experiments traditionally used to simulate Phase I metabolism, removal of matrix is often challenging [248]. The knowledge about possible transformation products can be used analytically for the identification of metabolites in drug development but also in a preparative manner to isolate transformation products which might be difficult to extract from matrix or whose lab synthesis is too complicated [249]. Knowledge of transformation products formed in living organisms is crucial for a thorough understanding of biodegradation processes and a better assessment of contaminants fate in the environment and in biota [250]. Although EC is only capable of simulating a limited number of processes compared to the ones occurring in organisms, it is still a useful tool to address metabolic routes. In Phase I metabolism, enzymatic processes might form different transformation products than those formed in EC experiments. The metabolites are to some extent further processed in Phase II metabolism by conjugation of the functionalized compounds to sugars or proteins for excretion [248, 250-251]. In the following paragraphs, the transformation routes by cytochrome P450 (CYP450) and electrochemistry are compared.

The main path of Phase I metabolism is functionalization by the enzyme CYP450. The underlying reactions include hydroxylation, alkene-epoxidation, dehydrogenation, aromatic hydroxylation, dehydration, N-, S- and O-desalkylation, N-hydroxylation, N- and S-oxidation and oxidative des-amination [1]. These reactions can partially be induced in electrochemical cells to simulate CYP450 oxidation processes [248]. The oxidation takes place at the iron in the porphyrin ring of CYP450 enzyme [252]. Cytochrome P enzymes are ubiquitous in nature, they are found in mammals, plants, bacteria, yeast and insects. These membrane-bound enzymes transform a large variety of xenobiotics. The transformation often leads to more polar compounds, which can be excreted more easily as they are more water soluble [1]. In some cases, however, inactive compounds become activated instead of detoxificated. This is sometimes used in drug development (where a pre-form may be more stable or easier to administer), but might sometimes also lead to carcinogenic activity of the transformation products [1]. Hydroxylation and oxidation processes are the main chemical transformation processes in the liver and are also often formed in electrochemical experiments [252].

Other possibilities to simulate CYP450 processes are Fenton chemistry and the use of synthetic metal-porphyrins [246, 249]. Reactions observed in electrochemical cells are limited to oxidation and reduction processes induced by single electron transfers [253]. Benzylic hydroxylation, hydroxylation at aromatic rings and electron donating groups, N-dealkylation, S-oxidation, dehydrogenation and in some cases N-oxidation and O-dealkylation are reactions often observed in oxidative EC experiments [249]. Products requiring a prior proton abstraction like aliphatic hydroxylation, N-oxidation, O-dealkylation or hydroxylation at unsubstituted aromatic compounds are not formed [251]. Continuous flow cells, used in medical drug development, are capable of transformations in the 10 – 100 mg scale [252]. Some pharmaceuticals are widely examined for electrochemical transformation products, for example carbamazepine was examined by reductive [254] and oxidative treatment [250].

The volume to surface ratio is important in electrochemical cells as the reaction takes place at the electrode surfaces [252]. Different electrode materials are used in oxidation and reduction processes. A common electrode material is the boron-doped diamond electrode (BDDE) used as working electrode. BDD electrodes generate hydroxyl radicals very effectively by water oxidation. The hydroxyl radicals interact (oxidatively) with the analytes. Advantages of BDD electrodes are the broad working range and minimal side-reactions compared to other electrodes [255]. BDDE electrodes allow application of higher potentials compared to glassy carbon electrodes [251]. An advantage of BDDE electrodes is the reduced adsorption of analytes on the electrode surface, especially in case of aromatic and hydrophobic compounds, compared to glassy carbon electrodes [255]. BDDE electrodes are so-called non-active electrodes and thus are more suitable for hydroxyl radical formation compared to active electrodes like platinum [247]. Hydroxylation of alkanes and alkenes was performed by platinum electrodes [256]. Diamond is an insulation material but introducing impurities with sp^3 hybridization makes diamond conductive. Mostly aromatic hydroxylation is induced by hydroxyl radicals [255]. Activated electrodes like glassy carbon electrodes allow surface reactions whereas non-active electrodes like diamond electrodes allow reactions with hydroxyl radicals in solution [254]. Graphite electrodes are used in Fenton reactions [253].

Common solvents are acetonitrile/water [248] and methanol/water mixtures [250] to enhance the analytes' solubility. Common acetonitrile concentrations range from 1 to 50 % [248]. Another advantage is that acetonitrile prevents compounds from being adsorbed by the cell surface [247]. Most oxidation reactions are pH depending [253]. Thus, application of different pH conditions (acidic, basic and neutral) reveals different types and ratios of oxidation products [248-249].

7.3 EXPERIMENTAL

7.3.1 Reagents, chemicals and consumables

HPLC solvents methanol hypergrade LC-MS (chromasolv), water hypergrade LC-MS (chromasolv) and formic acid (98%, eluent additive for LC-MS) were supplied by Sigma-Aldrich (Steinheim, Germany). Propiconazole, thiacloprid, epoxiconazole and imidacloprid were purchased by Dr. Ehrenstorfer GmbH (Augsburg, Germany). Terbutryn, naphazoline, clarithromycin, iohexol, 2,4-dichlorophenoxyacetic acid, MCPA, chloridazon, benzotriazole, irbesartan, sulfamethoxazole, ibuprofen, metformin and carbamazepine were purchased from Sigma-Aldrich (Steinheim, Germany). PTFE syringe filter 0.45 µm, 3 mm were supplied by Macherey-Nagel (Düren, Germany). Platinum wire (0.5 mm, 99.9 % trace metal basis) was purchased by Sigma-Aldrich (Steinheim, Germany).

7.3.2 Enrichment Experiments

With acetic acid buffer at pH 2.3 experiments were carried out at room temperature using a bare fused silica capillary (Agilent Technologies, Waldbronn, Germany) with 100 µm inner diameter in a CE instrument (Prince Technologies, Emmen, the Netherlands). Capillaries were conditioned by rinsing with water (3 min, 1000 mbar), sodium hydroxide (1 mol/L, 3 min, 1000 mbar), hydrochloric acid (1 mol/L, 3 min, 1000 bar), water (3 min, 1000 mbar) and buffer (3 min, 1000 mbar). The starting vessel contained 30 mL acetic acid buffer (750 mmol/L) with a mixture of the analyte at a concentration of 100 nM (terbutryn, naphazoline, clarithromycin, metformin, thiacloprid, propiconazole, sulfamethoxazole, imidacloprid, epoxiconazole, diclofenac, clothianidin, chloridazon, carbamazepine and benzotriazole). The sampling vessel contained 300 µL buffer. To induce electromigration, -20 kV were applied for 2 to 22 h. The capillary was flushed with 300 µL buffer to analyze the capillary content after application of voltage. All samples were filtered through 0.45 µm PTFE filters and subsequently analyzed by HPLC-MS.

To conduct anion enrichment experiments AAEE coated capillaries were used. An ammonium formate buffer (c = 100 mmol/L formic acid titrated to pH 9 using aqueous ammonia) was used for separation. The analyte mixture and concentration were the same as described for experiments at low pH. The applied voltage was 20 kV for 2 hours.

7.3.3 Electrochemical transformation by EC

For each compound tested a 50 µmol/L solution was prepared in acetonitrile/buffer (1/1) solution. Ammonium formate was used to prepare buffers at pH 9 and 7 and formic acid to prepare buffers at pH 2.8. Electrochemical experiments were carried out using a µPrep Cell 2.0 and a µPrep Cell (Antec Scientific, Zoeterwoude, the Netherlands) with two 100 µm spacer. The µPrep Cell 2.0 was equipped with GC/BDD electrode and the µPrep Cell with Ti electrode. For oxidation experiments a BDD electrode or a BDD glassy carbon electrode were used and for reductive experiments a WE TiBlue µPC 2.0 electrode produced by Antec. The counter electrode was Pd/H_2. The potentiostat was a Pontus 2 potentiostat by MK and the software Autopotus developed by AK Karst University of Münster. Massvoltammograms were obtained by direct injection with a flow rate of 20 µL/min and a microTOF (Bruker) equipped with an electrospray ionization (ESI) source in the positive ionization mode. The MS data acquisition took 7 minutes after applying voltage. For 250 sec a voltage ramp was applied starting at 0 mV and ending at 2500 mV with a rate of 10 mV per second in steps of 5 mV. For reductive experiments a voltage of 0 – 2500 mV was applied. The flow rate was 20 µL/min. For data analysis Origin 9.1.0 (OriginLab, Northampton, USA) was used.

7.3.4 Instrumental methods

HPLC-MS

For HPLC-MS analysis, a 1260 Infinity LC system coupled to a 6550 iFunnel QTOF LC/MS system (Agilent Technologies, Waldbronn, Germany) was used. Aliquots of 10 µL sample were injected onto a Zorbax Eclipse Plus C18 column (2.1 x 150 mm, 3.5-Micron, narrow bore, Agilent Technologies, Waldbronn, Germany) at 40 °C. A jet stream electrospray ionization (ESI) source was operated with a nebulizer pressure of 35 psig, drying gas temperature was set to 160 °C and the gas flow rate to 16 L/min and the fragmentor voltage to 360 V. In the positive ionization mode capillary voltage was set to -4000 V, skimmer voltage to 65 V and a nozzle voltage to -500 V. The mass range was 80 - 1200 m/z with a

data acquisition rate of 1 spectrum/s. The sheath gas temperature was set to 325 °C with a flow rate of 11 L/min. For internal calibration purine and HP0921 (Agilent Technologies, Waldbronn, Germany, m/z = 121.0508 and 922.0097) were used. A gradient elution at a flow rate of 0.3 mL/min using water containing 0.1 % formic acid and methanol was used. The initial content of 95 % water was decreased after 1 min to 5 % water over 7 min and after another 7 min at 5 % increased to 95 % water over 0.5 min. Data analysis was performed with MassHunter software (Agilent Technologies, Waldbronn, Germany).

CE-MS

Analysis was performed using a CE 7100 (Agilent Technologies, Waldbronn, Germany) coupled to a 6550 iFunnel mass spectrometer QTOF (Agilent Technologies, Waldbronn, Germany) as an autosampler and delivery system. Undiluted samples were injected hydrodynamically at 100 mbar for 20 sec and flushed with water at 1 bar for 30 sec using a bare fused silica capillary (50 µm diameter, 64.5 cm length) and transported through the capillary by 100 mbar for 3 min. The mass spectrometer was run by an ESI source in the positive ionization mode at 3500 V. A sheath liquid interface from Agilent Technologies was used with a mixture of isopropanol/water (1/1) containing 0.1 % formic acid with a flow rate of 0.5 mL/min. Gas temperature and flow rate was set to 150 °C and 11 L/min and nebulizer pressure to 5 psig. Data were analyzed by MassHunter Qualitative software (Agilent Technologies, Waldbronn, Germany). Sum formula of transformation products were calculated from exact masses by the software.

7.3.5 Data analysis

Data analysis was performed with Quantitative MassHunter Workstation software Version B.06.00 (Agilent Technologies, Waldbronn, Germany). Graphs and massvoltammograms were created with Origin. 9.1.0 (OriginLab, Northampton, USA). The retention time window was set to 1 min and mass range to 0.01 m/z for extraction of ion chromatograms.

7.4 Results and Discussion

7.4.1 Influence of pH

Experiments at pH 9 were performed with coated capillaries to prevent EOF. During measurement however, a strong EOF was observed in experiments with AAEE coated capillaries. AAEE capillaries were coated following the protocol developed in our working group [242]. The EOF caused a transfer of the starting solution from one vessel to the other, making preconcentration experiments at pH 9 impossible. Here, further work has to concentrate on optimization of the coating procedure. In experiments carried out at pH 2 with a bare fused silica capillary no EOF was observed, resulting in unchanged volumes in both vessels at the end of experiment. From a practical point of view and for proof of principle, the following experiments focused on pH 2. It was expected, that cationic analytes near the capillary-end enter the channel and migrate towards the cathode. Counter ions, in this case hydroxide from the buffer, migrate to the anode as a consequence of electroneutrality. During this process, the pH was expected to increase locally [26], but a stable pH was determined during the experiment in both vessels.

7.4.2 Assessment of experimental time and optimization strategy

To assess the transfer rate of cationic analytes (naphazoline, metformin, clarithromycin and terbutryn), voltage was applied and analyte concentrations were determined at the beginning and after 2 and 19 hours of voltage application. Exemplarily, the chromatograms for metformin and naphazoline recorded for the starting solution (standard), for the starting vessel solution (30 mL, inlet) and for the sampling vessel solution (300 µL, outlet) are shown in Figure 69.

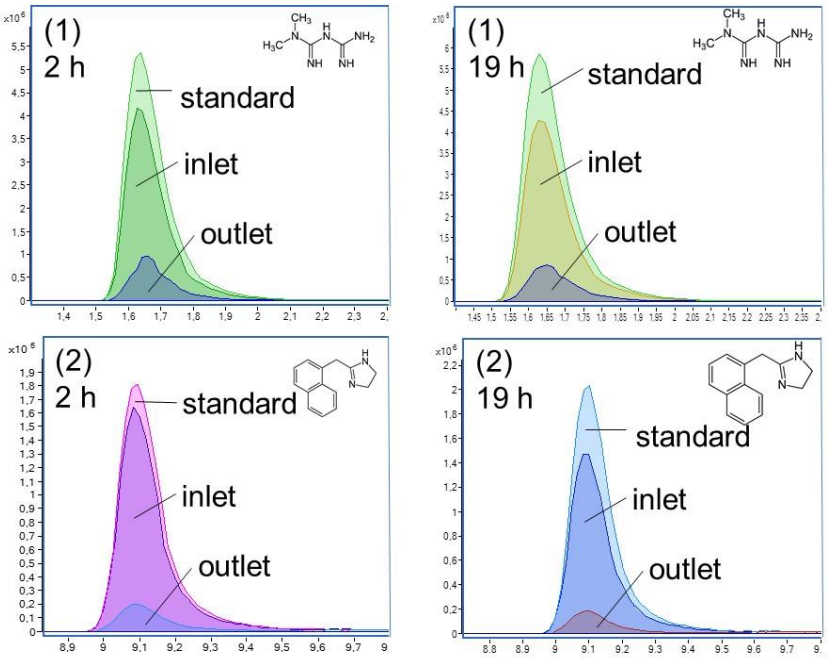


Figure 69: Extracted ion chromatograms for metformin (m/z = 130.1087) and naphazoline (m/z = 211.123), detected in the starting mixture (standard), starting vessel (inlet, 30 mL) and sampling vessel (outlet, 300 µL) after 2 and 19 hours at -20 kV, analyzed by HPLC-MS.

With pH 2 after 2 hours of voltage application only slight concentration changes were detected. Therefore, the experimental time was prolonged to 19 hours. After 19 hours of voltage application no concentration increase in the vessel was detected compared to the shorter experiment but the concentration in the starting vessel slightly decreased. This may be due to of limited injection capability for electrokinetic injection into capillaries, which was shown to be independent from injection time in a simulation by Karim et al. [26]. An equilibrium of concentrations between sampling, starting vessel and capillary might be an explanation for the observation that the concentration in the sampling vessel did not increase between 2 and 22 hours. The reduced overall recovery may be explained by electrochemical degradation of analytes (see Section 7.3.2).

Overall, the main fraction of the analyte remained in the starting vessel. To overcome this limitation different experimental setups were investigated. The first hypothesis was that the effective electric field lines do not cover the whole starting vessel and thus only analyte molecules present near the capillary are affected by the electric field and migrate into the capillary. Further injection would then only be possible upon diffusion of analyte into the effective electric field near the capillary inlet. Therefore, a twisted electrode was applied to extend the area covered by the electric field. The second hypothesis was that heating might accelerate the diffusion process. Therefore, the experiment was carried out at room temperature and at elevated temperature to compare both conditions. The third hypothesis

was that analytes close to the capillary inlet migrate into the capillary leading to a zone of lower analyte concentration around the capillary inlet which equilibrates by diffusion to the surrounding medium. Diffusion of analytes at such low concentrations might be slow [26]. Therefore, stirring of sample would lead to convection and analyte transfer would not be diffusion-controlled anymore.

Insertion of a water plug into the capillary between buffer and sample reduces the conductivity at the capillary inlet and increases the electric field at the capillary ending. This will lead to a stacking process in the capillary [257] and eliminates the formation of a high conductivity zone directly at the inlet which would prevent further injection. This process has successfully been applied in on-line preconcentration experiments [258]. Following the idea of conductivity enhancement, in a first approach a water plug of 5 sec was injected hydrodynamically before the experiment was started. In comparison of the experiment with water plug and the one without water, no differences were observed. In order to test the other hypotheses the influence of the described modifications on the migration behavior of analytes was investigated.

7.4.3 Influence of electrode geometry, convection and temperature

To determine the influence of the electric field spanned by the electrode, the experiment was performed with straight and twisted electrodes in the starting vessel, as shown in Figure 67. A larger volume was expected to be covered by field lines and thus a better transport of analytes into the capillary was expected. To assess the influence of temperature on diffusion controlled migration of analytes, experiments were performed at room temperature and at 50 °C. The last parameter investigated was convection via rigorous stirring. The results for these strategies were applied to the cationic compounds as above (clarithromycin, naphazoline, terbutryn and metformin) with a buffer of pH 2. The results are shown in Figure 70 to Figure 73. In all treatments less than 65 % of the initial compound concentration was detected after 22 hours of voltage application. Concentration factors differed between the analytes from 0 to 3.6-fold.

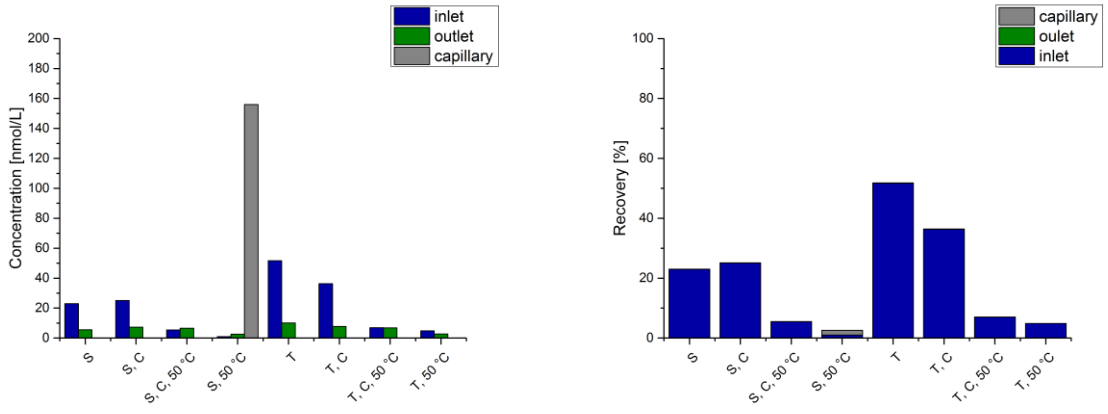


Figure 70: Clarithromycin concentrations detected in starting vessel (30 mL), sampling vessel (300 µL) and capillary (300 µL) by HPLC-MS (left) and recoveries calculated by amount of substance compared to the concentration in the initial solution of 100 nM (right) in samples enriched under different conditions and designs (S = straight electrode, T = twisted electrode, C = convection).

For clarithromycin, recoveries ranged between 5 and 52 % with the highest recovery in the experiment with the twisted electrode and the lowest recovery in the experiment with a straight electrode and elevated temperature. In every experimental condition in the sampling

vessel lower concentrations were detected than in the initial mixture. The only experimental condition under which clarithromycin was detectable in the capillary (1.6-fold higher concentrated) was the same conditions under which the lowest overall recoveries were detected. This finding indicates a stacking phenomenon in this experiment.

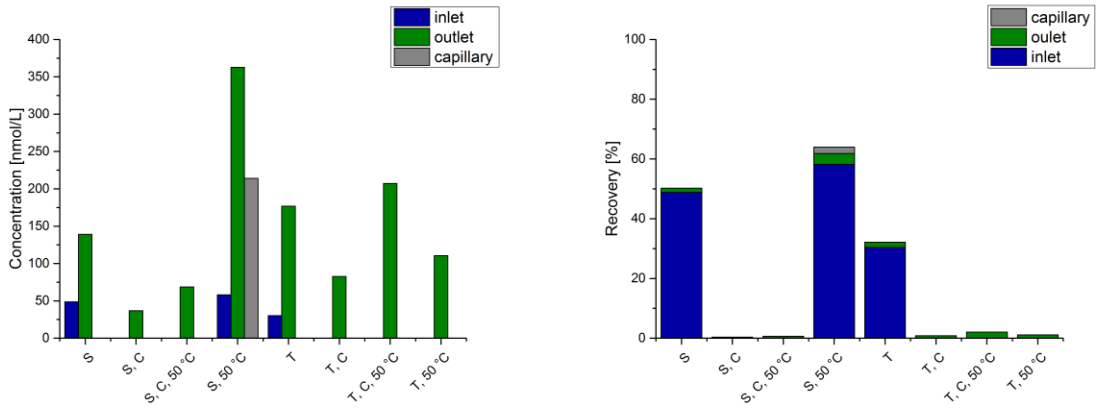


Figure 71: Metformin concentrations detected in starting vessel (30 mL), sampling vessel (300 µL) and capillary (300 µL) by HPLC-MS (left) and recoveries calculated by amount of substance compared to the concentration in the initial solution of 100 nM (right) in samples enriched under different conditions and designs (S = straight electrode, T = twisted electrode, C = convection).

Metformin showed a different behavior than clarithromycin. Under five of the eight experimental conditions a higher analyte concentration in the sampling vessel was detected than the concentration in the initial mixture. The highest enrichment factor of 3.6 was achieved in the experiment with higher temperature and a straight electrode. In addition, this was the only experiment in which analyte was detectable in the capillary in 2.1-fold higher concentration compared to the initial mixture. The higher enrichment can be related to the high electrophoretic mobility of metformin being small and a strong base. The total recoveries ranged from 0.4 to 60 %. In every experiment with convection and the one with higher temperature and a twisted electrode the recovery was below 2 %. At a higher stirring rate than migration rate convection could lead to a reduced migration rate of analytes into the capillary. But in this case the recovery should be higher in experiments with convection. Therefore, results indicate a higher transformation rate of metformin in experiments with convection

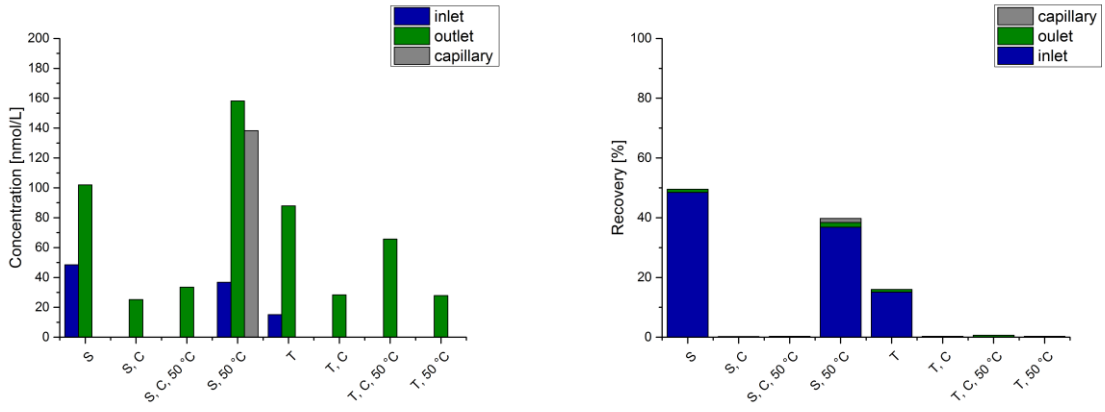


Figure 72: Naphazoline concentrations detected in starting vessel (30 mL), sampling vessel (300 µL) and capillary (300 µL) by HPLC-MS (left) and recoveries calculated by amount of substance compared to the concentration in the initial solution of 100 nM (right) in samples enriched under different conditions and desings (S = straight electrode, T = twisted electrode, C = convection).

The results in experiments with naphazoline were similar to those with metformin. Recoveries were in the range of 0.3 - 49 %. Recoveries below 1 % were observed in experiments with convection during the enrichment time and the one with higher temperature and a twisted electrode. As seen before, the only experimental condition with a higher analyte concentration detected in the sampling vessel by 1.6-fold and in the capillary by 1.4-fold than in the initial mixture was with straight electrode and elevated temperature.

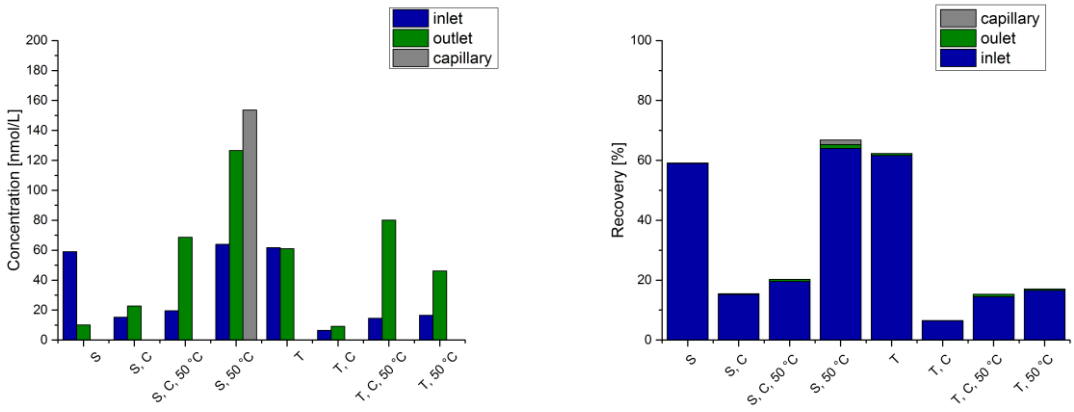


Figure 73: Terbutryn concentrations detected in starting vessel (30 mL), sampling vessel (300 µL) and capillary (300 µL) by HPLC-MS (left) and recoveries calculated by amount of substance compared to the concentration in the initial solution of 100 nM (right) in samples enriched under different conditions and designs (S = straight electrode, T = twisted electrode, C = convection).

The highest recoveries were determined for terbutryn, ranging from 6 to 65 %. The only experiment in which analyte was detected in the capillary in 1.5-fold higher concentration than in the initial mixture was the experiment with straight electrode and higher temperature. In every experiment with convection and in the experiment with the twisted electrode and elevated temperature recoveries were below 20 %. The higher overall recoveries indicate that terbutryn is more stable to electrochemical degradation than the other test compounds.

Summary: Under every condition tested the largest fraction of the analytes was detected in the starting vessel compared to the one in the capillary and in the sampling vessel even after 22 h of voltage application. However, the 100 times smaller volume in the sampling vessel causes a concentration effect already for an analyte transfer rate greater than 1 % from the starting to the sampling vessel. A transfer equal to 1 % or greater was observed for metformin in five experiments, for naphazoline in three and for terbutryn in one experiment but never for clarithromycin. The most striking observation was, that in most experiments only small fractions of analyte were detectable at the end of experiments, a large fraction was lost. Electrochemical degradation as reason for this observation is discussed later in this work.

Effects by changes in electrode geometry from straight to helical depended on the analyte. In experiments with metformin and naphazoline a decreased concentration in the starting vessel was observed for experiments with a helical electrode compared to a straight electrode. For clarithromycin, a higher concentration was detected in the starting vessel in experiments with a helical electrode compared to those conducted with a straight electrode. For terbutryn only negligible concentration changes were observed in the starting vessel regardless of the two electrode geometries. Considering all experiments the impact of electrode geometry seemed to be lower than the other tested parameters, temperature and convection. In the experiments with the helical electrode tendentially lower analyte recoveries were observed than in experiments with the straight electrode. To understand the reasons further investigations are necessary. Possible effects might by electrochemical

transformation, hydrolysis as well as adsorption phenomena. All effects are influenced by the effective electric field geometry with both the volume covered but also the strength. Thus, the hypothesis to obtain higher enrichment efficiencies by a more effective analyte injection cannot be proven regarding the results presented. Hirokawa et al. simulated a significant impact of electrode geometry on the effectivity of short-term electrokinetic sample injection, using a cylindrical electrode surrounding the vessel compared to a hollow electrode with the capillary inserted [26].

Application of heating (using a straight electrode) resulted in a higher concentration of analytes in the capillary. This finding corroborates published studies investigating the influence of temperature on electrokinetic sample injection [26]. Elevated temperature combined with a straight electrode geometry were the only parameters, where a significant analyte concentration was detectable in the capillary inner volume. This indicates an enhanced injection of analytes into the capillary, but no further or only very slow electrophoretic migration to the sampling vessel. In experiments with the helical electrode and elevated temperature, less than 10 % of the analyte were detectable after 22 h for all compounds. This indicates a faster transformation process induced by the helical electrode geometry. These results correlate with the findings discussed before.

Convection led to reduced terbutryn and clarithromycin recoveries and in case of metformin and naphazoline to nearly complete loss of analyte. The substance least affected by convection was terbutryn. This finding was independent from the electrode geometry. In comparison to the three investigated parameters convection showed the highest impact on substance recovery. In every experimental condition implying convection the recovery was drastically reduced. This shows that convection enhances the migration rate of analytes into the capillary, but in the same way facilitates degradation and desorption processes. Xu et al. simulated, that convection might increase the sample diffusion and thus accelerate the analyte supply to the effective electric field [259].

Xu et al. reported an increased amount of injected sample by heating to 75 °C, stirring and extending the sample volume [259], with a higher impact of heating compared to stirring. Comparing Figure 70 to Figure 73 shows that in this study, the combination of temperature and convection obviously led to lower analyte recoveries, independent of substance and electrode geometry. This indicates that the combination of both enhances degradation processes or the preconditions leading to degradation. To investigate if electrochemical degradation was the reason for reduced recoveries, transformation studies were conducted, explained in Section 7.3.3.

7.4.4 Experiment with straight electrode and elevated temperature

The highest analyte concentrations in the starting vessel, in the capillary and highest recoveries were detected for the following conditions: elevated temperature of 50 °C, straight electrode and no convection. In Figure 74 the relative analyte amounts detected and the concentrations in starting vessel, sampling vessel and capillary are shown for these conditions.

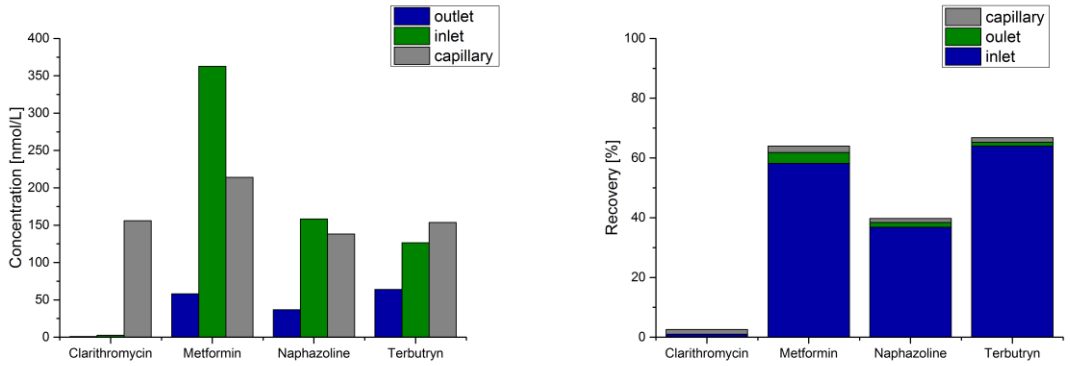


Figure 74: Concentrations in the starting vessel (30 mL), sampling vessel (300 µL) and capillary (300 µL) (left) and recoveries relative to initial analyte amount (right) detected in samples enriched at 50 °C with straight electrode and without convection.

For three of the four investigated compounds a higher concentration in the sampling vessel of 1.3-for terbutryn, 1.6-fold for naphazoline and 3.6-fold for metformin was detected compared to the initial mixture of 100 nM in 30 mL. Analyte was detected in 300 µL capillary rinsing solution in 1.5-fold to 2.1-fold concentrations of all four compounds. Obviously, under these conditions, more efficient injection and probably slower degradation processes occur. Possibly, the elevated temperature also reduces adsorption phenomena to glass vials and capillary. Thus, these are the most promising conditions for further investigations.

Taking all experimental conditions and substances into account, the lowest recoveries were observed in experiments with naphazoline followed by metformin. Higher recoveries in total were observed for terbutryn and clarithromycin, but for both compounds mostly in the starting vessel. The first two compounds are smaller and thus have a larger effective electrophoretic mobility. Clarithromycin is less stable and thus might be degraded faster than the other molecules if transferred to the sampling vessel. Terbutryn might be in the middle with respect to stability and electrophoretic mobility.

7.4.5 Transformation experiments

Electrochemical processes induced in an electrochemical cell are commonly oxidation processes [249]. Electrochemical oxidation normally involves one electron processes like oxidation at the aromatic ring or N-oxidation [253]. In order to show possible degradation processes, transformation products were investigated using an electrochemical cell (see Section 7.3.3). As shown below, the analyzed compounds were stable at the applied conditions up to a potential of 2 V. Alteration of pH, electrode material and reductive as well as oxidative conditions did not yield transformation products. For terbutryn oxidation of the thiol-group is likely to happen [248-249], but was not observed in the electrochemical cell experiments. Metformin and naphazoline bearing activated groups might need harsher conditions to be oxidized. A possibility to obtain electrochemical transformation products would be the use of Fenton chemistry as in these experiments radicals are generated, which tend to react also with stable compounds as shown by Bischoff and Bruins [253, 255].

7.4.6 Electrochemical transformation in literature

Transformation products are published in literature for all compounds except for naphazoline. Mass spectra detected in QTOF-MS measurements were analyzed for exact masses of transformation products known from literature. Chemical formulas of detected transformation products of terbutryn and metformin are given in Figure 75.

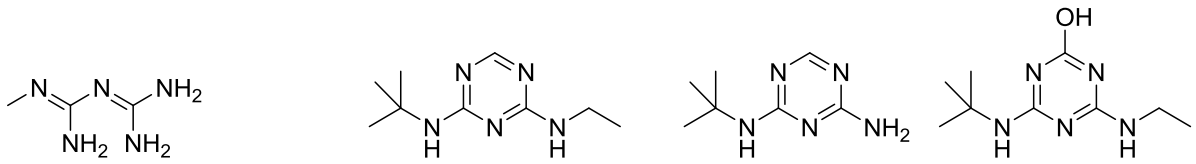


Figure 75: Transformation products of terbutryn detected in enrichment samples with retention time and exact mass [260 261]. TP(T)-1 N2-(tert-butyl)-N4-ethyl-1,3,5-triazine-2,4-diamine, TP(T)-2 N2-(tert-butyl)-1,3,5-triazine-2,4-diamine and TP(T)-3 4-(tert-butylamino)-6-(ethylamino)-1,3,5-triazine-2-ol. Transformation product 1-methylbiguanide and metformin [262].

An HPLC-MS method was developed to analyze these transformation products in the samples generated under different experimental conditions in order to investigate the hypothesis of electrochemical degradation in the enrichment experiments. The extracted ion chromatograms of parent compounds and the transformation products identified via exact mass are shown in Figure 76. Parameters used for compound identification are summarized in Table 22.

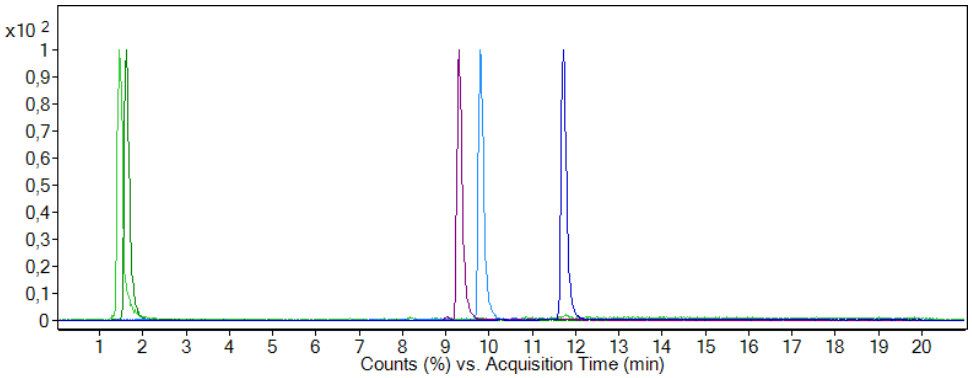


Figure 76: Extracted ion chromatograms of terbutryn (dark blue), TP(T)-1 (light blue), TP(T)-3 (purple) and metformin (dark green) and its metabolite TP(M)-1 (light green) analyzed in the sampling vessel solution after 22 h of voltage application with a straight electrode and without convection by HPLC-MS.

Table 22: Analytical parameters for HPLC-MS analysis of terbutryn with three metabolites and metformin with one metabolite detected in samples, with retention time and exact mass.

compound	formula	$[M+H]^+$	t_R [min]
terbutryn	$C_{10}H_{19}N_5S$	242.1434	11.7
TP(T)-1	$C_9H_{17}N_5$	196.1558	9.8
TP(T)-2	$C_7H_{13}N_5$	168.1228	not detected
TP(T)-3	$C_9H_{17}N_5O$	212.1516	9.4
metformin	$C_4H_{11}N_5$	130.1087	1.2
TP(M)-1	$C_3H_9N_5$	116.0930	1.5

The metformin transformation product TP(M)-1 was only detected in two samples among all samples from all experimental condition, both from enrichment experiments run at 50 °C (independent from electrode geometry). The signal area of the metabolite signal was 6 and 20 % compared to the metformin signal area in the same sampling vessel. After 22 hours several transformation products of terbutryn, known from literature [260-261], were detected. The relative signal areas of the transformation product peaks did not correlate to the signal area of the terbutryn peak in each sample, compare Figure 77. This might be due to fast mineralization of the transformation products or adsorption processes of transformation products.

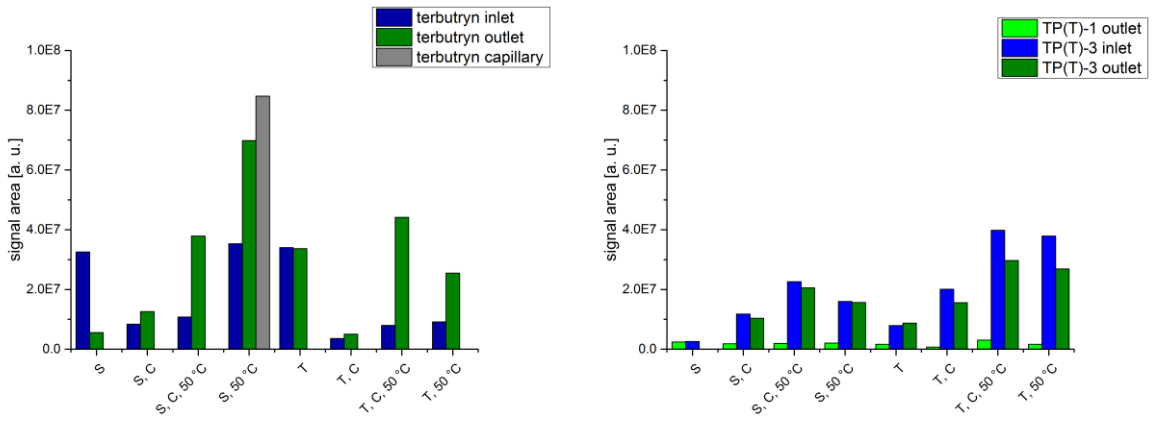


Figure 77: Signal areas in the starting vessel (blue), the sampling vessel (green) and the capillary (grey) for terbutryn (left) and two metabolites (right), measured by HPLC-MS after 22 h enrichment experiment with different conditions applied.

Terbutryn's transformation product TP(T)-2 was not detected in any sample. TP(T)-1 was detected in every sampling vessel in similar low concentrations as the parent compound, assuming similar ionization efficiency of terbutryn and the transformation product. TP(T)-3 was detected in every starting vessel and every sampling vessel except the one with the straight electrode (room temperature, without convection), but in this experiment the concentration in the starting vessel was lower than in all other samples and the concentration in the sampling vessel might have been below the limit of detection. Concentrations in the sampling vessel and the starting vessel were similar with slightly higher concentrations in the starting vessel. The thioether might be cleaved at acidic conditions in both vessels by hydrolysis. The triazine herbicide terbutryn might be electrochemically degraded as observed for conazole fungicides. These fungicides and triazole fungicides were completely mineralized to CO_2 by electrochemical treatment within 120 min at different pH values from 3 to 10 with a BDD electrode [16, 263].

Published transformation products of clarithromycin could not be detected in this study [94]. This might be explained by hydrolysis of clarithromycin and subsequent further mineralization at the applied experimental conditions of 22 hours at pH 2 and -20 kV. In water purification, electrochemical methods are applied to mineralize poorly biodegradable contaminants such as pharmaceuticals [264]. Anodic oxidation is the most common technique in wastewater treatment [265]. In these systems hydroxyl radicals are generated at the anode from water and organic pollutants are destroyed via reaction with hydroxyl radicals. Low hydroxyl radical concentrations led to only partial mineralization [266]. Another reason for the absence of transformation products may be adsorption to glassware or capillary inner surfaces.

To reduce possible degradation processes the addition of a sacrificial compounds being degraded instead of the analyte would be an opportunity or to spatially separate sampling analyte and electrode from one another as performed in electromembrane extraction [221].

7.5 Conclusion

Electromigrative enrichment experiments were conducted to investigate the applicability of electromigrative techniques for preparative preconcentration of ionizable analytes from water samples. The influence of pH value, electrode geometry, temperature and stirring on the migration behavior were investigated. For pH 9 the electroosmotic flow was shown to have a

high impact even in coated capillaries and further experiments were carried out at pH 2. For metformin, enrichment of 3.6-fold was achieved. The enhancing influence of convection and temperature on electrochemical decomposition were shown and a minor influence of electrode geometry. Overall, the developed approach demonstrates the ability of electromigrative techniques to enrich ionic compounds. Simultaneously, neutral or oppositely charged compounds are removed. Further work has to focus on a more complete transfer of analytes of interest in the electric field and avoidance of their degradation.

8 DISCUSSION AND CONCLUSION

The objectives of this thesis were divided into two major parts. The first part was the development of analytical methods and their application to analyze residues of the pharmaceutical carbamazepine and the neonicotinoid thiacloprid in midge larvae and the fungicide propiconazole in fungi mycelium, thus, to increase the understanding of their environmental impact. This was achieved by optimization of extraction as well as quantification methods to determine the pollutant burden in different organisms and then to apply these methods to samples from exposure studies. In environmental analysis methods are required to determine trace amounts of contaminants in surface waters. Therefore, in the second part, first steps were made towards the application of different electromigrative methods to preconcentrate ionic and ionizable analytes in wastewater samples.

8.1 ANALYTICAL DEVELOPMENTS FOR POLLUTANT ANALYSIS IN BIOTA

Although several extraction techniques were described in literature that are suitable to apply on different type of biota samples and contaminants, screening methods covering a broad range of compounds (parent compounds as well as their metabolites) are of interest. However, analysis of trace amounts of contaminants, especially, in small amount biota samples is still challenging. To analyze small amounts of biota samples suitable homogenization techniques are a crucial factors as well as removal of interfering matrix components and sensitive detection methods.

Generally, the initial step in sample preparation is sample homogenization. The suitable homogenization technique depends on different factors such as the type of sample matrix (e.g. rich in lipids or water content) and the available sample amount. Therefore, different homogenization techniques described in literature were compared. Different techniques were found suitable for homogenization of midge larvae and for fungi mycelium in this thesis.

Basic requirements for the extraction method were a simple and fast extraction procedure applicable to analyze a broad range of pollutants including their metabolites in a small number of individuals originating from various organisms. These criteria are generally fulfilled by the QuEChERS extraction [2] after miniaturization. Therefore, the QuEChERS extraction procedure was modified concerning the requirements of the samples and analytes. Different approaches for acid or base sensitive compounds and acidic analytes were described in Chapter 2. A crucial step during or after the extraction procedure is the application of different dispersed solid phase extraction (dSPE) sorbents to remove interfering matrix components. Considering to the requirements of the matrix and the physicochemical properties of the analytes different sorbents were used, in this thesis. Modified QuEChERS extractions were applied for analysis of thiacloprid in midge larvae (Chapter 4), carbamazepine in midges and midge larvae (Chapter 3) and propiconazole and its metabolites in fungi mycelium (Chapter 5) in this thesis. Further, details on homogenization and cleanup procedures are discussed in Section 8.1.2.

For separation and detection of these chemicals, HPLC coupled to ESI-QTOF was chosen. The extraction procedure was shown to cover a broad range of analyte polarity to enable simultaneous extraction of metabolites and parent compounds in a single extraction step. Care was taken to adapt the extraction procedure with respect to the applied homogenization technique. Cleanup steps including dSPE were implemented and optimized with regard to enhance both, the recoveries and the limits of quantification.

8.1.1 Method validation parameter of the HPLC-MS quantification methods

Extracts were separated and analyzed with HPLC coupled to MS. This instrumental application is well-known to enable the sensitivity, selectivity and matrix tolerance required for trace analysis of organic compounds in biota extracts. Thiacloprid and propiconazole were analyzed in the MS-only mode, due to identical quantifier fragmentation ions of analyte and isotopically labeled analyte. For carbamazepine and the isotopically labelled internal standard a MS/MS method was developed. The method validation parameters are shown in Table 23.

Table 23: HPLC-MS method parameters of thiacloprid, propiconazole and carbamazepine analysis of analytical standard material in methanol.

analyte	t_R [min]	LOD [µg/L]	LOQ [µg/L]
thiacloprid	10.1	0.2	0.5
propiconazole	13.2	0.1	0.2
carbamazepine	11.3	0.1	0.2

The three analytes were separated on a C18 column with retention times of 10 - 13 minutes, limits of detection of 0.1 - 0.2 µg/L and limits of quantification of 0.2 - 0.5 µg/L. The limit of detection was calculated based on a signal to noise ratio of 3 (S/N = 3) and the limit of quantification by a signal to noise ratio of 10 (S/N = 10). Internal standards are widely used in analysis of contaminant residues [31, 37, 44-45, 47, 50, 52, 54, 59]. During workup and ionization the isotopically labeled compounds experience the same loss during workup and impairments of ionization efficiency during analysis. The quantification methods developed in this thesis, were based on isotopically labelled standards as they are known to account for recovery and matrix effects of analytes particularly in complex matrices.

8.1.2 Quantification of pesticides and pharmaceuticals in different matrices

Extraction and cleanup procedures

The QuEChERS extraction procedure is based on a liquid-liquid extraction step with acetonitrile and water, followed by addition of salts to induce phase separation. If acquired a dSPE cleanup step can be implemented. For this, different sorbents are applicable. The applied methods were optimized with regard to the homogenization technique and the sorbent used in the cleanup step.

Choice of homogenization methods of different biota samples

Homogenization has two main impacts on the extraction procedure: 1) a more effective and faster extraction of analytes is aimed at achievement of higher and reproducible recoveries, and 2) a simultaneous effective co-extraction of matrix components, which potentially could interfere in MS analysis. For all samples approximately 20 mg biota sample were analyzed with different homogenization techniques, listed in Table 24.

Table 24: Homogenization techniques for the different samples and analytes.

matrix	condition	analyte	homogenization	conditions
3 -5 midge larvae	frozen	thiacloprid	micro-homogenizator	liquid nitrogen
20 mg mycelium	dried	propiconazole	mortar and pestle	addition of NaCl
20 mg mycelium	frozen	propiconazole	mortar and pestle	liquid nitrogen
10 mg midge larvae	frozen	carbamazepine	micro-homogenizator	liquid nitrogen
10 mg midges	frozen	carbamazepine	micro-homogenizator	liquid nitrogen

3 - 5 midge larvae were homogenized for quantification of thiacloprid and 10 mg larvae or midges were homogenized in case of quantification of carbamazepine. Frozen larvae and midges were homogenized in liquid nitrogen with a micro-homogenizator directly in the extraction vessel, which minimalized tissue loss as no transfer to another extraction vessel was required. 20 mg wet weight frozen mycelium from fungi experiments on agar plates were homogenized with mortar and pestle in liquid nitrogen, which is common for biota analysis [35, 47, 53]. Mycelium was more difficult to homogenize than larvae samples due to texture and higher sample amounts. Therefore, mortar and pestle were chosen instead of the micro-homogenizator approach used for the larvae and midges samples. 20 mg dried mycelium samples from liquid culture experiments were homogenized with mortar and pestle after addition of sodium chloride to avoid electrostatic effects. Liquid nitrogen was not used here. Direct homogenization of dried samples was considered due to propiconazoles temperature stabile properties as proven in previous experiments. For fungi, between 3 and 50 mg mycelium were homogenized covering a broader range of matrix amount compared to midge larvae samples (10 - 20 mg) and thus the mortar and pestle approach was chosen. In all three optimized extraction procedures analyte signal intensities in the raw extract were in the range of 34 - 70 % compared to the signal intensity of the standard in methanol at the same concentrations. To enhance these signal intensities by reduction of interfering matrix components different dSPE materials were evaluated.

Choice of dSPE materials

A possibility to remove interfering matrix components, and thus reduce the matrix effect during analysis is the implementation of a dSPE step. Different sorbent materials are available according to the physicochemical properties of analyte and sample matrix. The sorbent should retain co-extracted matrix components while ideally not removing analyte. Common dSPE materials are PSA and C18. PSA removes fatty acids and organic acids [2]. C18 (non-endcapped) removes nonpolar matrix components by its functionalized octadecylsilane groups and polar basic compounds with non-endcapped silanol groups [193]. The different extracts were cleaned with PSA and C18 (non-endcapped) and the signal intensities of analytes were compared in cleaned and raw extracts. The dSPE materials applied in the optimized method and method validation parameters are shown in Table 25.

Table 25: Applied dSPE material and quantification method validation parameters: recovery, limit of quantification and matrix effect calculated from spiked extract samples.

matrix	analyte	dSPE	recovery	matrix effect	LOQ	mass
midge larvae	thiacloprid	PSA	100 ± 5 %	27 %	12 ng/g	20 mg
dried mycelium	propiconazole	none	100 ± 7 %	20 %	25 ng/g dw	20 mg
frozen mycelium	propiconazole	none	85 ± 6 - 93 ± 7 %	36 %	5 ng/g ww	20 mg
midge larvae	carbamazepine	C18	95 ± 15 %	23 %	12 ng/g	10 mg

For mycelium extracts, implementation of the cleanup was not beneficial to enhance the signal intensity. In agar plate experiments, the signal intensity of propiconazole was 66 ± 6 % in dSPE cleaned extracts compared to 62 ± 7 % in raw extracts. Considering that an additional step during sample processing could lead to loss of chemicals and that the recoveries were not significantly increased, the use of dSPE was not beneficial. Hence, quantification of propiconazole was achieved in raw extracts. For thiacloprid extraction from midge larvae dSPE cleanup with PSA enhanced signal intensities to 67 ± 2 % compared to 34 ± 4 % in the raw extract, resulting in a matrix effect with 27 ± 2 % signal suppression in the cleaned extract. For carbamazepine extraction from midge larvae and midges cleanup

with C18 enhanced signal intensities to 50 % and reduced the matrix effect to 23 % compared to signal intensities in the raw extract (recovery 46 %, matrix effect 24 %). PSA cleaned extracts showed a similar matrix effects of 22 % but reduced recoveries of 34 % compared to C18 cleaned extracts. This indicates that interaction of PSA with carbamazepine is greater than with non-endcapped C18. To assess analyte losses during workup and matrix effects, quantification was based on isotopically labeled internal standards.

Method validation parameters of quantification methods in biota tissue

The method validation parameters of the four methods are summarized in Table 25. For fungi from liquid culture medium experiments, 1 µg/L was the limit of detection and 2 µg/L the limit of quantification for 20 mg dried fungi mycelium corresponding to 25 ng/g dry weight. The limit of quantification for propiconazole in extract of mycelium from agar plates was 0.2 µg/L corresponding to 5 ng propiconazole per g wet weight mycelium. Recovery in agar plate experiments was 100 ± 7 % and in liquid culture experiments 85 ± 6 % to 93 ± 7 % based on deuterated propiconazole as internal standard.

The recovery for thiacloprid in midge larvae extract was 100 ± 5 % based on internal standard and the limit of detection 1 µg/L. The limit of quantification is in accordance with applications of QuEChERS extraction for thiacloprid from bumble bees [53], honey bees [35] and gammarids [40] , where the limit of quantification was in the low ng/g range. The method developed in this thesis required 20 mg matrix, whereas in the mentioned publications 100 - 200 mg matrix were consumed. Compared to a nano-LC-MS method published by Berlioz-Barbier et al. to analyze diuron and spinosad in 3 - 4 chironomid larvae with limits of quantification of 1 - 50 ng/g wet weight [21], the method developed here reveals comparable values.

Detection limit for carbamazepine was 5 µg/L in 10 mg extracted tissue corresponding to approximately 3 midges or 2 larvae and 12 ng/g wet weight. Quantification based on deuterated internal standard was performed with a recovery of 95 ± 15 %. The method validation parameters are in accordance with literature. Miller et al. analyzed carbamazepine in 100 mg freeze dried gammarids with a limit of quantification of 6 ng/g and Dussault et al. investigated matrix effects in extracts of 9 - 12 spiked chironomids while achieving a limit of quantification of 2.9 µg/L [65].

Overall, the developed methods allowed the analysis of pollutants in very small amounts of sample (10 - 20 mg), which were provided from laboratory exposure studies. The limits of quantification were in the ng/g range (5 - 25 ng/g), and therefore uptake at environmentally relevant concentrations (ng/L - µg/L) is quantifiable with the developed methods. The results demonstrate that the miniaturization of QuEChERS is possible for various sample types but requires the adaption of the methodology to the analyte and organism of interest.

Analysis of metabolites

To know the fate of chemicals, analysis of their metabolites is crucial. In some cases metabolites of compounds are more active to organisms than their parent compounds or inactive compounds are activated during transformation [1]. To extract metabolites, which are often more polar than the parent compounds [1] together with the parent compound using the same extraction procedure, a method covering a broad range of polarity is required. The ability of the QuEChERS extraction to extract parent compound and metabolites was shown for the fungicide boscalid from honey bees [43] and diclofenac from mussel [45] and fish [48]. The method developed in this thesis for extraction of propiconazole from fungi mycelium comprised the extraction of three of its main metabolites, details are presented in Chapter 5.

Extracts of midge larvae were analyzed for transformation products of thiacloprid and carbamazepine, however, they were not detected. This could be due to concentrations of the transformation products in the investigated organisms below the limit of detection or to an insufficient capacity of the extraction method. Synthesis, electrochemical production or photolysis followed by purification would be required to obtain standards of transformation products for a more detailed investigation of the extraction efficiency.

8.2 ECOTOXICOLOGICAL EFFECTS

The optimized quantification methods were applied to determine the internal pesticide and pharmaceutical concentrations in organisms from exposure studies. These analyses were performed to understand (1) the correlation of internal and exposure concentration, (2) the effects of nanoparticles on pollutant transformation, (3) the uptake rate and transformation of pollutants, (4) the effects of nanoparticles on the bioavailability, and (5) the transfer of incorporated compounds from larvae to midges and thus between ecosystems.

8.2.1 Correlation of internal concentration and exposure concentration

The internal pollutant concentration in biota from exposure experiments was analyzed to test the hypothesis that the internal concentration reflects biological effects more closely than nominal exposure concentrations. In experiments with nonylphenol, the internal concentration correlated more accurately to dose-response concentrations than water concentrations [10]. Therefore, samples from different exposure experiments were analyzed: (1) larvae exposed to thiacloprid, (2) fungi exposed to propiconazole and (3) larvae and midges exposed to carbamazepine.

(1) The internal thiacloprid concentration in midge larvae from mortality studies was analyzed. In these experiments, only for the lowest exposure concentration of 0.03 µg/L thiacloprid a reduced internal concentration of 12 ± 5 ng/g in larvae after 96 h was observed. Higher exposure concentrations always resulted in internal concentrations of 47 ± 7 ng/g. The concentration in larvae which died during the exposure time was independent of exposure time, and determined to be in the range of 54 ± 11 ng/g. This concentration is only slightly higher than the one found in larvae which survived the treatment, and thus, possibly represents the lethal thiacloprid concentration for chironomids.

(2) The concentration of propiconazole and three of its metabolites were analyzed in mycelium from different fungi species and in the medium. Mycelium samples originated from experiments on agar plates with *L. bicolor* and in liquid culture *A. muscaria* at different exposure concentrations. In a second approach samples originated from experiments with three different fungi species (*A. muscaria*, *L. bicolor* and *C. geophilum*) which were exposed to different propiconazole concentrations (0.1, 2 and 5 mg/L) in liquid culture.

In agar plate experiments with *L. bicolor* and reduced bioavailable propiconazole concentrations by addition of nanoparticles the detected internal propiconazole concentration clearly increased with the bioavailable fungicide concentration. The internal concentration increased from 0.2 to 7 µg/g ww mycelium for an exposure range of 20 - 100 µg/L. The internal concentration showed a strong correlation to the endpoint of reduced growth of mycelium. In samples exposed to 20 µg/L 40 ± 7 mg mycelium were available at the end of exposure time whereas for an exposure at 100 µg/L a mycelium amount of only 2 ± 1 mg was available (for details see Chapter 5). An increase in fungal growth was expected,

because propiconazole is an inhibitor of lanosterol biosynthesis by inhibiting C-14 demethylation of lanosterol [15-16].

In liquid culture experiments with different fungi species significantly different internal concentrations were observed between species. Likewise, the inhibition of growth differed between the tested species. In *A. muscaria* mycelium, which was exposed to the highest concentration of 5 mg/L, the lowest internal concentration of 33 ± 21 µg/g was detected combined with the strongest growth inhibition with 7 ± 2 mg available. In *L. bicolor* exposed to the lowest concentration of 100 µg/L the detected internal concentration was intermediate with 284 ± 80 µg/g. Here, the growth inhibition was observed with 12 ± 5 mg mycelium available. In *C. geophilum* exposed to a medium concentration of 2 mg/L the highest internal concentration of 785 ± 47 µg/g was detected, correlating with the lowest inhibition of growth with 42 ± 3 mg mycelium being present at the end of exposure time. In these experiments the internal concentration inversely correlated to growth inhibition. On the first glimpse, the hypothesis on a correlation of internal concentration and effect, may be rejected. However, the differences may be better explained by different metabolic activity, which is discussed in Section 5.5.4.

In liquid culture experiments with *A. muscaria* exposed to 1 - 5 mg propiconazole an increasing internal concentration of 1 ± 0.6 µg/g to 5 ± 1 µg/g was detected. These internal concentrations were not reflected in reduced growth, because at the lowest exposure concentration 6 ± 2 mg fungi mycelium were available and at the highest exposure concentration 5 ± 1 mg.

3) The internal carbamazepine concentration in midge larvae and midges from an emergence test was quantified. With increasing concentration levels for exposure an increase in body-burden for larvae and emerged midges was observed in all treatment groups. In the exposure range of 0.025 - 3.2 mg/L the internal concentration in larvae increased from 8 ± 2 to 96 ± 30 ng/g in larvae and in midges from 19 ± 4 to 204 ± 10 ng/g.

Internal propiconazole concentrations in fungi correlated well to the exposure concentrations in experiment on agar plates and in liquid culture. In the liquid culture experiments an increase in the internal concentration was observed. In experiments with agar plates inhibition of growth and internal concentration correlated very well. In mortality studies with chironomids larvae exposed to thiacloprid, only in larvae exposed to the lowest thiacloprid concentration a reduced internal concentration was detected. This indicates the sensitivity of analysis of the internal concentration in a range, where mortality was not observed but the toxic load was present. For chironomid larvae exposed to carbamazepine in an emergence test an increase in body-burden was observed over the whole test concentration range. These observations revealed that in emergence studies, investigating a more sensitive endpoint than mortality, even at lower concentrations a marked difference in internal concentrations was observed. The results indicate that the internal concentration in biota may correspond to sublethal or chronic effects more precisely than to acute endpoints.

Calculated concentration factors between exposure concentration and determined internal concentration in fungi mycelium ranged from 5 L/kg (*A. muscaria* in liquid to culture) to 400 L/kg (*C. geophilum* in liquid culture) and approximately 3000 L/kg (*L. bicolor* 56 L/kg in agar plate experiments and 4600 ± 1600 L/kg in liquid culture experiments). This shows the bioaccumulation potential of the fungicide propiconazole and that bioconcentration factors highly depend on the analyzed species. In experiments with midge larvae an accumulation factor of 70 ± 10 L/kg was observed for thiacloprid between exposure medium and midge larvae whereas the ratio between the carbamazepine exposure concentration and the

internal concentration in larvae and midges was below 1. However, even at the low carbamazepine exposure concentration a strong correlation between internal and exposure concentration was observed.

8.2.2 Metabolism of pesticides

The ability of fungi to degrade biomolecules is essential for the carbon cycle in forests [151]. Due to their ability to degrade not only biological but also anthropogenic organic compounds (co-metabolism) fungi are used in remediation of contaminated soil [14]. To degrade large biomolecules fungi excrete extracellular enzymes into the soil [166-167]. This shows, that for fungi two metabolic routes have to considered, metabolism in the fungi to excrete compounds and metabolism by external enzymes to take up metabolites for nutrition. Therefore, different metabolite profiles between fungi mycelium extracts and medium extracts may evolve and different effects may be expected with regard to the fungi's reaction to pesticides. The objective was to investigate differences in uptake and the metabolic transformation rate of propiconazole by different fungi species and to determine the bioaccumulation and metabolic fate of propiconazole in fungi by analysis of propiconazole and three of its metabolites. Biotransformation products of propiconazole are known in literature from transformation studies with rainbow trouts [203] and gammarids [204]. Rösch et al. detected two main metabolites in gammarids, the first one resulting from hydroxylation and the second one by ether cleavage from propiconazole [204].

In analysis of *L. bicolor* mycelium grown on agar plates, signal areas of the metabolites were 10-fold lower compared to the ones of propiconazole. The observation of metabolites in mycelium shows a metabolic activity of the fungus towards propiconazole and its capability to metabolize the fungicide at exposure concentrations up to 100 µg/L in agar plates. In the analysis of agar plate extracts propiconazole was detectable but no metabolites.

The analysis of mycelium extracts of fungi grown in liquid culture revealed no metabolites. In contrast metabolites were detected in the liquid culture medium of the same three fungi species except for the medium of *L. bicolor*. For *L. bicolor*, no propiconazole was observed in the medium at the end of the experiment. This indicates that propiconazole was fully metabolized or incorporated by the fungi. These findings corroborate literature observations: In experiments on agar plates *A. muscaria* and *C. geophilum* were able to tolerate up to 0.1 ppm propiconazole and one strain of *C. geophilum* was even stimulated in growth [161]. In medium samples from *L. bicolor* all analytes were detected. In *C. geophilum*, three metabolites but not propiconazole were detected and samples of *A. muscaria* did not reveal any of the compounds. Detected metabolite concentrations clearly differed between species. Therefore, taking the species sensitivity into account is relevant in effect based exposure studies. In case of *A. muscaria*, concentration levels were below the limit of detection for all metabolites and thus significantly lower than in *L. bicolor* and *C. geophilum* ($p > 0.05$). Obviously, *A. muscaria* is capable of metabolizing propiconazole faster than the other two fungi species (excluding abiotic transformation, see Section 5.5.2).

8.2.3 Effects of nanoparticles

In toxicity studies, the effects of active substances are investigated to assess their risk imposed on the environment. Laboratory based exposure studies with mostly investigating only single substance experiments, do not fully reflect environmental exposure conditions as mixtures of compounds and particulate matter are present and might lead to combined effects. Although in soil and surface waters natural and artificial nanoparticles are known to be present in significant amounts, the interaction of possible toxic substances with them is

rarely considered. Sorption processes of compounds to particles might alter their bioavailability. Nanoparticles are characterized by their size of 1 - 100 nm [112]. Due to this definition, only by size the physicochemical properties of nanoparticles are very diverse. The most important general property is the large surface of these particles, displaying a large reactive interfacial area [19]. This leads to relatively higher sorption capacities of nanoparticles compared to larger particles. Benthic living organisms are strongly exposed to both, contaminants bound to sediment and to contaminants dissolved in the water phase. Thus, stronger effects than expected from just pollutant concentrations detected in water can be anticipated [17]. Pertubating activities and filtering of sediments might release bound contaminants upon changes of sorption equilibria, and therefore, enhance the exposure concentration [17]. Especially, hydrophobic compounds sorb to sediments and an uptake by benthic invertebrates was observed although 99.99 % of the investigated hydrophobic compounds were bound to particles [18]. Uptake via ingestion of particulate matter was also observed as an important uptake route for other sediment bound contaminants [4]. Nanoparticles may serve as vectors or sinks for various organic compounds including pollutants. Accordingly, different effects of nanoparticles on the bioavailability were observed (1) enhanced bioavailability by transfer process, (2) reduced bioavailability by strong sorption, and (3) reduced transformation rates.

(1) Bioavailability of pollutants in the presence of nanoparticles

Nanoparticles can act as vectors and transport pollutants and possibly aid uptake, when they are taken up by organisms. For example filtering invertebrates ingest particles depending on their size and carbon content [116]. Aquatic insect larvae might ingest pesticides adsorbed to sediment as part of their diet [119]. By this, adsorbed pollutants might be transported into organisms. These carrier effects have been observed in experiments with diuron sorbed to carbon nanotubes and algae [20]. A reduced photosynthetic activity was observed at reduced bioavailable diuron concentrations. This was explained by locally enhanced concentrations of diuron bound to nanotubes, and higher concentrations of these nanotubes near the algae. In the chironomids mortality experiments presented in this thesis nanoparticles were present in the larvae at the end of the 96 h experiment. Nanoparticles remained in the larvae after keeping them in water without nanoparticles for 24 h. This indicates the vector properties of nanoparticles. But mortality was still reduced in comparison to experiments without nanoparticles [141-142]. This shows that the high sorption capacity of zeolite nanoparticles for thiacloprid reduced the bioavailability of the insecticide concentration.

In experiments with solely thiacloprid, only for the lowest exposure concentration of 0.03 µg/L a reduced internal concentration of 12 ± 5 ng/g was observed compared to higher exposure concentrations resulting in internal concentrations of 47 ± 7 ng/g. In experiments with nanoparticles, the detected concentration was in the same range with 36 - 53 ng/g. However, when adding nanoparticles to the exposure medium even at the lowest exposure concentration, an internal concentration of 42 ± 1 ng/g was detected. It has to be stressed, that the concentration of directly incorporated thiacloprid could not be distinguished from particle bound thiacloprid which may desorb during the extraction process with the applied method. Therefore, the higher internal thiacloprid concentration detected in the experiment at 0.03 µg/L, bioavailable thiacloprid concentration could (partly) stem from incorporated nanoparticles, which would underline the property of nanoparticles serving as a vector. Further methodological work is necessary to discriminate between the two uptake processes. The mortality decreased in experiments with zeolite nanoparticles indicating a reduced bioavailability of thiacloprid upon sorption, which emphasizes the hypothesis of vector-based uptake [141]. Similarly, a reduced toxicity by reduced bioavailability upon sorption

of carbamazepine to sediment was observed in a study with chironomids in comparison of tests with and without sediment [17].

In agar plate experiments with fungi clearly lower internal concentrations were observed in the presence of nanoparticles resulting from lower bioavailable concentrations due to sorption processes. The concentration of propiconazole and three of its metabolites was analyzed in fungi mycelium and medium. The fungi were exposed to propiconazole and nanoparticles. The influence of nanoparticles on the uptake by different fungi species and their ability to metabolize the fungicide were investigated under different growing conditions. In agar plate experiments *L. bicolor* was exposed to 100 µg/L propiconazole and different nanoparticle concentrations reducing the bioavailable propiconazole concentration. In the analyzed mycelium the propiconazole uptake clearly decreased from 7 ± 2 to 0.2 ± 0.1 µg/g wet weight with increasing nanoparticle concentrations. This shows a strong effect of sorption to nanoparticles on the bioavailability of propiconazole.

In contrast, the results from liquid culture experiments revealed a different aspect of reduced bioavailability upon sorption to nanoparticles: In liquid culture experiments *A. muscaria* was exposed to 5 mg/L propiconazole and different nanoparticle concentrations, which reduced the bioavailable propiconazole concentration. Compared to experiments without nanoparticles in these experiments higher propiconazole concentrations were detected at the end of exposure time. Furthermore, the detected propiconazole concentration increased with increasing nanoparticle concentrations. When nanoparticles were added to the liquid culture medium containing 5 mg/L fungicide, the internal concentration was up to five times higher compared to experiments without any nanoparticles. Experiments with *A. muscaria* exposed to solely propiconazole indicated a strong ability of *A. muscaria* to degrade propiconazole in liquid culture. Therefore, the higher detected concentrations at the end of exposure time in experiments with nanoparticles point towards a reduced transformation of propiconazole due to reduced bioavailability upon sorption to nanoparticles. The metabolic activity of these experiments is discussed in Section 5.5.4.

(2) Effects on uptake and transformation of contaminants in the presence of nanoparticles

Sorption of pollutants to nanoparticles may result in reduced bioavailability and this reduces the metabolism in the organisms or physical and chemical degradation in the environment. Persistence of several pollutants can be explained by sorption. For ecotoxicological studies, the reduced transformation in the environment has to taken into account as it may result in changes in equilibrium conditions and lead to chronic exposure scenarios. This effect was demonstrated for metal ions, where combined sorption and desorption processes resulted in a steady release of metal ions, leading to a chronic exposure of aquatic organisms [22]. The propiconazole concentrations detected in liquid culture experiments analyzed in this thesis indicate similar processes.

In liquid culture experiments with nanoparticles, the most abundant metabolite in all samples was metabolite BTP 4, detected at higher concentrations than propiconazole, followed by metabolite BTP 2 in about 10-fold lower amount assuming similar ionization efficiencies. All samples revealed very similar ratios in metabolic composition. Surprisingly, all analytes showed decreasing concentrations at higher nominal initial propiconazole concentrations. Metabolites BTP 2 and 3 were at the limit of detection in mycelium extracts, however, at quantifiable concentrations in exposure medium. In the previous experiment with different fungi species metabolites were detected neither in the *A. muscaria* mycelium nor in the medium. The calculated propiconazole concentration detected in mycelium was less than 10 % of the initial concentration. This indicates fast degradation of propiconazole. In the experiment with nanoparticles, propiconazole and metabolites were detected at the end of

the exposure time in medium and mycelium. Clearly, nanoparticle sorption inhibits the degradation of propiconazole by the fungus.

The propiconazole concentration during the experiment was not monitored. But it can be speculated that propiconazole desorbed from the nanoparticles at decreasing propiconazole concentrations resulting in an almost steady propiconazole exposure concentration over a long time. This would lead to relatively higher metabolite concentrations at lower exposure concentrations, if propiconazole is constantly desorbed after bioavailable propiconazole is metabolized. At higher exposure concentrations the metabolism would be faster as propiconazole is available already at the start of the experiment. The potential toxicity of the formed metabolites was not investigated in this study. As metabolites were detected at higher concentrations than the parent compound, further experiments with isolated metabolites are required to assess their toxicity.

Overall, different effects of pollutant sorption to nanoparticles were observed in the samples analyzed in this thesis. In experiments with the neonicotinoid, thiacloprid, and zeolite nanoparticles, reduced mortality was observed but the nanoparticles were still present in the gut of the larvae at the end of experiment [141]. This shows the ability of nanoparticles to be a vector for contaminants. Thus, the bioavailability of the contaminants sorbed to particles depends on the conditions in the organisms' gut. In agar plate experiments, a reduced bioavailability upon sorption to nanoparticles was observed which resulted in a significantly reduced incorporated propiconazole concentration in *L. bicolor* mycelium. In liquid culture experiment *A. muscaria* was able to degrade propiconazole during the exposure time. This ability was reduced in experiments with nanoparticles due to a reduced bioavailability of the fungicide. This reduced degradation rate resulted in higher propiconazole concentrations being present at the end of the experiment. Exposure was therefore, at lower concentrations but over a prolonged time.

These observations show that prediction of the effects of nanoparticles are challenging as they depend on (i) the analyte and nanoparticle sorption capacity as shown for midge larvae and different nanoparticle, and (ii) on the organisms metabolic activity as observed in experiments with fungi and the environmental conditions reflected by agar plate and liquid culture experiments.

8.2.4 Transfer of incorporated contaminants from aquatic to the terrestrial environment

Midge larvae and midges are an important source of prey for insectivorous predators in the aquatic and terrestrial environment [3]. Bioaccumulated contaminants in prey represent an important way of exposure for their predators [4], thus, through the foodweb. The transport of pollutants via body burden from the aquatic to the terrestrial environment after emergence by holometabolic insects with aquatic larvae and terrestrial imagines, has been investigated for trace metals [6] and polychlorinated biphenyls (PCBs) [5]. Furthermore, the transfer of PCBs from contaminated midge larvae to tree swallows has been researched in field studies [82]. Metamorphosis can affect the internal contaminant concentration of midges in two ways: by increasing or decreasing concentrations. For example, 3-fold higher PCB concentrations were detected in adult midges compared to concentrations in larvae [5]. The biomass of adult midges is approximately one third of the biomass of midges, therefore, the PCB burden changes, however, only if normalized based on biomass but not when calculated per individual. For polycyclic aromatic hydrocarbons and metals, predominantly lower concentrations (2 to 125-fold) were observed in midges compared to the one in larvae [83]. The impact of midges to work as vectors from aquatic to terrestrial ecosystems has been

investigated for different metals in *Chironomus riparius*. Trace metals among them zinc, copper and cadmium, are known to accumulate in larvae but have a different fate upon metamorphosis. In case of copper, the metal was almost completely excreted from larvae over pupae to imagines. In case of zinc, the body-burden decreased from larvae over pupae to imagines [6]. For arsenic similar results were observed in laboratory experiments. For instance, 72% of the arsenic burden was excreted between larval and adult stages [84].

In this thesis larvae and midges from exposure studies with carbamazepine were analyzed to assess the transfer of incorporated pharmaceutical burden from larvae to midges during the pupation process. The removal rate of carbamazepine in sewage treatment plants can be as low as or even below 10 % [7]. The internal concentrations were determined in midge larvae and midges originating from an emergence study with different carbamazepine concentrations, to investigate the fate of carbamazepine during the metamorphosis from *Chironomus riparius* larvae to imagines.

The results showed, that larvae accumulated considerable amounts of carbamazepine and these were transferred to midges during metamorphosis. In the exposure range of 0.025 - 3.2 mg/L the internal concentration in larvae increased from 8 ± 2 to 96 ± 30 ng/g in larvae and in midges from 19 ± 4 to 204 ± 10 ng/g. This indicates that larvae do not have efficient excretory mechanisms for carbamazepine. The adult midges emerging from exposed larvae had a significantly approximately 3-fold higher body-burden regardless of the exposure concentration. When interpreting the body-burden of larvae and midges, it has to be considered that biomass reduces by approximately 70 % during metamorphosis. An average weight of 2 mg and 6 mg were determined for midges and larvae, respectively. If all carbamazepine taken up by larvae is retained upon metamorphosis, a 3-fold bioconcentrated burden in midges is expected and was observed in the experiments. This finding is in accordance with published studies, where an increase by 3-fold has been observed in experiments with PCBs [83] as discussed above. Results indicate a transfer of 100 % body burden from larvae to midges and indicate that this route is relevant for exposure of predators, and thus incorporation into the food web. Overall, the results demonstrate a transfer of aquatic pharmaceutical trace contaminants to terrestrial ecosystems due to uptake in aquatic larvae. The results presented here, show that the transfer route from aquatic living larvae to terrestrial imagines has to be considered in case of bioaccumulating contaminants, like in this case carbamazepine which is only partly removed in wastewater treatment plants.

8.3 PRECONCENTRATION AND FRACTIONATION OF IONIC AND IONIZABLE ANALYTES BY ELECTROMIGRATIVE TECHNIQUES

8.3.1 Water analysis and concentration

The growing number of chemicals on the market and their presence in surface waters give rise to concerns about their environmental fate and behavior. The occurrence of these contaminants at trace amounts in surface waters make analysis challenging and enrichment steps necessary. Chapter 6 and 7 of this thesis focus on sample preparation techniques for ionic and ionizable analytes. The detection of ionic and highly polar compounds is of special interest as these often include metabolites or transformation products, which are mostly more polar than their parent compounds [1]. In this thesis, the applicability of two approaches was investigated to fractionate (Chapter 6) and preconcentrate (Chapter 7) ionic and ionizable compounds by electromigrative techniques. The methods investigated can be

considered environmentally friendly as only simple aqueous electrolytes are required. The idea is to transfer solely charged compounds from the sample to an injection solution. Therefore, neutral and oppositely charged matrix components at given pH including sample solvents and interfering matrix components are eliminated simultaneously. These water samples preparation steps are expected to enhance the reliability of ionic and highly polar contaminant quantification as classical RPLC-MS experiences strong quenching effects with compounds elution close to the void volume.

8.3.2 Water analysis

In order to analyze several contaminants relevant in wastewater effluents a multi-component method comprising 92 substances was developed, with identification based on the criteria retention time and exact mass. With the developed method identification and quantification of analytes in the low µg/L was possible by HPLC-MS. For further information see Chapter 6.

8.3.3 Investigation of effects on the electromigrative behavior of charged analytes

The electromigrative enrichment experiments were intended to investigate the applicability of long term electrokinetic injection on preconcentration of ionizable analytes from water samples. To achieve the most suitable conditions, the influence of pH value, electrode geometry, temperature and stirring on the migration behavior were investigated.

The process of analyte transfer was observed to be largely time-independent and maximal transfer was achieved already within 2 hours. This finding corroborates simulations from Karim et al. for electrokinetic injection into capillaries [26]. However, after 22 hours of voltage application, on total less than 63 % of the analytes were recovered and different approaches were made to investigate the limiting processes for the ion transfer. Three hypothesis were tested with different experimental setups to enhance analyte transfer: electrode geometry, temperature and convection. In literature it was shown, that only analytes present in the effective electric field may be introduced into the capillary [26]. Therefore, different electrode geometries (straight and helical) were used to extend the effective electric field. If the injection is limited by diffusion processes heating and stirring would accelerate the diffusion process leading to a higher analyte transfer. Thus, the influence of these conditions was also investigated.

In each experimental setup, the highest concentration of analyte was detected in the starting vessel, although they were significantly lower than the initial concentration. Electrochemical transformation, adsorption phenomena and hydrolysis are possible processes to explain the reduced overall analyte concentrations in starting and accepting vessels as well as in the capillary. The impact of electrode geometry was lower than stirring and heating. Elevated temperature combined with a straight electrode resulted in significant analyte concentrations in the capillary inner volume, whereas combination with a helical electrode resulted in less than 10 % analyte recovery. In experiments where the starting vessel was stirred, the recovery was drastically reduced independently from other experimental parameters. Similar observations were reported by Hirokawa et al. for electrokinetic injection [26]. The results indicate that the effect of the helical electrode on the effective electric field and of convection on the diffusion process, led to enhanced degradation processes or conditions leading to degradation, e.g. hydrolysis. The analysis of transformation products revealed the presence of one metabolite of metformin and of two transformation products of terbutryn. With the optimized conditions (elevated temperature of 50 °C, a straight electrode and no convection in the starting vessel) enrichment factors of 1.3 in case of terbutryn, 1.6 for naphazoline and 3.6 in case of metformin were achieved. The developed approach demonstrates the ability of

electromigrative techniques to enrich ionic compounds. To overcome degradation processes of analytes, further work has to focus on analyte stability and a more complete transfer of analytes of interest.

8.3.4 Electromigrative fractionation and preconcentration techniques

Free flow electrophoresis is a continuous preparative isolation and purification technique based on electromigrative processes, which is commonly applied to fractionate large biomolecules [25]. In this thesis, to my knowledge this technique was applied the first time to fractionate micro-pollutants in surface water samples downstream to wastewater treatment plants. Fractionation by free flow electrophoresis rises the possibility to obtain fractions compatible with appropriate further separation techniques such as liquid chromatography or capillary electrophoresis. The combination of both techniques brings together the high separation capacity of ionic compounds by electromigrative separation with the high sensitivity and further selectivity of HPLC-MS analysis. Combining these two orthogonal separation techniques (electrophoretic fractionation based on the electrophoretic mobility of analytes, and liquid chromatography, based on the polarity of analytes) would enhance the overall capacity and selectivity of analysis. To combine these advantages and investigate the suitability of this technique wastewater samples were fractionated with free flow isotachophoresis and free flow interval zone electrophoresis and analyzed for contaminants by HPLC-MS. The advantages of fractionation of water samples by free flow electrophoresis would be the simultaneous concentration of analytes in smaller fraction volumes, the reduction of sample complexity, and the possibility to work in a continuous mode. Analytes are separated according to their electrophoretic mobility (FFITP) or isoelectric point (FFIZE pH) in up to 96 fractions. The fractions were further analyzed by HPLC-ESI-QTOF-MS via screening for 92 micropollutants.

In the ITP system fractionation was carried out with Cl^- and OH^- at pH 9.3 as leader and terminator, resulting in one of the broadest mobility windows possible to give the broadest coverage of micropollutants being anions at this pH. The results showed that the experimental time of 7 min, which was the longest separation time available at the instrumentation, was not enough to complete the separation processes. However, higher peak areas of compounds in fractions compared to raw water samples observed in base peak chromatograms indicate the potential of the methodology for preconcentration of ionic analytes. A disadvantage of ITP is that the separation depends on the number and concentration of compounds present in the sample. To overcome this limitation, FFIZE-pH was applied for fractionation.

In the experiment with FFIZE-pH a pH gradient of pH 5 - 9 was implemented. Amphoteric analytes were expected to migrate until reaching a zone with a pH at their point while, other analytes would migrate until reaching a zone where neutralization occurs. Due to the limited number of analytes comprised in the multi-component method, only two compounds which are amphoteric in the applied pH range were identified. These were either detected in the raw water sample (gabapentin) or in every sample (phenylalanine). To fully judge the applicability of this system for fractionation of wastewater contaminants, further experiments are required using standards with a broad distribution of pK_a values and electrophoretic mobilities. However, the preliminary results indicate the ability of FFE to fractionate ionic and ionizable wastewater contaminants.

8.4 CONCLUSION

In this thesis three extraction protocols were developed based on a modified QuEChERS extraction in order to determine pesticide and pharmaceutical residues in biota samples. The extraction procedures were optimized with regard to homogenization and cleanup of extracts by different sorbents if required by matrix and analytes. The optimized methods enabled to extract the contaminants and in case of propiconazole the simultaneous extraction of three of its metabolites. Limits of quantification by HPLC-MS in extracts from approximately 20 mg tissue were achieved in the low ng/g wet or dry weight range. The developed methods show the suitability of the extraction procedure coupled to sensitive detection methods for quantification of trace levels of organic contaminants and their metabolites in small amounts of biota.

The optimized methods were then applied to biota samples from exposure studies with the purpose to assess the ecotoxicological relevance of contaminants regarding different aspects of their environmental behavior: the bioavailability of contaminants in the presence of nanoparticles, the possibility of a transfer of pollutants during metamorphosis from midge larvae to midges and the metabolic transformation of analytes. The method was shown to be suitable to quantify carbamazepine and thiacloprid residues in midge larvae and midges. The complete transfer of incorporated carbamazepine from larvae to midges was observed. In experiments with nanoparticles reduced uptake from agar plates due to sorption of propiconazole to nanoparticles and a reduced metabolic transformation rate in liquid culture experiments with fungi were observed. These results cover different aspects of the nvironmental fate of contaminants depending on their physicochemical properties and thus give rise to a more complete understanding of the interplay of pollutants and particulate matter with regard to ecotoxicology.

The investigation of electromigrative techniques was intended to preconcentrate ionic analytes, reduce wastewater sample complexity for further analysis by HPLC-MS and enhance overall selectivity and capacity. The results indicated that electromigrative methodologies are a suitable alternative for preconcentration and fractionation of ionic or ionizable micro-pollutants. The work thus addresses those highly polar and charged micropollutants difficult to analyze with classical techniques.

REFERENCES

1. Sono, M.; Roach, M. P.; Coulter, E. D.; Dawson, J. H., Heme-containing oxygenases. *Chemical Reviews* **1996,** *96* (7), 2841-2887.

2. Michelangelo Anastassiades, S. J. L., Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *Journal of AOAC International* **2003,** *86* (2), 412-431.

3. Azevedo-Pereira, H. M. V. S.; Lemos, M. F. L.; Soares, A. M. V. M., Effects of imidacloprid exposure on Chironomus riparius meigen larvae: linking acetylcholinesterase activity to behaviour. *Ecotoxicology and Environmental Safety* **2011,** *74* (5), 1210-1215.

4. Croce, V.; De Angelis, S.; Patrolecco, L.; Polesello, S.; Valsecchi, S., Uptake and accumulation of sediment-associated 4-nonylphenol in a benthic invertebrate (Lumbriculus variegatus, freshwater oligochaete). *Environmental Toxicology and Chemistry* **2005,** *24* (5), 1165-1171.

5. Larsson, P., Transport of C-14-labeled PCB compounds from sediment to water and from water to air in laboratory model systems. *Water Research* **1983,** *17* (10), 1317-1326.

6. Timmermans, K. R.; Walker, P. A., The fate of trace-metals during the metamorphosis of chironomids (diptera, chironomidae). *Environmental Pollution* **1989,** *62* (1), 73-85.

7. Heberer, T., Tracking persistent pharmaceutical residues from municipal sewage to drinking water. *Journal of Hydrology* **2002,** *266* (3-4), 175-189.

8. Dussault, E. B.; Balakrishnan, V. K.; Sverko, E.; Solomon, K. R.; Sibley, P. K., Toxicity of human pharmaceuticals and personal care products to benthic invertebrates. *Environmental Toxicology and Chemistry* **2008,** *27* (2), 425-432.

9. Oetken, M.; Nentwig, G.; Löffler, D.; Ternes, T.; Oehlmann, J., Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug carbamazepine. *Archives of Environmental Contamination and Toxicology* **2005,** *49* (3), 353-361.

10. Maenpaa, K.; Kukkonen, J. V. K., Bioaccumulation and toxicity of 4-nonylphenol (4-NP) and 4-(2-dodecyl)-benzene sulfonate (LAS) in Lumbriculus variegatus (oligochaeta) and Chironomus riparius (Insecta). *Aquatic Toxicology* **2006,** *77* (3), 329-338.

11. Escher, B. I.; Hermens, J. L. M., Peer reviewed: internal exposure: linking bioavailability to effects. *Environmental Science & Technology* **2004,** *38* (23), 455A-462A.

12. Simon-Delso, N.; Amaral-Rogers, V.; Belzunces, L. P.; Bonmatin, J. M.; Chagnon, M.; Downs, C.; Furlan, L.; Gibbons, D. W.; Giorio, C.; Girolami, V.; Goulson, D.; Kreutzweiser, D. P.; Krupke, C. H.; Liess, M.; Long, E.; McField, M.; Mineau, P.; Mitchell, E. A. D.; Morrissey, C. A.; Noome, D. A.; Pisa, L.; Settele, J.; Stark, J. D.; Tapparo, A.; Van Dyck, H.; Van Praagh, J.; Van der Sluijs, J. P.; Whitehorn, P. R.; Wiemers, M., Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental Science and Pollution Research* **2015,** *22* (1), 5-34.

13. Martin, F.; Aerts, A.; Ahrén, D.; Brun, A.; Danchin, E. G. J.; Duchaussoy, F.; Gibon, J.; Kohler, A.; Lindquist, E.; Pereda, V.; Salamov, A.; Shapiro, H. J.; Wuyts, J.; Blaudez, D.; Buée, M.; Brokstein,

P.; Canbäck, B.; Cohen, D.; Courty, P. E.; Coutinho, P. M.; Delaruelle, C.; Detter, J. C.; Deveau, A.; DiFazio, S.; Duplessis, S.; Fraissinet-Tachet, L.; Lucic, E.; Frey-Klett, P.; Fourrey, C.; Feussner, I.; Gay, G.; Grimwood, J.; Hoegger, P. J.; Jain, P.; Kilaru, S.; Labbé, J.; Lin, Y. C.; Legué, V.; Le Tacon, F.; Marmeisse, R.; Melayah, D.; Montanini, B.; Muratet, M.; Nehls, U.; Niculita-Hirzel, H.; Secq, M. P. O.-L.; Peter, M.; Quesneville, H.; Rajashekar, B.; Reich, M.; Rouhier, N.; Schmutz, J.; Yin, T.; Chalot, M.; Henrissat, B.; Kües, U.; Lucas, S.; Van de Peer, Y.; Podila, G. K.; Polle, A.; Pukkila, P. J.; Richardson, P. M.; Rouzé, P.; Sanders, I. R.; Stajich, J. E.; Tunlid, A.; Tuskan, G.; Grigoriev, I. V., The genome of Laccaria bicolor provides insights into mycorrhizal symbiosis. *Nature* **2008,** *452*, 88.

14. Meharg, A. A.; Cairney, J. W. G., Ectomycorrhizas - extending the capabilities of rhizosphere remediation? *Soil Biology & Biochemistry* **2000,** *32* (11-12), 1475-1484.

15. Weete, J. D., Wise, M. L., Effects of triazoles on fungi. *Experimental Mycology* **1987,** *11*, 2014-222.

16. Urzua, J.; Gonzalez-Vargas, C.; Sepulveda, F.; Ureta-Zanartu, M. S.; Salazar, R., Degradation of conazole fungicides in water by electrochemical oxidation. *Chemosphere* **2013,** *93* (11), 2774-2781.

17. Gilroy, E. A. M.; Balakrishnan, V. K.; Solomon, K. R.; Sverko, E.; Sibley, P. K., Behaviour of pharmaceuticals in spiked lake sediments - Effects and interactions with benthic invertebrates. *Chemosphere* **2012,** *86* (6), 578-584.

18. Gaskell, P. N.; Brooks, A. C.; Maltby, L., Variation in the bioaccumulation of a sediment-sorbed hydrophobic compound by benthic macroinvertebrates: Patterns and mechanisms. *Environmental Science & Technology* **2007,** *41* (5), 1783-1789.

19. Navarro, E.; Baun, A.; Behra, R.; Hartmann, N. B.; Filser, J.; Miao, A. J.; Quigg, A.; Santschi, P. H.; Sigg, L., Environmental behavior and ecotoxicity of engineered nanoparticles to algae, plants, and fungi. *Ecotoxicology* **2008,** *17* (5), 372-386.

20. Schwab, F.; Bucheli, T. D.; Camenzuli, L.; Magrez, A.; Knauer, K.; Sigg, L.; Nowack, B., Diuron sorbed to carbon nanotubes exhibits enhanced toxicity to Chlorella vulgaris. *Environmental Science & Technology* **2013,** *47* (13), 7012-7019.

21. Berlioz-Barbier, A.; Bulete, A.; Fabure, J.; Garric, J.; Cren-Olive, C.; Vulliet, E., Multi-residue analysis of emerging pollutants in benthic invertebrates by modified micro-quick-easy-cheap-efficient-rugged-safe extraction and nanoliquid chromatography-nanospray-tandem mass spectrometry analysis. *Journal of Chromatography A* **2014,** *1367*, 16-32.

22. Schultz, C.; Powell, K.; Crossley, A.; Jurkschat, K.; Kille, P.; Morgan, A. J.; Read, D.; Tyne, W.; Lahive, E.; Svendsen, C.; Spurgeon, D. J., Analytical approaches to support current understanding of exposure, uptake and distributions of engineered nanoparticles by aquatic and terrestrial organisms. *Ecotoxicology* **2015,** *24* (2), 239-261.

23. Cotton, J.; Leroux, F.; Broudin, S.; Poirel, M.; Corman, B.; Junot, C.; Ducruix, C., Development and validation of a multiresidue method for the analysis of more than 500 pesticides and drugs in water based on on-line and liquid chromatography coupled to high resolution mass spectrometry. *Water Research* **2016,** *104*, 20-27.

24. Bieber, S.; Greco, G.; Grosse, S.; Letzel, T., RPLC-HILIC and SFC with Mass Spectrometry: Polarity-Extended Organic Molecule Screening in Environmental (Water) Samples. *Analytical Chemistry* **2017,** *89* (15), 7907-7914.

25. Weber, G.; Bocek, P., Optimized continuous flow electrophoresis. *Electrophoresis* **1996,** *17* (12), 1906-1910.

26. Hirokawa, T.; Koshimidzu, E.; Xu, Z., Electrokinetic sample injection for high-sensitivity capillary zone electrophoresis (part 1): Effects of electrode configuration and setting. *Electrophoresis* **2008,** *29* (18), 3786-3793.

27. Bruzzoniti, M. C.; Checchini, L.; De Carlo, R. M.; Orlandini, S.; Rivoira, L.; Del Bubba, M., QuEChERS sample preparation for the determination of pesticides and other organic residues in environmental matrices: a critical review. *Analytical and Bioanalytical Chemistry* **2014,** *406* (17), 4089-4116.

28. Wang, D. G.; de Solla, S. R.; Lebeuf, M.; Bisbicos, T.; Barrett, G. C.; Alaee, M., Determination of linear and cyclic volatile methylsiloxanes in blood of turtles, cormorants, and seals from Canada. *Science of the Total Environment* **2017,** *574*, 1254-1260.

29. Fidalgo-Used, N.; Blanco-Gonzalez, E.; Sanz-Medel, A., Sample handling strategies for the determination of persistent trace organic contaminants from biota samples. *Analytica Chimica Acta* **2007,** *590* (1), 1-16.

30. Huerta, B.; Jakimska, A.; Gros, M.; Rodriguez-Mozaz, S.; Barcelo, D., Analysis of multi-class pharmaceuticals in fish tissues by ultra-high-performance liquid chromatography tandem mass spectrometry. *Journal of Chromatography A* **2013,** *1288*, 63-72.

31. Bueno, M. J. M.; Boillot, C.; Fenet, H.; Chiron, S.; Casellas, C.; Gomez, E., Fast and easy extraction combined with high resolution-mass spectrometry for residue analysis of two anticonvulsants and their transformation products in marine mussels. *Journal of Chromatography A* **2013,** *1305*, 27-34.

32. Miller, T. H.; McEneff, G. L.; Brown, R. J.; Owen, S. F.; Bury, N. R.; Barron, L. P., Pharmaceuticals in the freshwater invertebrate, Gammarus pulex, determined using pulverised liquid extraction, solid phase extraction and liquid chromatography-tandem mass spectrometry. *Science of the Total Environment* **2015,** *511*, 153-160.

33. Andreu, V.; Pico, Y., Determination of currently used pesticides in biota. *Analytical and Bioanalytical Chemistry* **2012,** *404* (9), 2659-2681.

34. Masia, A.; Vasquez, K.; Campo, J.; Pico, Y., Assessment of two extraction methods to determine pesticides in soils, sediments and sludges. Application to the Turia River Basin. *Journal of Chromatography A* **2015,** *1378*, 19-31.

35. Kiljanek, T.; Niewiadowska, A.; Semeniuk, S.; Gawel, M.; Borzecka, M.; Posyniak, A., Multi-residue method for the determination of pesticides and pesticide metabolites in honeybees by liquid and gas chromatography coupled with tandem mass spectrometry-Honeybee poisoning incidents. *Journal of Chromatography A* **2016,** *1435*, 100-114.

36. Sapozhnikova, Y.; Lehotay, S. J., Evaluation of different parameters in the extraction of incurred pesticides and environmental contaminants in fish. *Journal of Agricultural and Food Chemistry* **2015,** *63* (21), 5163-5168.

37. Walorczyk, S.; Gnusowski, B., Development and validation of a multi-residue method for the determination of pesticides in honeybees using acetonitrile-based extraction and gas

chromatography-tandem quadrupole mass spectrometry. *Journal of chromatography. A* **2009,** *1216* (37), 6522-31.

38. Mullin, C. A.; Frazier, M.; Frazier, J. L.; Ashcraft, S.; Simonds, R.; vanEngelsdorp, D.; Pettis, J. S., High levels of miticides and agrochemicals in north american apiaries: implications for honey bee health. *PLOS ONE* **2010,** *5* (3), e9754.

39. Paya, P.; Anastassiades, M.; Mack, D.; Sigalova, I.; Tasdelen, B.; Oliva, J.; Barba, A., Analysis of pesticide residues using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) pesticide multiresidue method in combination with gas and liquid chromatography and tandem mass spectrometric detection. *Analytical and Bioanalytical Chemistry* **2007,** *389* (6), 1697-1714.

40. Inostroza, P. A.; Wicht, A.-J.; Huber, T.; Nagy, C.; Brack, W.; Krauss, M., Body burden of pesticides and wastewater-derived pollutants on freshwater invertebrates: Method development and application in the Danube River. *Environmental pollution* **2016,** *214*, 77-85.

41. Kurth, D.; Krauss, M.; Schulze, T.; Brack, W., Measuring the internal concentration of volatile organic compounds in small organisms using micro-QuEChERS coupled to LVI-GC-MS/MS. *Analytical and Bioanalytical Chemistry* **2017,** *409* (25), 6041-6052.

42. He, Z. Y.; Wang, L.; Peng, Y.; Luo, M.; Wang, W. W.; Liu, X. W., Determination of selected polychlorinated biphenyls in soil and earthworm (Eisenia fetida) using a QuEChERS-based method and gas chromatography with tandem MS. *Journal of Separation Science* **2015,** *38* (21), 3766-3773.

43. Jabot, C.; Daniele, G.; Giroud, B.; Tchamitchian, S.; Belzunces, L. P.; Casabianca, H.; Vulliet, E., Detection and quantification of boscalid and its metabolites in honeybees. *Chemosphere* **2016,** *156*, 245-251.

44. Al Naggar, Y.; Codling, G.; Vogt, A.; Naiem, E.; Mona, M.; Seif, A.; Giesy, J. P., Organophosphorus insecticides in honey, pollen and bees (Apis mellifera L.) and their potential hazard to bee colonies in Egypt. *Ecotoxicology and Environmental Safety* **2015,** *114*, 1-8.

45. Daniele, G.; Fieu, M.; Joachim, S.; James-Casas, A.; Andres, S.; Baudoin, P.; Bonnard, M.; Bonnard, I.; Geffard, A.; Vulliet, E., Development of a multi-residue analysis of diclofenac and some transformation products in bivalves using QuEChERS extraction and liquid chromatography-tandem mass spectrometry. Application to samples from mesocosm studies. *Talanta* **2016,** *155*, 1-7.

46. Berge, A.; Vulliet, E., Development of a method for the analysis of hormones and pharmaceuticals in earthworms by quick, easy, cheap, effective, rugged and safe (QuEChERS) extraction followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). *Analytical and Bioanalytical Chemistry* **2015,** *407* (26), 7995-8008.

47. Berlioz-Barbier, A.; Baudot, R.; Wiest, L.; Gust, M.; Garric, J.; Cren-Olive, C.; Bulete, A., MicroQuEChERS-nanoliquid chromatography-nanospray-tandem mass spectrometry for the detection and quantification of trace pharmaceuticals in benthic invertebrates. *Talanta* **2015,** *132*, 796-802.

48. Daniele, G.; Fieu, M.; Joachim, S.; Bado-Nilles, A.; Baudoin, P.; Turies, C.; Porcher, J. M.; Andres, S.; Vulliet, E., Rapid analysis of diclofenac and some of its transformation products in the three-spined stickleback, Gasterosteus aculeatus, by liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry* **2016,** *408* (16), 4435-4444.

49. Groz, M. P.; Bueno, M. J. M.; Rosain, D.; Fenet, H.; Casellas, C.; Pereira, C.; Maria, V.; Bebianno, M. J.; Gomez, E., Detection of emerging contaminants (UV filters, UV stabilizers and musks) in marine mussels from Portuguese coast by QuEChERS extraction and GC-MS/MS. *Science of the Total Environment* **2014,** *493*, 162-169.

50. Barganska, Z.; Slebioda, M.; Namiesnik, J., Determination of pesticide residues in honeybees using modified QUEChERS sample work-up and liquid chromatography-tandem mass spectrometry. *Molecules* **2014,** *19* (3), 2911-2924.

51. Wiest, L.; Bulete, A.; Giroud, B.; Fratta, C.; Amic, S.; Lambert, O.; Pouliquen, H.; Arnaudguilhem, C., Multi-residue analysis of 80 environmental contaminants in honeys, honeybees and pollens by one extraction procedure followed by liquid and gas chromatography coupled with mass spectrometric detection. *Journal of Chromatography A* **2011,** *1218* (34), 5743-5756.

52. Lazartigues, A.; Fratta, C.; Baudot, R.; Wiest, L.; Feidt, C.; Thomas, M.; Cren-Olive, C., Multiresidue method for the determination of 13 pesticides in three environmental matrices: water, sediments and fish muscle. *Talanta* **2011,** *85* (3), 1500-1507.

53. David, A.; Botias, C.; Abdul-Sada, A.; Goulson, D.; Hill, E. M., Sensitive determination of mixtures of neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down QuEChERS method for exposure assessment. *Analytical and Bioanalytical Chemistry* **2015,** *407* (26), 8151-8162.

54. Cunha, S. C.; Fernandes, J. O.; Vallecillos, L.; Cano-Sancho, G.; Domingo, J. L.; Pocurull, E.; Borrull, F.; Maulvault, A. L.; Ferrari, F.; Fernandez-Tejedor, M.; Van den Heuvel, F.; Kotterman, M., Co-occurrence of musk fragrances and UV-filters in seafood and macroalgae collected in European hotspots. *Environmental Research* **2015,** *143*, 65-71.

55. Sordet, M.; Berlioz-Barbier, A.; Bulete, A.; Garric, J.; Vulliet, E., Quantification of of emerging micropollutants in an amphipod crustacean by nanoliquid chromatography coupled to mass spectrometry using multiple reaction monitoring cubed mode. *Journal of Chromatography A* **2016,** *1456*, 217-225.

56. Belenguer, V.; Martinez-Capel, F.; Masia, A.; Pico, Y., Patterns of presence and concentration of pesticides in fish and waters of the Jucar River (Eastern Spain). *Journal of Hazardous Materials* **2014,** *265*, 271-279.

57. Calatayud-Vernich, P.; Calatayud, F.; Simo, E.; Pico, Y., Efficiency of QuEChERS approach for determining 52 pesticide residues in honey and honey bees. *Methodsx* **2016,** *3*, 452-458.

58. Kretschmann, A.; Cedergreen, N.; Christensen, J. H., Measuring internal azole and pyrethroid pesticide concentrations in Daphnia magna using QuEChERS and GC-ECD-method development with a focus on matrix effects. *Analytical and Bioanalytical Chemistry* **2016,** *408* (4), 1055-1066.

59. Cunha, S. C.; Pena, A.; Fernandes, J. O., Mussels as bioindicators of diclofenac contamination in coastal environments. *Environmental Pollution* **2017,** *225*, 354-360.

60. Stöckelhuber, M.; Müller, C.; Vetter, F.; Mingo, V.; Lötters, S.; Wagner, N.; Bracher, F., Determination of pesticides adsorbed on arthropods and gastropods by a micro-QuEChERS approach and GC-MS/MS. *Chromatographia* **2017,** *80* (5), 825-829.

61. Madureira, T. V.; Velhote, S.; Santos, C.; Cruzeiro, C.; Rocha, E.; Rocha, M. J., A step forward using QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) based extraction and gas

chromatography-tandem mass spectrometry-levels of priority polycyclic aromatic hydrocarbons in wild and commercial mussels. *Environmental Science and Pollution Research* **2014,** *21* (9), 6089-6098.

62. Taliansky-Chamudis, A.; Gomez-Ramirez, P.; Leon-Ortega, M.; Garcia-Fernandez, A. J., Validation of a QuECheRS method for analysis of neonicotinoids in small volumes of blood and assessment of exposure in Eurasian eagle owl (Bubo bubo) nestlings. *Science of the Total Environment* **2017,** *595*, 93-100.

63. Haroune, L.; Cassoulet, R.; Lafontaine, M. P.; Belisle, M.; Garant, D.; Pelletier, F.; Cabana, H.; Bellenger, J. P., Liquid chromatography-tandem mass spectrometry determination for multiclass pesticides from insect samples by microwave-assisted solvent extraction followed by a salt-out effect and micro-dispersion purification. *Analytica Chimica Acta* **2015,** *891*, 160-170.

64. Yang, M.; Ma, Y. N.; Gui, W. J.; Ren, Y. P.; Zhu, G. N.; Liu, Y. H., Determination of 26 endocrine disrupting chemicals in fish and water using modified QuEChERS combined with solid-phase extraction and UHPLC-MS/MS. *Analytical Methods* **2015,** *7* (19), 8418-8431.

65. Dussault, E. B.; Balakrishnan, V. K.; Solomon, K. R.; Sibley, P. K., Matrix effects on mass spectrometric determinations of four pharmaceuticals and personal care products in water, sediments, and biota. *Canadian Journal of Chemistry-Revue Canadienne De Chimie* **2009,** *87* (5), 662-672.

66. Van De Steene, J. C.; Mortier, K. A.; Lambert, W. E., Tackling matrix effects during development of a liquid chromatographic-electrospray ionisation tandem mass spectrometric analysis of nine basic pharmaceuticals in aqueous environmental samples. *Journal of Chromatography A* **2006,** *1123* (1), 71-81.

67. Stahnke, H.; Kittlaus, S.; Kempe, G.; Alder, L., Reduction of matrix effects in liquid chromatography-electrospray ionization-mass spectrometry by dilution of the sample extracts: how much dilution is needed? *Analytical Chemistry* **2012,** *84* (3), 1474-1482.

68. Rajski, L.; Lozano, A.; Ucles, A.; Ferrer, C.; Fernandez-Alba, A. R., Determination of pesticide residues in high oil vegetal commodities by using various multi-residue methods and clean-ups followed by liquid chromatography tandem mass spectrometry. *Journal of Chromatography A* **2013,** *1304*, 109-120.

69. Lopez-Fernandez, O.; Rial-Otero, R.; Simal-Gandara, J., High-throughput HPLC-MS/MS determination of the persistence of neonicotinoid insecticide residues of regulatory interest in dietary bee pollen. *Analytical and Bioanalytical Chemistry* **2015,** *407* (23), 7101-7110.

70. Lehotay, S.; O. Neil, M.; Tully, J.; Valverde, A.; Contreras, M.; Mol, H.; Heinke, V.; Anspach, T.; Lach, G.; Fussell, R.; Mastovska, K.; Poulsen, M.; Brown, A.; Hamamck, W.; Cook, J.; Lader, L.; Lindtner, K.; Gamon Vila, M.; Hopper, M.; Parker, A., Determination of Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate: Collaborative Study. **2007,** *90*, 485-520.

71. DIN EN 15662:2009-02 Foods of plant origin - Multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partiotioning and clean-up by dispersive SPE - Modular QuEChERS method. **2009**.

72. EFSA, Evaluation of the data on clothianidin, imidacloprid and thiamethoxam for the updated risk assessment to bees for seed treatments and granules in the EU. *EFSA Supporting Publications* **2018,** *15* (2).

73. Lazartigues, A.; Wiest, L.; Baudot, R.; Thomas, M.; Feidt, C.; Cren-Olive, C., Multiresidue method to quantify pesticides in fish muscle by QuEChERS-based extraction and LC-MS/MS. *Analytical and Bioanalytical Chemistry* **2011,** *400* (7), 2185-2193.

74. Meyer, W.; Reich, M.; Beier, S.; Behrendt, J.; Gulyas, H.; Otterpohl, R., Measured and predicted environmental concentrations of carbamazepine, diclofenac, and metoprolol in small and medium rivers in northern Germany. *Environmental Monitoring and Assessment* **2016,** *188* (8), 487.

75. Berge, A.; Giroud, B.; Wiest, L.; Domenjoud, B.; Gonzalez-Ospina, A.; Vulliet, E., Development of a multiple-class analytical method based on the use of synthetic matrices for the simultaneous determination of commonly used commercial surfactants in wastewater by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **2016,** *1450*, 64-75.

76. Anastassiades, M.; Kolberg, D.; Benkenstein, A.; Zechmann, S.; Mack, D.; Barth, A.; Wildgrube, C.; Dörk, D., Quick method for the analysis of residues of numerous highly polar pesticides in food commodities involving simultaneous extraction with methanol and determination via LC-MS/MS (QuPPe-AO-Method) II. food of animal origin. *EU Reference Laboratories for Residues of Pesticides* **2016**.

77. Kolberg, D. I. S.; Mack, D.; Anastassiades, M.; Hetmanski, M. T.; Fussell, R. J.; Meijer, T.; Mol, H. G. J., Development and independent laboratory validation of a simple method for the determination of paraquat and diquat in potato, cereals and pulses. *Analytical and Bioanalytical Chemistry* **2012,** *404* (8), 2465-2474.

78. OECD, Test No. 218: sediment-water chironomid toxicity using spiked sediment.

79. OECD, Test No. 219: sediment-water chironomid toxicity using spiked water.

80. OECD, Test No. 233: sediment-water chironomid life-cycle toxicity test using spiked water or spiked sediment.

81. OECD, Test No. 235: chironomus sp., acute immobilisation test.

82. Maul, J. D.; Belden, J. B.; Schwab, B. A.; Whiles, M. R.; Spears, B.; Farris, J. L.; Lydy, M. J., Bioaccumulation and trophic transfer of polychlorinated biphenyls by aquatic and terrestrial insects to tree swallows (Tachycineta bicolor). *Environmental Toxicology and Chemistry* **2006,** *25* (4), 1017-1025.

83. Kraus, J. M.; Walters, D. M.; Wesner, J. S.; Stricker, C. A.; Schmidt, T. S.; Zuellig, R. E., Metamorphosis alters contaminants and chemical tracers in insects: implications for food webs. *Environmental Science & Technology* **2014,** *48* (18), 10957-10965.

84. Mogren, C. L.; von Kiparski, G. R.; Parker, D. R.; Trumble, J. T., Survival, reproduction, and arsenic body burdens in Chironomus riparius exposed to arsenate and phosphate. *Science of the Total Environment* **2012,** *425*, 60-65.

85. Rodina, T. A.; Mel'nikov, E. S.; Sokolov, A. V.; Prokof'ev, A. B.; Arkhipov, V. V.; Aksenov, A. A.; Pozdnyakov, D. L., Rapid HPLC-MS/MS determination of carbamazepine and carbamazepine-10,11-epoxide. *Pharmaceutical Chemistry Journal* **2016,** *50* (6), 419-423.

86. Zhang, Y. J.; Geissen, S. U.; Gal, C., Carbamazepine and diclofenac: Removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere* **2008,** *73* (8), 1151-1161.

87. Garcia, S. N.; Foster, M.; Constantine, L. A.; Huggett, D. B., Field and laboratory fish tissue accumulation of the anti-convulsant drug carbamazepine. *Ecotoxicology and Environmental Safety* **2012,** *84* (Supplement C), 207-211.

88. Bernus, I.; Dickinson, R. G.; Hooper, W. D.; Eadie, M. J., Dose-dependent metabolism of carbamazepine in humans. *Epilepsy Research* **1996,** *24* (3), 163-172.

89. Kot-Wasik, A.; Jakimska, A.; Sliwka-Kaszynska, M., Occurrence and seasonal variations of 25 pharmaceutical residues in wastewater and drinking water treatment plants. *Environmental Monitoring and Assessment* **2016,** *188* (12), 661.

90. Jarvis, A. L.; Bernot, M. J.; Bernot, R. J., The effects of the psychiatric drug carbamazepine on freshwater invertebrate communities and ecosystem dynamics. *Science of the Total Environment* **2014,** *496*, 461-470.

91. Jarvis, A. L.; Bernot, M. J.; Bernot, R. J., Relationships between the psychiatric drug carbamazepine and freshwater macroinvertebrate community structure. *Science of the Total Environment* **2014,** *496*, 499-509.

92. Meredith-Williams, M.; Carter, L. J.; Fussell, R.; Raffaelli, D.; Ashauer, R.; Boxall, A. B. A., Uptake and depuration of pharmaceuticals in aquatic invertebrates. *Environmental Pollution* **2012,** *165*, 250-258.

93. Heye, K.; Becker, D.; Lütke Eversloh, C.; Durmaz, V.; Ternes, T. A.; Oetken, M.; Oehlmann, J., Effects of carbamazepine and two of its metabolites on the non-biting midge Chironomus riparius in a sediment full life cycle toxicity test. *Water Research* **2016,** *98*, 19-27.

94. Calza, P.; Medana, C.; Padovano, E.; Giancotti, V.; Baiocchi, C., Identification of the unknown transformation products derived from clarithromycin and carbamazepine using liquid chromatography/high-resolution mass spectrometry. *Rapid Communications in Mass Spectrometry* **2012,** *26* (15), 1687-1704.

95. Harkey, G. A.; Landrum, P. F.; Klaine, S. J., Preliminary studies on the effect of feeding during whole sediment bioassays using chironomus-riparius larvae. *Chemosphere* **1994,** *28* (3), 597-606.

96. Hwang, H.; Fisher, S. W.; Landrum, P. F., Identifying body residues of HCBP associated with 10-d mortality and partial life cycle effects in the midge, Chironomus riparius. *Aquatic Toxicology* **2001,** *52* (3-4), 251-267.

97. Miller, T. H.; McEneff, G. L.; Stott, L. C.; Owen, S. F.; Bury, N. R.; Barron, L. P., Assessing the reliability of uptake and elimination kinetics modelling approaches for estimating bioconcentration factors in the freshwater invertebrate, Gammarus pulex. *Science of the Total Environment* **2016,** *547*, 396-404.

98. Hendriks, A. J.; van der Linde, A.; Cornelissen, G.; Sijm, D. T., The power of size. 1. Rate constants and equilibrium ratios for accumulation of organic substances related to octanol-water partition ratio and species weight. *Environmental Toxicology and Chemistry* **2001,** *20* (7), 1399-420.

99. Hillebrand, T.; Tettenborn, F.; Fuchs, S.; Toshovski, S.; Metzger, S.; Tjoeng, I.; Wermter, P.; Kersting, M.; Hecht, D.; Werbeck, N.; Wunderlin, P., Maßnahmen zur Verminderung des Eintrages von Mikroschadstoffen in die Gewässer - Phase 2. *Umweltbundesamt* **2016,** *60*.

100. Bonmatin, J. M.; Giorio, C.; Girolami, V.; Goulson, D.; Kreutzweiser, D. P.; Krupke, C.; Liess, M.; Long, E.; Marzaro, M.; Mitchell, E. A. D.; Noome, D. A.; Simon-Delso, N.; Tapparo, A., Environmental fate and exposure; neonicotinoids and fipronil. *Environmental Science and Pollution Research* **2015,** *22* (1), 35-67.

101. Jeschke, P.; Nauen, R.; Schindler, M.; Elbert, A., Overview of the status and global strategy for neonicotinoids. *Journal of Agricultural and Food Chemistry* **2011,** *59* (7), 2897-2908.

102. Brühl, C.; Alscher, A.; Hahn, M.; Berger, G.; Bethwell, C.; Gaef, F.; Schmidt, T.; Weber, B., Protection of biodiversity in the risk assessment and risk management of pesticides (plant protection products & biocides) with a focus on arthropods, soil organisms and amphibians. *Umweltbundesamt* **2015,** *76*.

103. LANUV, ECHO-Stoffbericht Neonicotinoide. *Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen* **2015**.

104. Taghavi, A.; Heine, K.; Kaberlah, F., Synopse zu Wirkungsmechanismen von Spurenstoffen im Säureorganismus als Grundlage der Bewertung des Wirkungspotentials von Stoffsummen und der Erkennung "neuer" toxischer Endpunkte. *Umweltbundesamt* **2011,** *47*.

105. EU; commission, Review report for the active substance thiacloprid directive 91/414/EEC. *E1-plant health* **2004**.

106. Pena, A.; Rodriguez-Liebana, J. A.; Mingorance, M. D., Persistence of two neonicotinoid insecticides in wastewater, and in aqueous solutions of surfactants and dissolved organic matter. *Chemosphere* **2011,** *84* (4), 464-470.

107. Banic, N. D.; Abramovic, B. F.; Sojic, D. V.; Krstic, J. B.; Fincur, N. L.; Bakovic, I. P., Efficiency of neonicotinoids photocatalytic degradation by using annular slurry reactor. *Chemical Engineering Journal* **2016,** *286*, 184-190.

108. Banic, N. D.; Sojic, D. V.; Krstic, J. B.; Abramovic, B. F., Photodegradation of neonicotinoid active ingredients and their commercial formulations in water by different advanced oxidation processes. *Water Air and Soil Pollution* **2014,** *225* (5).

109. Dai, Y. J.; Ji, W. W.; Chen, T.; Zhang, W. J.; Liu, Z. H.; Ge, F.; Yuan, S., Metabolism of the neonicotinoid insecticides acetamiprid and thiacloprid by the yeast Rhodotorula mucilaginosa strain IM-2. *Journal of Agricultural and Food Chemistry* **2010,** *58* (4), 2419-2425.

110. Liu, Z. H.; Dai, Y. J.; Huang, G. D.; Gu, Y. Y.; Ni, J.; Wei, H.; Yuan, S., Soil microbial degradation of neonicotinoid insecticides imidacloprid, acetamiprid, thiacloprid and imidaclothiz and its effect on the persistence of bioefficacy against horsebean aphid Aphis craccivora Koch after soil application. *Pest Management Science* **2011,** *67* (10), 1245-1252.

111. Hommen, U.; Schäfers, S.; Roß-Nickoll, M.; Ratte, T., Auswertung der wichtigsten in Deutschland durchgeführten Monitoringstudien zu Auswirkungen von Pflanzenschutzmitteln auf Nichtzielorganismen. *Fraunhofer Institut Molekularbiologie und Angewandte Ökologie* **2004**.

112. Hartland, A.; Lead, J. R.; Slaveykova, V.; O'Carroll, D.; Valsami-Jones, E., The environmental significance of natural nanoparticles. *Nature Education Knowledge* **2013,** *4* (8), 7.

113. Nowack, B.; Bucheli, T. D., Occurrence, behavior and effects of nanoparticles in the environment. *Environmental Pollution* **2007,** *150* (1), 5-22.

114. Pakrashi, S.; Kumar, D.; Iswarya, V.; Bhuvaneshwari, M.; Chandrasekaran, N.; Mukherjee, A., A comparative ecotoxicity analysis of alpha- and gamma-phase aluminium oxide nanoparticles towards a freshwater bacterial isolate Bacillus licheniformis. *Bioprocess and Biosystems Engineering* **2014,** *37* (12), 2415-2423.

115. Rajala, J. E.; Maenpaa, K.; Vehniainen, E. R.; Vaisanen, A.; Scott-Fordsmand, J. J.; Akkanen, J.; Kukkonen, J. V. K., Toxicity testing of silver nanoparticles in artificial and natural sediments using the benthic organism Lumbriculus variegatus. *Archives of Environmental Contamination and Toxicology* **2016,** *71* (3), 405-414.

116. Kukkonen, J.; Landrum, P. F., Distribution of organic carbon and organic xenobiotics among different particle-size fractions in sediments. *Chemosphere* **1996,** *32* (6), 1063-1076.

117. De Hoogh, C. J.; Wagenvoort, A. J.; Jonker, F.; van Leerdam, J. A.; Hogenboom, A. C., HPLC-DAD amd Q-TOF MS techniques identify cause of daphnia biomonitor alarms in the river Meuse. *Environmental Science & Technology* **2006,** *40*, 2678-2685.

118. Schaller, J., Bioturbation/bioirrigation by Chironomus plumosus as main factor controlling elemental remobilization from aquatic sediments? *Chemosphere* **2014,** *107*, 336-343.

119. Katagi, T.; Tanaka, H., Metabolism, bioaccumulation, and toxicity of pesticides in aquatic insect larvae. *Journal of Pesticide Science* **2016,** *41* (2), 25-37.

120. Stoughton, S. J.; Liber, K.; Culp, J.; Cessna, A., Acute and chronic toxicity of imidacloprid to the aquatic invertebrates Chironomus tentans and Hyalella azteca under constant- and pulse-exposure conditions. *Archives of Environmental Contamination and Toxicology* **2008,** *54* (4), 662-673.

121. Langer-Jaesrich, M.; Köhler, H.-R.; Gerhardt, A., Assessing toxicity of the insecticide thiacloprid on Chironomus riparius (Insecta: Diptera) using multiple end points. *Archives of Environmental Contamination and Toxicology* **2010,** *58* (4), 963-972.

122. Langer-Jaesrich, M.; Köhler, H.-R.; Gerhardt, A., Can mouth part deformities of Chironomus riparius serve as indicators for water and sediment pollution? A laboratory approach. *Journal of Soils and Sediments* **2010,** *10* (3), 414-422.

123. Pestana, J. L. T.; Loureiro, S.; Baird, D. J.; Soares, A. M. V. M., Fear and loathing in the benthos: Responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. *Aquatic Toxicology* **2009,** *93* (2-3), 138-149.

124. Azevedo-Pereira, H. M. V. S.; Lemos, M. F. L.; Soares, A. M. V. M., Behaviour and growth of Chironomus riparius meigen (Diptera: Chironomidae) under imidacloprid pulse and constant exposure scenarios. *Water Air and Soil Pollution* **2011,** *219* (1-4), 215-224.

125. Berghahn, R.; Mohr, S.; Hübner, V.; Schmiediche, R.; Schmiedling, I.; Svetich-Will, E.; Schmidt, R., Effects of repeated insecticide pulses on macroinvertebrate drift in indoor stream mesocosms. *Aquatic Toxicology* **2012,** *122*, 56-66.

126. Mohan, C.; Kumar, Y.; Madan, J.; Saxena, N., Multiresidue analysis of neonicotinoids by solid-phase extraction technique using high-performance liquid chromatography. *Environmental Monitoring and Assessment* **2010,** *165* (1-4), 573-576.

127. Sharma, K. K.; Mukherjee, I.; Singh, B.; Sahoo, S. K.; Mandal, K.; Mohapatra, S.; Ahuja, A. K.; Sharma, D.; Parihar, N. S.; Sharma, B. N.; Kale, V. D.; Walunj, A. R., Dissipation pattern and risk assessment of flubendiamide on chili at different agro-climatic conditions in India. *Environmental Monitoring and Assessment* **2015,** *187* (5).

128. Seo, E.-K.; Kwon, H.; Hong, S.-M.; Kim, T.-K., Simultaneous determination of flonicamid, imidacloprid, and its metabolites in paprika by QuEChERS and tandem mass spectrometry. *Journal of the Korean Society for Applied Biological Chemistry* **2015,** *58* (4), 603-610.

129. Abdel-Ghany, M. F.; Hussein, L. A.; El Azab, N. F.; El-Khatib, A. H.; Linscheid, M. W., Simultaneous determination of eight neonicotinoid insecticide residues and two primary metabolites in cucumbers and soil by liquid chromatography-tandem mass spectrometry coupled with QuEChERS. *Journal of Chromatography B* **2016,** *1031*, 15-28.

130. Rodriguez-Cabo, T.; Casado, J.; Rodriguez, I.; Ramil, M.; Cela, R., Selective extraction and determination of neonicotinoid insecticides in wine by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **2016,** *1460*, 9-15.

131. Konatu, F. R. B.; Breitkreitz, M. C.; Jardim, I., Revisiting quick, easy, cheap, effective, rugged, and safe parameters for sample preparation in pesticide residue analysis of lettuce by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **2017,** *1482*, 11-22.

132. Dasenaki, M. E.; Bletsou, A. A.; Hanafi, A. H.; Thomaidis, N. S., Liquid chromatography-tandem mass spectrometric methods for the determination of spinosad, thiacloprid and pyridalyl in spring onions and estimation of their pre-harvest interval values. *Food Chemistry* **2016,** *213*, 395-401.

133. Brox, S.; Ritter, A. P.; Küster, E.; Reemtsma, T., A quantitative HPLC-MS/MS method for studying internal concentrations and toxicokinetics of 34 polar analytes in zebrafish (Danio rerio) embryos. *Analytical and Bioanalytical Chemistry* **2014,** *406* (20), 4831-4840.

134. Gbylik-Sikorska, M.; Sniegocki, T.; Posyniak, A., Determination of neonicotinoid insecticides and their metabolites in honey bee and honey by liquid chromatography tandem mass spectrometry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2015,** *990*, 132-140.

135. Sanchez-Hernandez, L.; Hernandez-Dominguez, D.; Bernal, J.; Neusüß, C.; Martin, M. T.; Bernal, J. L., Capillary electrophoresis-mass spectrometry as a new approach to analyze neonicotinoid insecticides. *Journal of Chromatography A* **2014,** *1359*, 317-324.

136. Jovanov, P.; Guzsvany, V.; Lazic, S.; Franko, M.; Sakac, M.; Saric, L.; Kos, J., Development of HPLC-DAD method for determination of neonicotinoids in honey. *Journal of Food Composition and Analysis* **2015,** *40*, 106-113.

137. Giroud, B.; Vauchez, A.; Vulliet, E.; Wiest, L.; Buleté, A., Trace level determination of pyrethroid and neonicotinoid insecticides in beebread using acetonitrile-based extraction followed by analysis with ultra-high-performance liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A* **2013,** *1316*, 53-61.

138. Akoijam, R.; Singh, B.; Mandal, K., Development and validation of a Quick, Easy, Cheap, Effective, Rugged and Safe Method for the determination of imidacloprid and its metabolites in soil. *Journal of Chromatographic Science* **2015,** *53* (4), 542-547.

139. Akoijam, R.; Singh, B., Metabolic degradation of imidacloprid in paddy field soil. *Environmental Monitoring and Assessment* **2014,** *186* (10), 5977-5984.

140. Rossini, D.; Ciofi, L.; Ancillotti, C.; Checchini, L.; Bruzzoniti, M. C.; Rivoira, L.; Fibbi, D.; Orlandini, S.; Del Bubba, M., Innovative combination of QuEChERS extraction with on-line solid-phase extract purification and pre-concentration, followed by liquid chromatography-tandem mass spectrometry for the determination of non-steroidal anti-inflammatory drugs and their metabolites in sewage sludge. *Analytica Chimica Acta* **2016,** *935*, 269-281.

141. Lorenz, C. S.; Wicht, A. J.; Guluzada, L.; Crone, B.; Karst, U.; Lee, H. J.; Triebskorn, R.; Haderlein, S. B.; Huhn, C.; Köhler, H.-R., Nano-sized zeolites as modulators of thiacloprid toxicity on Chironomus riparius. *Peerj* **2017,** *5*.

142. Lorenz, C. S.; Wicht, A. J.; Guluzada, L.; Luo, L. L.; Jäger, L.; Crone, B.; Karst, U.; Triebskorn, R.; Liang, Y.; Anwander, R.; Haderlein, S. B.; Huhn, C.; Köhler, H.-R., Nano-sized Al2O3 reduces acute toxic effects of thiacloprid on the non-biting midge Chironomus riparius. *Plos One* **2017,** *12* (5).

143. Ernst, W.; Schaefer, R. G.; Goerke, H.; Eder, G., A method for working up marine organisms for the determination of PCB's, DDT, DDE, DDD, γ-HCH and HCB. *Fresenius' Zeitschrift für analytische Chemie* **1974,** *272* (5), 358-363.

144. Kruve, A.; Leito, I., Comparison of different methods aiming to account for/overcome matrix effects in LC/ESI/MS on the example of pesticide analyses. *Analytical Methods* **2013,** *5* (12), 3035-3044.

145. Burns, D. T.; Danzer, K.; Townshend, A., Use of the terms "recovery" and "apparent recovery" in analytical procedures - (IUPAC recommendations 2002). *Pure and Applied Chemistry* **2002,** *74* (11), 2201-2205.

146. Bauer, A.; Luetjohann, J.; Hanschen, F. S.; Schreiner, M.; Kuballa, J.; Jantzen, E.; Rohn, S., Identification and characterization of pesticide metabolites in Brassica species by liquid chromatography travelling wave ion mobility quadrupole time-of-flight mass spectrometry (UPLC-TWIMS-QTOF-MS). *Food Chemistry* **2018,** *244*, 292-303.

147. Jantunen, A. P. K.; Tuikka, A.; Akkanen, J.; Kukkonen, J. V. K., Bioaccumulation of atrazine and chlorpyrifos to Lumbriculus variegatus from lake sediments. *Ecotoxicology and Environmental Safety* **2008,** *71* (3), 860-868.

148. Maenpaa, K. A.; Sormunen, A. J.; Kukkonen, J. V. K., Bioaccumulation and toxicity of sediment associated herbicides (ioxynil, pendimethalin, and bentazone) in Lumbriculus variegatus (Oligochaeta) and Chironomus riparius (Insecta). *Ecotoxicology and Environmental Safety* **2003,** *56* (3), 398-410.

149. Dabrin, A.; Durand, C. L.; Garric, J.; Geffard, O.; Ferrari, B. J. D.; Coquery, M., Coupling geochemical and biological approaches to assess the availability of cadmium in freshwater sediment. *Science of the Total Environment* **2012,** *424*, 308-315.

150. van der Heijden, M. G. A.; Martin, F. M.; Selosse, M.-A.; Sanders, I. R., Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytologist* **2015,** *205* (4), 1406-1423.

151. Martinez, A. T.; Speranza, M.; Ruiz-Duenas, F. J.; Ferreira, P.; Camarero, S.; Guillen, F.; Martinez, M. J.; Gutierrez, A.; del Rio, J. C., Biodegradation of lignocellulosics: microbial, chemical, and enzymatic aspects of the fungal attack of lignin. *International Microbiology: The Official Journal of the Spanish Society for Microbiology* **2005,** *8* (3), 195-204.

152. Raudaskoski, M.; Kothe, E., Novel findings on the role of signal exchange in arbuscular and ectomycorrhizal symbioses. *Mycorrhiza* **2015,** *25* (4), 243-252.

153. Gorzelak, M. A.; Asay, A. K.; Pickles, B. J.; Simard, S. W., Inter-plant communication through mycorrhizal networks mediates complex adaptive behaviour in plant communities. *AoB Plants* **2015,** *7*.

154. Schrey, S. D.; Erkenbrack, E.; Fruh, E.; Fengler, S.; Hommel, K.; Horlacher, N.; Schulz, D.; Ecke, M.; Kulik, A.; Fiedler, H. P.; Hampp, R.; Tarkka, M. T., Production of fungal and bacterial growth modulating secondary metabolites is widespread among mycorrhiza-associated streptomycetes. *Bmc Microbiology* **2012,** *12*.

155. Deveau, A.; Barret, M.; Diedhiou, A. G.; Leveau, J.; de Boer, W.; Martin, F.; Sarniguet, A.; Frey-Klett, P., Pairwise transcriptomic analysis of the interactions between the ectomycorrhizal fungus Laccaria bicolor S238N and three beneficial, neutral and antagonistic soil bacteria. *Microbial Ecology* **2015,** *69* (1), 146-159.

156. Högberg, M. N.; Högberg, P., Extramatrical ectomycorrhizal mycelium contributes one-third of microbial biomass and produces, together with associated roots, half the dissolved organic carbon in a forest soil. *New Phytologist* **2002,** *154* (3), 791-795.

157. Courty, P. E.; Buee, M.; Diedhiou, A. G.; Frey-Klett, P.; Le Tacon, F.; Rineau, F.; Turpault, M. P.; Uroz, S.; Garbaye, J., The role of ectomycorrhizal communities in forest ecosystem processes: New perspectives and emerging concepts. *Soil Biology & Biochemistry* **2010,** *42* (5), 679-698.

158. Tedersoo, L.; May, T.; E Smith, M., Ectomycorrhizal lifestyle in fungi: Global diversity, distribution, and evolution of phylogenetic lineages. **2009,** *20*, 217-63.

159. di Pietro, M.; Churin, J. L.; Garbaye, J., Differential ability of ectomycorrhizas to survive drying. *Mycorrhiza* **2007,** *17* (6), 547-550.

160. Trocha, L. K.; Rudawska, M.; Leski, T.; Dabert, M., Genetic diversity of naturally established ectomycorrhizal fungi on Norway spruce seedlings under nursery conditions. *Microbial Ecology* **2006,** *52* (3), 418-425.

161. Laatikainen, T.; Heinonen-Tanski, H., Mycorrhizal growth in pure cultures in the presence of pesticides. *Microbiological Research* **2002,** *157* (2), 127-137.

162. Michelot, D.; Melendez-Howell, L. M., Amanita muscaria: chemistry, biology, toxicology, and ethnomycology. *Mycological Research* **2003,** *107*, 131-146.

163. Lopez, M. F.; Dietz, S.; Grunze, N.; Bloschies, J.; Weiss, M.; Nehls, U., The sugar porter gene family of Laccaria bicolor: function in ectomycorrhizal symbiosis and soil-growing hyphae. *New Phytologist* **2008,** *180* (2), 365-378.

164. Nuutinen, J. T.; Timonen, S., Identification of nitrogen mineralization enzymes, L-amino acid oxidases, from the ectomycorrhizal fungi Hebeloma spp. and Laccaria bicolor. *Mycological Research* **2008,** *112*, 1453-1464.

165. Ren, Z. S.; Mallik, A. U., Selected ectomycorrhizal fungi of black spruce (Picea mariana) can detoxify phenolic compounds of Kalmia angustifolia. *Journal of Chemical Ecology* **2006,** *32* (7), 1473-1489.

166. Burns, R.; Deforest, J.; Marxsen, J.; Sinsabaugh, R.; Stromberger, M.; Wallenstein, M.; Weintraub, M.; Zoppini, A., Soil enzymes in a changing environment: Current knowledge and future directions. *Soil Biology and Biochemistry* **2013,** *58*, 216-234.

167. Gianfreda, L.; Rao, M. A., Potential of extra cellular enzymes in remediation of polluted soils: a review. *Enzyme and Microbial Technology* **2004,** *35* (4), 339-354.

168. ECHA, Regulation (EU) No 528/2012 concerning the making available on the market and use of biocidal products, Propiconazole. *Evaluation of active substances, Assessment Report* **2015**.

169. Gartiser, S.; Lüskow, H.; Groß, R., Thematic strategy on sustainable use of plant production products. *Umweltbundesamt* **2012,** *6*.

170. Woo, C.; Daniels, B.; Stirling, R.; Morris, P., Tebuconazole and propiconazole tolerance and possible degradation by Basidiomycetes: A wood-based bioassay. *International Biodeterioration & Biodegradation* **2010,** *64* (5), 403-408.

171. Satapute, P.; Kaliwal, B., Biodegradation of the fungicide propiconazole by Pseudomonas aeruginosa PS-4 strain isolated from a paddy soil. *Annals of Microbiology* **2016,** *66* (4), 1355-1365.

172. Sarkar, S.; Seenivasan, S.; Premkumar, R., Biodegradation of propiconazole by Pseudomonas putida isolated from tea rhizosphere. *Plant Soil and Environment* **2009,** *55* (5), 196-201.

173. Dawson-Andoh, B. E., Ergosterol content as a measure of biomass of potential biological control fungi in liquid cultures. *Holz als Roh- und Werkstoff* **2002,** *60*, 115 - 117.

174. Skolness, S. Y.; Blanksma, C. A.; Cavallin, J. E.; Churchill, J. J.; Durhan, E. J.; Jensen, K. M.; Johnson, R. D.; Kahl, M. D.; Makynen, E. A.; Villeneuve, D. L.; Ankley, G. T., Propiconazole inhibits steroidogenesis and reproduction in the fathead minnow (Pimephales promelas). *Toxicological Sciences* **2013,** *132* (2), 284-297.

175. Kjeldsen, L. S.; Ghisari, M.; Bonefeld-Jorgensen, E. C., Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity. *Toxicology and Applied Pharmacology* **2013,** *272* (2), 453-464.

176. Ghisari, M.; Long, M.; Tabbo, A.; Bonefeld-Jorgensen, E. C., Effects of currently used pesticides and their mixtures on the function of thyroid hormone and aryl hydrocarbon receptor in cell culture. *Toxicology and Applied Pharmacology* **2015,** *284* (3), 292-303.

177. Sind Biozideinträge in die Umwelt von besorgniserregendem Ausmaß? Empfehlungen des Umweltbundesamtes für eine Vorgehensweise zur Untersuchung der Umweltbelastung durch Biozide. *Umweltbundesamt* **2016,** *15*.

178. Rüdel, H.; Fliedner, A.; Schwarzbauer, J.; Wluka, A.-K., Development of cornerstones for a monitoring programme for the assessment of biocide emissions into the environment. *Umweltbundesamt* **2017,** *24*.

179. Cao, X.; Liu, S.; Yang, X.; Liu, Z.; Liu, L., A modified QuEChERS sample preparation method for simultaneous determination of 62 pesticide residues in edible fungi using gas chromatography-triple quadrupole mass spectrometry. *Food Analytical Methods* **2016,** *9* (1), 263-274.

180. Franco, A. R.; Ferreira, A. C.; Castro, P. M. L., Co-metabolic degradation of mono-fluorophenols by the ectomycorrhizal fungi Pisolithus tinctorius. *Chemosphere* **2014,** *111*, 260-265.

181. Huang, Y.; Wang, J., Degradation and mineralization of DDT by the ectomycorrhizal fungi, Xerocomus chrysenteron. *Chemosphere* **2013,** *92* (7), 760-764.

182. Tixier, C.; Sancelme, M.; Ait-Aissa, S.; Widehem, P.; Bonnemoy, F.; Cuer, A.; Truffaut, N.; Veschambre, H., Biotransformation of phenylurea herbicides by a soil bacterial strain, Arthrobacter sp N2: structure, ecotoxicity and fate of diuron metabolite with soil fungi. *Chemosphere* **2002,** *46* (4), 519-526.

183. Pinto, A. P.; Serrano, C.; Pires, T.; Mestrinho, E.; Dias, L.; Martins Teixeira, D.; Caldeira, A. T., Degradation of terbuthylazine, difenoconazole and pendimethalin pesticides by selected fungi cultures. *Science of the Total Environment* **2012,** *435*, 402-410.

184. Sanyal, D.; Kulshrestha, G., Metabolism of metolachlor by fungal cultures. *Journal of Agricultural and Food Chemistry* **2002,** *50* (3), 499-505.

185. Garon, D.; Krivobok, S.; Seigle-Murandi, F., Fungal degradation of fluorene. *Chemosphere* **2000,** *40* (1), 91-97.

186. Nan, J.; Wang, J.; Piao, X.; Yang, C.; Wu, X.; Quinto, M.; Li, D., Novel and rapid method for determination of organophosphorus pesticide residues in edible fungus using direct gas purge microsyringe extraction coupled on-line with gas chromatography-mass spectrometry. *Talanta* **2015,** *142*, 64-71.

187. Teng, Z.; Wang, S.; Su, X.; Chen, G.; Liu, Y.; Luo, Z.; Luo, W.; Tang, Y.; Ju, H.; Zhao, D.; Lu, G., Facile synthesis of Yolk–shell structured inorganic–organic hybrid spheres with ordered radial mesochannels. *Advanced Materials* **2014,** *26* (22), 3741-3747.

188. Teng, Z.; Su, X.; Lee, B.; Huang, C.; Liu, Y.; Wang, S.; Wu, J.; Xu, P.; Sun, J.; Shen, D.; Li, W.; Lu, G., Yolk–shell structured mesoporous nanoparticles with thioether-bridged organosilica frameworks. *Chemistry of Materials* **2014,** *26* (20), 5980-5987.

189. Honda, A.; Miyazaki, T.; Ikegami, T.; Iwamoto, J.; Yamashita, K.; Numazawa, M.; Matsuzaki, Y., Highly sensitive and specific analysis of sterol profiles in biological samples by HPLC-ESI-MS/MS. *Journal of Steroid Biochemistry and Molecular Biology* **2010,** *121* (3-5), 556-564.

190. Trosken, E. R.; Bittner, N.; Volkel, W., Quantitation of 13 azole fungicides in wine samples by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A* **2005,** *1083* (1-2), 113-119.

191. Nurmi, J.; Pellinen, J., Multiresidue method for the analysis of emerging contaminants in wastewater by ultra performance liquid chromatography-time-of-flight mass spectrometry. *Journal of Chromatography A* **2011,** *1218* (38), 6712-6719.

192. Skladanka, J.; Dohnal, V.; Jezkova, A., Fibre and ergosterol contents in forage of Arrhenatherum elatius, Dactylis glomerata and Festulolium at the end of the growing season. *Czech Journal of Animal Science* **2008,** *53* (8), 320-329.

193. Agilent; Technologies, Agilent's new octadecylsilane (ODS) (C18) solid-phase extraction cartridges: sampliQ C18. *Technical Note* **2008**.

194. Robles-Molina, J.; Lara-Ortega, F. J.; Gilbert-Lopez, B.; Garcia-Reyes, J. F.; Molina-Diaz, A., Multi-residue method for the determination of over 400 priority and emerging pollutants in water and wastewater by solid-phase extraction and liquid chromatography-time-of-flight mass spectrometry. *Journal of Chromatography A* **2014,** *1350*, 30-43.

195. Jedlickova, L.; Gadas, D.; Havlova, P.; Havel, J., Determination of ergosterol levels in barley and malt varieties in the Czech Republic via HPLC. *Journal of Agricultural and Food Chemistry* **2008,** *56* (11), 4092-4095.

196. Kadakal, C.; Artik, N., Degradation kinetics of ergosterol in tomato paste serum. *European Food Research and Technology* **2008,** *227* (3), 683-688.

197. Horbik, D.; Lowinska-Kluge, A.; Gorski, Z.; Stanisz, E.; Zgola-Grzeskowiak, A., Microwave-assisted extraction combined with HPLC-MS/MS for diagnosis of fungal contamination in building materials. *Journal of the Brazilian Chemical Society* **2013,** *24* (9), 1478-1486.

198. Barreira, J. C. M.; Oliveira, M. B. P. P.; Ferreira, I. C. F. R., Development of a novel methodology for the analysis of ergosterol in mushrooms. *Food Analytical Methods* **2014,** *7* (1), 217-223.

199. Beni, A.; Soki, E.; Lajtha, K.; Fekete, I., An optimized HPLC method for soil fungal biomass determination and its application to a detritus manipulation study. *Journal of Microbiological Methods* **2014,** *103*, 124-130.

200. Honda, A.; Yamashita, K.; Hara, T.; Ikegami, T.; Miyazaki, T.; Shirai, M.; Xu, G.; Numazawa, M.; Matsuzaki, Y., Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS. *Journal of Lipid Research* **2009,** *50* (2), 350-357.

201. Honda, A.; Yamashita, K.; Miyazaki, H.; Shirai, M.; Ikegami, T.; Xu, G.; Numazawa, M.; Hara, T.; Matsuzaki, Y., Highly sensitive analysis of sterol profiles in human serum by LC-ESI-MS/MS. *Journal of Lipid Research* **2008,** *49* (9), 2063-2073.

202. Horlacher, N., Die stofflichen Grundlagen der Interaktion zwischen dem phytopathogenen Pilz Heterobasidion, Pflanzen und Streptomyces sp. AcH 505 aus der Rhizosphäre. *Dissertation* **2012**.

203. Konwick, B. J.; Garrison, A. W.; Avants, J. K.; Fisk, A. T., Bioaccumulation and biotransformation of chiral triazole fungicides in rainbow trout (Oncorhynchus mykiss). *Aquatic Toxicology* **2006,** *80* (4), 372-381.

204. Rösch, A.; Anliker, S.; Hollender, J., How biotransformation influences toxicokinetics of azole fungicides in the aquatic invertebrate Gammarus pulex. *Environmental Science & Technology* **2016,** *50* (13), 7175-7188.

205. Chen, P. J.; Moore, T.; Nesnow, S., Cytotoxic effects of propiconazole and its metabolites in mouse and human hepatoma cells and primary mouse hepatocytes. *Toxicology in Vitro* **2008,** *22* (6), 1476-1483.

206. Vialaton, D.; Pilichowski, J. F.; Baglio, D.; Paya-Perez, A.; Larsen, B.; Richard, C., Phototransformation of propiconazole in aqueous media. *Journal of Agricultural and Food Chemistry* **2001,** *49* (11), 5377-5382.

207. Davies, L. O.; Bramke, I.; France, E.; Marshall, S.; Oliver, R.; Nichols, C.; Schafer, H.; Bending, G. D., Non-UV light influences the degradation rate of crop protection products. *Environmental Science & Technology* **2013,** *47* (15), 8229-8237.

208. Owen, W. J.; Donzel, B., Oxidative-degradation of chlortoluron, propiconazole, and metylaxyl in suspension-cultures of various crop plants. *Pesticide Biochemistry and Physiology* **1986,** *26* (1), 75-89.

209. Nehls, U.; Gohringer, F.; Wittulsky, S.; Dietz, S., Fungal carbohydrate support in the ectomycorrhizal symbiosis: a review. *Plant Biology* **2010,** *12* (2), 292-301.

210. Wiese, J.; Kleber, R.; Hampp, R.; Nehls, U., Functional characterization of the Amanita muscaria monosaccharide transporter, AmMst1. *Plant Biology* **2000,** *2* (3), 278-282.

211. Common implementation strategy for the water framework directive (2000/60/EC) guidance document No. 27 technical guidance for deriving environmental quality standards. 2011.

212. Directive 2000/60/EC of the European Parliament and of the Council, of 23October 2000, establishing a framework for Community action in the field of water policy. *Official Journal of the European Union* **2000,** *L327*.

213. Directive 2013/39/EU of the European Parliament and of the Council, of 12August 2013, amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy. *Official Journal of the European Union* **2013,** *L 226/1*.

214. Krauss, M.; Singer, H.; Hollender, J., LC-high resolution MS in environmental analysis: from target screening to the identification of unknowns. *Analytical and Bioanalytical Chemistry* **2010,** *397* (3), 943-951.

215. Schymanski, E. L.; Singer, H. P.; Slobodnik, J.; Ipolyi, I. M.; Oswald, P.; Krauss, M.; Schulze, T.; Haglund, P.; Letzel, T.; Grosse, S.; Thomaidis, N. S.; Bletsou, A.; Zwiener, C.; Ibanez, M.; Portoles, T.; de Boer, R.; Reid, M. J.; Onghena, M.; Kunkel, U.; Schulz, W.; Guillon, A.; Noyon, N.; Leroy, G.; Bados, P.; Bogialli, S.; Stipanicev, D.; Rostkowski, P.; Hollender, J., Non-target screening with high-resolution mass spectrometry: critical review using a collaborative trial on water analysis. *Analytical and Bioanalytical Chemistry* **2015,** *407* (21), 6237-6255.

216. Rajski, L.; Gomez-Ramos, M. D.; Fernandez-Alba, A. R., Large pesticide multiresidue screening method by liquid chromatography-Orbitrap mass spectrometry in full scan mode applied to fruit and vegetables. *Journal of Chromatography A* **2014,** *1360*, 119-127.

217. Gómez, M. J.; Gómez-Ramos, M. M.; Malato, O.; Mezcua, M.; Férnandez-Alba, A. R., Rapid automated screening, identification and quantification of organic micro-contaminants and their main transformation products in wastewater and river waters using liquid chromatography–quadrupole-time-of-flight mass spectrometry with an accurate-mass database. *Journal of Chromatography A* **2010,** *1217* (45), 7038-7054.

218. Wode, F.; Reilich, C.; van Baar, P.; Dunnbier, U.; Jekel, M.; Reemtsma, T., Multiresidue analytical method for the simultaneous determination of 72 micropollutants in aqueous samples with ultra high performance liquid chromatography-high resolution mass spectrometry. *Journal of Chromatography A* **2012,** *1270*, 118-126.

219. Roman, M. C.; Brown, P. R., Free-flow electrophoresis. *Analytical Chemistry* **1994,** *66* (2), A86-A94.

220. Alvarez, G.; Montero, L.; Llorens, L.; Castro-Puyana, M.; Cifuentes, A., Recent advances in the application of capillary electromigration methods for food analysis and Foodomics. *Electrophoresis* **2018,** *39* (1), 136-159.

221. Oliveira, A. M.; Loureiro, H. C.; de Jesus, F. F. S.; de Jesus, D. P., Electromembrane extraction and preconcentration of carbendazim and thiabendazole in water samples before capillary electrophoresis analysis. *Journal of Separation Science* **2017,** *40* (7), 1532-1539.

222. Bol'shakova, D. S.; Amelin, V. G., Determination of pesticides in environmental materials and food products by capillary electrophoresis. *Journal of Analytical Chemistry* **2016,** *71* (10), 965-1013.

223. Reddy, A. V. B.; Yusop, Z.; Jaafar, J.; Jamil, N. H.; Majid, Z. A.; Aris, A. B., Development and validation of capillary electrophoresis method for simultaneous determination of six pharmaceuticals in different food samples combining on-line and off-line sample enrichment techniques. *Food Analytical Methods* **2018,** *11* (2), 533-545.

224. Paul, P.; Duchateau, T.; Sanger-van de Griend, C.; Adams, E.; Van Schepdael, A., Capillary electrophoresis with capacitively coupled contactless conductivity detection method development and validation for the determination of azithromycin, clarithromycin, and clindamycin. *Journal of Separation Science* **2017,** *40* (17), 3535-3544.

225. Turgeon, R. T.; Bowser, M. T., Micro free-flow electrophoresis: theory and applications. *Analytical and Bioanalytical Chemistry* **2009,** *394* (1), 187-198.

226. Weber, G.; Bocek, P., Recent developments in preparative free flow isoelectric focusing. *Electrophoresis* **1998,** *19* (10), 1649-1653.

227. Nath, S.; Schutte, H.; Weber, G.; Hustedt, H.; Deckwer, W. D., Separation of enzymes from candida-boidinii crude extract by contious-flow zone electrophoresis. *Electrophoresis* **1990,** *11* (11), 937-941.

228. Nath, S.; Schutte, H.; Hustedt, H.; Deckwer, W. D.; Weber, G., Separation of enzymes from microorganism crude extracts by free-flow zone electrophoresis. *Biotechnology and Bioengineering* **1996,** *51* (1), 15-22.

229. Clifton, M. J.; Jouve, N.; Debalmann, H.; Sanchez, V., Conditions for purification of proteins by free-flow zone electrophoresis. *Electrophoresis* **1990,** *11* (11), 913-919.

230. Heydt, A.; Wagner, H.; Paul, H. L., Concentration and purification of plant pathogenic viruses by field step electrophoresis. *Journal of Virological Methods* **1988,** *19* (1), 13-22.

231. Knisley, K. A.; Rodkey, L. S., Comparative-studies of recycling isoelectric-focusing and continous-flow electrophoresis - separation of proteins with minor charge differences. *Electrophoresis* **1990,** *11* (11), 927-931.

232. Kohlheyer, D.; Eijkel, J. C. T.; van den Berg, A.; Schasfoort, R. B. M., Miniaturizing free-flow electrophoresis - a critical review. *Electrophoresis* **2008,** *29* (5), 977-993.

233. Rhodes, P. H.; Snyder, R. S., Sample band spreading phenomena in ground and space-based electrophoretic separation. *Electrophoresis* **1986,** *7* (3), 113-120.

234. Poggel, M.; Melin, T., Free-flow zone electrophoresis: A novel approach and scale-up for preparative protein separation. *Electrophoresis* **2001,** *22* (6), 1008-1015.

235. Nunez, O.; Gallart-Ayala, H.; Martins, C. P. B.; Lucci, P., New trends in fast liquid chromatography for food and environmental analysis. *Journal of Chromatography A* **2012,** *1228*, 298-323.

236. Pedersen-Bjergaard, S.; Rasmussen, K. E., Electrokinetic migration across artificial liquid membranes: New concept for rapid sample preparation of biological fluids. *Journal of Chromatography A* **2006,** *1109* (2), 183-190.

237. Breadmore, M. C., Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips. *Electrophoresis* **2007,** *28* (1-2), 254-281.

238. Karim, N. I.; Ibrahim, W. A. W.; Sanagi, M. M.; Keyon, A. S. A., Online preconcentration by electrokinetic supercharging for separation of endocrine disrupting chemical and phenolic pollutants in water samples. *Electrophoresis* **2016,** *37* (20), 2649-2656.

239. Xu, Z.; Kawahito, K.; Ye, X.; Timerbaev, A. R.; Hirokawa, T., Electrokinetic supercharging with a system-induced terminator and an optimized capillary versus electrode configuration for parts-per-trillion detection of rare-earth elements in CZE. *Electrophoresis* **2011,** *32* (10), 1195-1200.

240. Breadmore, M. C., Unlimited-volume stacking of ions in capillary electrophoresis. Part 1: Stationary isotachophoretic stacking of anions. *Electrophoresis* **2008,** *29* (5), 1082-1091.

241. Götz, C.; Otto, J.; Singer, H., Überprüfung des Reinigungseffekts. Auswahl geeigneter organischer Spurenstoffe. *Aqua & Gas* **2015,** *95* (2), 34-40.

242. Frohnhöfer, H. G.; Geiger-Rudolph, S.; Pattky, M.; Meixner, M.; Huhn, C.; Maischein, H.-M.; Geisler, R.; Gehring, I.; Maderspacher, F.; Nüsslein-Volhard, C.; Irion, U., Spermidine, but not spermine, is essential for pigment pattern formation in zebrafish. *Biology Open* **2016**.

243. Ali, I.; Aboul-Enein, H. Y.; Gupta, V. K., Analysis of metformin dosage formulations by capillary electrophoresis at nano scale detection. *Combinatorial Chemistry & High Throughput Screening* **2007,** *10* (7), 611-615.

244. Ribeiro, M. M. A. C.; Oliveira, T. C.; Batista, A. D.; Muñoz, R. A. A.; Richter, E. M., A sub-minute electrophoretic method for simultaneous determination of naphazoline and zinc. *Journal of Chromatography A* **2016,** *1472*, 134-137.

245. Directive 2013/39/EU of the european parliament and of the council. Official journal of the european union: 2013.

246. Lohmann, W.; Karst, U., Biomimetic modeling of oxidative drug metabolism. *Analytical and Bioanalytical Chemistry* **2008,** *391* (1), 79-96.

247. Faber, H.; Lutze, H.; Lareo, P. L.; Frensemeier, L.; Vogel, M.; Schmidt, T. C.; Karst, U., Liquid chromatography/mass spectrometry to study oxidative degradation of environmentally relevant pharmaceuticals by electrochemistry and ozonation. *Journal of Chromatography A* **2014,** *1343*, 152-159.

248. Ruokolainen, M.; Gul, T.; Permentier, H.; Sikanen, T.; Kostiainen, R.; Kotiaho, T., Comparison of TiO2 photocatalysis, electrochemically assisted Fenton reaction and direct electrochemistry for simulation of phase I metabolism reactions of drugs. *European Journal of Pharmaceutical Sciences* **2016,** *83*, 36-44.

249. Johansson, T.; Weidolf, L.; Jurva, U., Mimicry of phase I drug metabolism - novel methods for metabolite characterization and synthesis. *Rapid Communications in Mass Spectrometry* **2007,** *21* (14), 2323-2331.

250. Seiwert, B.; Golan-Rozen, N.; Weidauer, C.; Riemenschneider, C.; Chefetz, B.; Hadar, Y.; Reemtsma, T., Electrochemistry combined with LC-HRMS: elucidating transformation products of the recalcitrant pharmaceutical compound carbamazepine generated by the white-rot fungus pleurotus ostreatus. *Environmental Science & Technology* **2015,** *49* (20), 12342-12350.

251. Faber, H.; Melles, D.; Brauckmann, C.; Wehe, C. A.; Wentker, K.; Karst, U., Simulation of the oxidative metabolism of diclofenac by electrochemistry/(liquid chromatography/)mass spectrometry. *Analytical and Bioanalytical Chemistry* **2012,** *403* (2), 345-354.

252. Stalder, R.; Roth, G. P., Preparative microfluidic electrosynthesis of drug metabolites. *ACS Medicinal Chemistry Letters* **2013,** *4* (11), 1119-1123.

253. Jurva, U.; Wikstrom, H. V.; Weidolf, L.; Bruins, A. P., Comparison between electrochemistry/mass spectrometry and cytochrome P450 catalyzed oxidation reactions. *Rapid Communications in Mass Spectrometry* **2003,** *17* (8), 800-810.

254. König, A.; Weidauer, C.; Seiwert, B.; Reemtsma, T.; Unger, T.; Jekel, M., Reductive transformation of carbamazepine by abiotic and biotic processes. *Water Research* **2016,** *101*, 272-280.

255. Roeser, J.; Alting, N. F. A.; Permentier, H. P.; Bruins, A. P.; Bischoff, R., Boron-doped diamond electrodes for the electrochemical oxidation and cleavage of peptides. *Analytical Chemistry* **2013,** *85* (14), 6626-6632.

256. Baumann, A.; Lohmann, W.; Schubert, B.; Oberacher, H.; Karst, U., Metabolic studies of tetrazepam based on electrochemical simulation in comparison to in vivo and in vitro methods. *Journal of Chromatography A* **2009,** *1216* (15), 3192-3198.

257. Chien, R. L.; Burgi, D. S., Field amplified sample injection in high-performance capillary electrophoresis. *Journal of Chromatography* **1991,** *559* (1-2), 141-152.

258. Quirino, J. P.; Terabe, S., Approaching a million-fold sensitivity increase in capillary electrophoresis with direct ultraviolet detection: cation-selective exhaustive injection and sweeping. *Analytical Chemistry* **2000,** *72* (5), 1023-1030.

259. Xu, Z.; Koshimidzu, E.; Hirokawa, T., Electrokinetic sample injection for high-sensitivity CZE (part 2): Improving the quantitative repeatability and application of electrokinetic supercharging-CZE to the detection of atmospheric electrolytes. *Electrophoresis* **2009,** *30* (20), 3534-3539.

260. Luft, A.; Wagner, M.; Ternes, T. A., Transformation of biocides irgarol and terbutryn in the biological wastewater treatment. *Environmental Science & Technology* **2014,** *48* (1), 244-254.

261. Ibanez, M.; Sancho, J. V.; Pozo, O. J.; Hernandez, F., Use of quadrupole time-of-flight mass spectrometry in environmental analysis: Elucidation of transformation products of triazine herbicides in water after UV exposure. *Analytical Chemistry* **2004,** *76* (5), 1328-1335.

262. Tisler, S.; Zwiener, C., Formation and occurrence of transformation products of metformin in wastewater and surface water. *Science of The Total Environment* **2018,** *628-629*, 1121-1129.

263. Han, W.; Zhong, C.; Liang, L.; Sun, Y.; Guan, Y.; Wang, L.; Sun, X.; Li, J., Electrochemical degradation of triazole fungicides in aqueous solution using TiO2-NTs/SnO2-Sb/PbO2 anode: Experimental and DEF studies. *Electrochimica Acta* **2014,** *130*, 179-186.

264. Zhang, H.; Liu, F.; Wu, X. G.; Zhang, J. H.; Zhang, D. B., Degradation of tetracycline in aqueous medium by electrochemical method. *Asia-Pacific Journal of Chemical Engineering* **2009,** *4* (5), 568-573.

265. Lizama-Bahena, C.; Alvarez-Gallegos, A.; Hernandez, J. A.; Silva-Martinez, S., Elimination of bio-refractory chlorinated herbicides like atrazine, alachlor, and chlorbromuron from aqueous effluents by Fenton, electro-Fenton, and peroxi-coagulation methods. *Desalination and Water Treatment* **2015,** *55* (13), 3683-3693.

266. Brillas, E.; Calpe, J. C.; Casado, J., Mineralization of 2,4-D by advanced electrochemical oxidation processes. *Water Research* **2000,** *34* (8), 2253-2262.

SUPERVISION AND PROJECT PARTNERS

Anna-Jorina Wicht - Institute for Physical and Theoretical Chemistry, Faculty of Science, University of Tübingen (Tübingen, Germany):
I conducted all analytical measurements, sample preparation steps and evaluation of analytical data presented in this thesis. Ideas for development and optimization of the pesticide and pharmaceutical extraction procedures from biota and detection methods for analytes in biota and water by HPLC-MS and ideas for setup of electromigrative enrichment experiments stem from me. Joint discussions, especially with biologists supported reflection of intermediate results for further planning of the next experimental phases. During the project I was responsible for project management and communication. Finally, I summarized the work conducted in the framework of the presented thesis. Details on the contribution in the different chapter are given below. Results presented in Chapter 3 and 4 were achieved in the EXPAND project financed by the University of Tübingen within the Excellence Initiative.

Elisabeth Früh - Institute of Evolution and Ecology, Faculty of Science, University of Tübingen (Tübingen, Germany)
Elisabeth Früh carried out the fungi exposure experiments under supervision of Dr. Sarah Dietz. Further experimental designs were based on joint discussions of intermediate results. Elisabeth Früh provided fungi tissue, agar plate and liquid culture samples originating from exposure experiments and blank samples for method development.

Leyla Guluzada - Center for Applied Geosciences, Environmental Mineralogy and Chemistry, Faculty of Science, University of Tübingen (Tübingen, Germany)
Leyla Guluzada conducted sorption experiments of nanoparticles and pesticides. The calculated sorption coefficients were used for calculation of exposure concentrations in biological experiments. Lelya Guluzada provided extracts of fungi liquid culture medium.

Leilei Luo - Institute for Inorganic Chemistry, Faculty of Science, University of Tübingen (Tübingen, Germany):
Non-commercially available nanoparticles used in fungi exposure experiments were synthesized by Leilei Luo under supervision of Prof. Reiner Anwander.

Katharina Heye - Department of Aquatic Ecotoxicology, University Frankfurt (Frankfurt, Germany)
Katharina Heye designed and conducted the exposure experiments with midge larvae and carbamazepine. Larvae, midges and exposure medium samples were provided for analysis.

Prof. Carolin Huhn – Institute of Physical and Theoretical Chemistry, Faculty of Science, University of Tübingen, (Tübingen Germany)
Prof. Carolin Huhn was the supervisor of my PhD project and contributed basic ideas, expertise and scientific network for setup, measurements and discussions presented in this thesis.

Prof. Uwe Karst, University of Münster (Münster, Germany)

Prof. Uwe Karst provided equipment and expertise for electrochemical cell experiments and Simon Scheeren contributed with his expertise in experimental implementation of electrochemical transformation experiments.

Dr. Carla Lorenz - Institute of Evolution and Ecology, Faculty of Science, University of Tübingen, (Tübingen, Germany)
Dr. Carla Lorenz conducted midge larvae exposure experiments to thiacloprid under supervision of Prof. Heinz-Rüdiger Köhler. Larvae tissue and exposure medium samples were provided for analysis. Two publications with a focus on the biological endpoints were submitted by Dr. Lorenz from the shared project:
Lorenz, C. S., Wicht, A. J., Guluzada, L., Luo, L., Jäger, L. Crone, B., Karst, U. Triebskorn, R. Liang, Y., Anwander, R., Haderlein, S. B, Huhn, C. Köhler, H. R. Nano-sized Al_2O_3 reduces acute toxic effects of thiacloprid on the non-biting midge *Chironomus riparius*. Plos One, 2017, 12 (5)
Lorenz, C. S., Wicht, A. J., Guluzada, L., Crone, B., Karst, U., Lee, H. J., Triebskorn, R. Haderlein, S. B., Huhn, C., Köhler, H. R., Nano-sized zeolites as modulators of thiacloprid toxicity on *Chironomus riparius*, Peerj, 2017, 5.

Dr. Wolfgang Schulz - Zweckverband Landeswasserversorgung Langenau, (Langenau, Germany)
Dr. Wolfgang Schulz provided the reference standard used for the development of the multi-component HPLC-MS water analysis method.

Dr. Gerhard Weber , FFE-Service GmbH (Feldkirchen, Germany)
Dr. Gerhard Weber and his company provided surface water samples fractionated by FFE techniques.

OWN CONTRIBUTIONS IN THE CHAPTERS:

Chapter 2: QuEChERS extraction in ecotoxicology: analytical procedure and applications to biota - a review

Anna-Jorina Wicht, Carolin Huhn

The literature search and writing was done to 100 % by me. The manuscript was corrected by Carolin Huhn.

Chapter 3: Determination of carbamazepine transfer from exposed midge larvae to adult midges by QuEChERS extraction and HPLC-MS/MS analysis

Anna-Jorina Wicht, Katharina Heye, Anja Schmidt, Carolin Huhn

Ideas for the extraction procedure development and optimization are to 100 % my own work. Development of the extraction procedure, sample preparation, evaluation of data and writing of the manuscript was my contribution (100 %). Ecotoxicological tests were designed by Katharina Heye (90 %) and conducted by Anja Schmidt under her supervision. She also collected organisms for extraction and HPLC-MS analysis. The project was supervised by Carolin Huhn.

Chapter 4: Quantification of thiacloprid in chironomids exposed to the neonicotinoid thiacloprid in the presence of nanoparticles with a miniaturized QuEChERS procedure by HPLC-MS

Anna-Jorina Wicht, Carla S. Lorenz, Leyla Guluzada, Stefan Haderlein, Heinz R. Köhler, Carolin Huhn

I provided ideas for the development of the extraction procedure and its optimization and implemented the procedure including the development of the extraction procedure, sample preparation, evaluation of data and writing (100 %). Optimization of the sample homogenization step was performed by Christoph Schäfer in his Bachelor thesis under my supervision. Ecotoxicological tests were designed and conducted by Carla S. Lorenz under supervision of Heinz R. Köhler. Sorption experiments were performed by Leyla Guluzada under supervision of Stefan Haderlein. The project was supervised by Carolin Huhn. Data interpretation was achieved together with coautors.

Chapter 5: Determination of propiconazole and metabolites in mycelium from different fungi species from exposure studies in the presence of nanoparticles by HPLC-MS

Anna-Jorina Wicht, Elisabeth Früh, Leilei Luo, Leyla Guluzada, Stefan Haderlein, Reiner Anwander, Sandra Dietz, Carolin Huhn

I conducted the development of the extraction procedure, sample preparation and analysis based on my strategy (100 %). I was also in charge for evaluation of data and writing (100 %). Ecotoxicological tests were designed and conducted by Elisabeth Früh under supervision of Sandra Dietz. I contributed basic ideas for further experimental design from discussing intermediate results (ca. 5 %). Sorption experiments were performed by Leyla Guluzada under supervision of Stefan Haderlein. Synthesis of nanoparticles was done by

Leilei Luo. The project was supervised by Carolin Huhn. Data interpretation was done together with Carla S. Lorenz, Heinz R. Köhler and Carolin Huhn.

Chapter 6: Analysis of wastewater and surface water samples fractionated by different free flow electrophoretic techniques

Anna-Jorina Wicht, Gerhard Weber, Carolin Huhn

Ideas for development of the analytical method is to 100 % my own work. Development of the HPLC-MS method, analysis, evaluation of data and writing were done to 100 % by me. Sample collection and fractionation experiments were performed by Gerhard Weber (100 %). The project was supervised by Carolin Huhn. Data interpretation was done together with Carolin Huhn.

Chapter 7: Investigation of the influence of electrode geometry, temperature and convection on the transfer of ionic analytes by electromigrative enrichment processes

Anna-Jorina Wicht, Manuel Klein, Carolin Huhn

I conducted 70 % of the experiments and 30 % were performed by Manual Klein during his internship under my supervision. Sample analysis, data evaluation and writing were my task (100 %). The project was supervised by Carolin Huhn. Electrochemical transformation experiments were performed with help of Simon Scheerer and discussion of results together with Uwe Karst.

ABBREVIATIONS

abbreviation	
2,4-D	2,4-dichlorophenoxyacetic acid
AAEE	N-acryloylamido-ethoxyethanol
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
BAF	bioaccumulation factor
BCF	bioconcentration factor
BDDE	boron-doped diamond electrode
BPC	base peak chromatogram
BSAF	biota-soil accumulation factor
BTP	bio-transformation product
CBZ	carbamazepine
CE	capillary electrophoresis
CYP450	cytochrome P450
DEET	N,N-diethyl-toluamide
DLLME	dispersed liquid-liquid micro extraction
dSPE	dispersive solid phase extraction
dw	dry weight
EA	ethyl acetate
EC	electrochemical cell
EIC	extracted ion chromatograms
EOF	electroosmotic flow
ESI	electrospray ionization
FA	formic acid
FFE	free flow electrophoresis
FFITP	free flow isotachophoresis
FFIZE	free flow interval zone electrophoresis
GC	gas chromatography
GCB	graphiticed carbon black
HILIC	hydrophilic interaction chromatography
HPLC	high performance liquid chromatography
HRMS	high-resolution mass spectrometry
IEF	isoelectric focusing
ISTD	internal standard
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantification
MAE	microwave-assisted extraction
MCPA	2-methyl-4-chlorophenoxyacetic acid
MeCN	acetonitrile
MeOH	methanol
MS	mass spectrometry
MSPD	matrix solid phase dispersion

abbreviation	
NMP	N-methyl-2-pyrrolidone
NP	nanoparticle
OD	optical density
PAH	polycyclic aromatic hydrocarbon
PCB	polychlorinated biphenyl
pI	isoelectric point
PLE	pressurized liquid extraction
P_{ow}	octanol-water coefficient
PSA	primary secondary amines
PTP	photo-transformation product
QuEChERS	Quick Easy Cheap Rugged and Safe
QuPPE	Quick Polar Pesticides Extraction
RPLC	reversed phase liquid chromatography
rpm	rounds per minute
S/N	signal to noise
SFE	step-field electrophoresis
SI	signal intensity
SPE	solid phase extraction
TOF	time of flight
t_R	retention time
UHPLC	ultra HPLC
USE	ultrasonic extraction
VMS	volatile methyl siloxanes
ww	wet weight

ACKNOWLEDGEMENT

The work conducted in the presented thesis was done at the Eberhard Karls University at the Institute for Physical and Theoretical Chemistry in the working group for effect-based environmental analysis.

My thanks go to the following persons who supported me during the processing of my dissertation.

First of all I would like to thank my supervisor Prof. Dr. Carolin Huhn, who encouraged me to look at the topic from different perspectives. I would like to thank her for her advice, scientific and methodical support and the opportunities to present my work at different interesting and exciting conferences and meet experts from industry and academia. Further I would like to thank Prof. Dr. Heinz-R. Köhler my co-supervisor for discussions and support.

I am grateful for the support of everyone involved in my studies. I wish to express my gratitude to the members of the EXPAND team for discussion and cooperation, Prof. Dr. Carolin Huhn, Prof. Dr. Heinz-R. Köhler, Prof. Dr. Stefan Haderlein, Prof. Dr. Reiner Anwander, Dr. Sandra Dietz, Dr. Carla Lorenz, Elisabeth Früh, Leyla Guluzada and Leilei Luo. Further I want to thank my project partner Katharina Heye at the Goethe University Frankfurt for discussions and cooperation and my project partner Prof. Dr. Uwe Karst and Lisa, Kevin, Simon and Robin at Westfälische Wilhelms-University Münster.

Of course, I want to thank my fellow students, Ben, Benedikt, Martin, Sarah, Tanja and Tobias who have accompanied and supported me during my studies.

Last but not least I thank my family and close friends for their tireless strengthening and motivation, as well as for the always open ear for mine thoughts.

LIST OF PUBLICATIONS AND SCIENTIFIC CONTRIBUTIONS

Talks:

2017 Doktorandenseminar, Hohenroda:
"Investigation of the influence of pesticide sorption to nanoparticles on the mortality of chironomids and toxicity in fungi"
YES-Meeting, Stockholm, Sweden:
"Quantification of pesticides in fungi and midge larvae in the presence of nanoparticles"

2016 LC-MS, Leipzig:
"Capillary electrophoresis: A suitable tool for the analysis of ionogenic compounds in the environment"

Poster:

2017 SETAC, Brussels, Belgium:
"Internal concentration of pesticides in midge larvae and fungi in the presence of nanoparticles"
Wasser 2017, Donaueschingen:
„Vergleich der elektromigrativen Anreicherungsmöglichkeiten der Kapillarelektrophorese und Free-flow Elektrophorese"
ANAKON Tübingen:
"Quantification of pesticides in fungi and midge larvae in the presence of nanoparticles"

2016 Wasser 2016, Bamberg:
"CE-MS in water analysis: suitability and applications for ionic compounds"
SETAC-GLB 2016 Tübingen:
"Determination of the neonicotinoid thaicloprid in *Chironomus riparius* larvae and toxicity changes upon its binding to nanoparticles". Posterpreis

Publications:

Lorenz, C. S.; Wicht, A. J.; Guluzada, L.; Luo ,L.; Jager, L.; Crone, B.; Karst, U.; Triebskorn, R.; Liang, Y.; Anwander, R.; Haderlein, S. B.; Huhn, C.; Köhler, H. R., Nano-sized Al_2O_3 reduces acute toxic effects of thiacloprid on the non-biting midge *Chironomus riparius*. *Plos One* **2017**, *12 (5)*.

Lorenz, C. S.; Wicht, A. J.; Guluzada, L.; Luo ,L.; Jager, L.; Crone, B.; Karst, U.; Lee, H. J..; Liang, Y.; Triebskorn, R.; Anwander, R.; Haderlein, S. B.; Huhn, C.; Köhler, H. R., Nano-sized zeolites as modulators of thiacloprid toxicity on *Chironomus riparius*. *PeerJ* **2017**, *17402*.