Occurrence and fate of emerging organic micropollutants in biological wastewater treatment

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Referent: PD Dr. Thomas A. Ternes

Koreferenten: Prof. Dr. Joachim Scholz
Prof. Dr. Hansruedi Siegrist (Eawag, Dübendorf, Schweiz)

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Summary

More than 10,000 organic chemicals such as pharmaceuticals, ingredients of personal care products and biocides are ubiquitously used in every day life. After their application, many of these chemicals enter the domestic sewer. Research has shown that conventional biological wastewater treatment in municipal wastewater treatment plants (WWTPs) is an insufficient barrier for the release of most of these anthropogenic chemicals into the receiving waters. This bears unforeseen risks for aquatic wildlife and drinking water resources. Especially for recently introduced and/or detected compounds (so called emerging micropollutants), there is a growing need to investigate the occurrence and fate in WWTPs.

In order to get a comprehensive picture on the behavior in municipal wastewater treatment, the following groups of emerging organic micropollutants, spanning a broad range of applications and physico-chemical properties, were selected as target compounds: pharmaceuticals (beta blockers, psycho-active drugs), UV-filters, vulcanization accelerators (benzothiazoles), biocides (anti-dandruffs, preservatives, disinfectants) and pesticides (phenylurea and triazine herbicides). In literature, several analytical methods are already available for the determination of some particular classes of contaminants in specific environmental matrices. However, the **development of multi-residue analytical methods** is the prerequisite to cope with the steadily increasing number of emerging micropollutants present in the environment and to provide a reliable and wider knowledge about their **occurrence** and **partitioning** (sorption), as well as for **monitoring the overall full-scale removal**, and **transformation** of these pollutants in WWTPs.

Consequently, a sensitive **multi-residue method** was developed for the detection of various emerging micropollutants such as biocides, UV-filters and vulcanization accelerators in surface water, wastewater and activated sludge. Special focus was placed on avoiding and compensating matrix effects (ion suppression or enhancement), which significantly influence the sensitivity and reproducibility of analytical methods utilizing liquid chromatography coupled to tandem mass spectrometry (LC tandem MS). The final validated method was based on solid-phase extraction (SPE), followed by analyte detection via LC tandem MS operated with electrospray ionization (ESI) in the positive and negative ionization mode. Due to the use of stable isotope-labeled surrogate standards, most of the 36 selected target compounds were detected with relative recoveries between 70 and 130% down to the low ng L^{-1} and ng g^{-1}

range in the aqueous media and sludge, respectively. Almost all compounds detected in rivers and streams, were also found to be present in WWTP effluents confirming their widespread **occurrence** and the importance of WWTPs as point sources. Maximum concentrations up to 5.1 and 3.9 µg L^{-1} were observed in raw wastewater for the water-soluble and up to now rarely investigated UV-filters benzophenone-4 (BZP-4) and phenylbenzimidazole sulfonic acid (PBSA), respectively. For the first time, the anti-dandruff climbazole was detected in raw wastewater and in activated sludge with concentrations as high as 1.4 µg L^{-1} and 1.2 µg g_{TSS}^{-1}, respectively.

The fate of non-volatile organic micropollutants in WWTPs is partly determined by **partitioning** to activated sludge. The developed analytical method was the basis to examine the sorption behavior of 41 organic micropollutants including biocides, UV-filters as well as phenylurea and triazine herbicides in laboratory-scale sorption batch experiments with activated sludge. Sludge-water distribution coefficients ($K_{d,sec}$) and Freundlich sorption isotherms were determined analyzing the soluble and sorbed compound concentrations. For most compounds, sorption isotherms were linear and hence the $K_{d,sec}$ values independent from the compound concentration. Based on the $K_{d,sec}$ values, which were mainly below 500 L kg^{-1}, removal by sorption onto activated sludge in WWTPs was predicted to be negligible (< 10%). However, higher $K_{d,sec}$ values and predicted removal efficiencies were obtained for the biocides triclocarban ($K_{d,sec}$ = 19000 ± 7000 L kg^{-1}; removal efficiency: 80-95%), triclosan (6400 ± 1400 L kg^{-1}; 55-85%), chlorophene (2200 ± 300 L kg^{-1}; 30-60%), imazalil (2200 ± 600 L kg^{-1}; 25-55%), fenpropimorph (1800 ± 500 L kg^{-1}; 15-40%) and the UV-filter benzophenone-3 (670 ± 220 L kg^{-1}; 5-20%).

The results confirmed that sorption is dominated by ionic (Coloumb) interactions for compounds being charged at the ambient pH of activated sludge (pH 6-8). Furthermore, for non-charged compounds a good correlation was observed between the $K_{d,sec}$ and the octanol-water partition coefficients (K_{OW}). It can therefore be concluded that the sorption affinity of the examined non-charged compounds is mainly influenced by hydrophobic interactions with constituents of the sludge flocs, such as the cell membranes of the microorganisms, and that K_{OW} values allow for an estimation of the full-scale removal of these compounds.

Beta blockers and psycho-active drugs were chosen as target compounds to investigate the **overall full-scale removal** by long-term measurement campaigns along the different

treatment processes of a state-of-the-art WWTP. For both compound groups a potential environmental hazard is indicated from ecotoxicological studies. Beta blockers are used for the treatment of hypertension and coronary artery disease, whereas the class of psycho-active drugs include opium analgesics, tranquilizers and antiepileptics. Therefore, they are among the most prescribed pharmaceuticals and are consequently ubiquitously present in WWTP influents up to the low $\mu g\ L^{-1}$ range.

For beta blockers and psycho-active drugs a partial removal was restricted to the second biological treatment unit with an elevated sludge retention time (SRT) of 18 d (reactor with nitrification), whereas removal in the first biological treatment unit with a SRT of only 0.5 d (reactor for removal of dissolved organic carbon (DOC)) was negligible. However, the removal efficiencies were below 60% for all compounds except for the opium alkaloids codeine and morphine, which were removed by more than 80%, and confirmed the insufficient capacity of current WWTPs for the removal of emerging organic micropollutants. Accompanying laboratory sorption and transformation batch experiments confirmed that the removal of the selected beta blockers and psycho-active drugs in WWTPs is completely attributable to primary biological transformation. The transformation of the selected contaminants in the batch reactors could be well described by pseudo-first order kinetics. Furthermore, the modeling of the biological removal in the full-scale activated sludge reactor, using the biological transformation constants (k_{biol}) determined in the batch experiments, revealed a good agreement with the measured values. For most compounds the removal efficiencies measured on the full-scale WWTP were within the 95% confidence intervals predicted by the model.

The studies of this thesis confirmed that the ultimate fate of emerging micropollutants, besides sorption, is primarily characterized by **transformation** rather than by complete mineralization, resulting in the formation of unknown transformation products (TPs). Consequently, incubation experiments under aerobic conditions were performed with diluted activated sludge to investigate the formation of TPs. The opium alkaloids (codeine, morphine, dihydrocodeine, hydrocodon) were chosen as target compounds due to their extensive transformation observed in the full-scale WWTP sampling campaigns. Focusing on codeine, the chemical structures of TPs were elucidated using a multi-step approach based on TP isolation by semi-preparative high performance liquid chromatography (HPLC) coupled to a fraction collector, followed by high-resolution mass spectrometry (HR-MS) and nuclear magnetic

resonance (NMR) experiments (^{1}H-NMR, ^{13}C-NMR, ^{1}H,^{1}H-COSY, ^{1}H,^{13}C-HSQC). Thereby, the chemical structures of 8 TPs of codeine were unambiguously identified and potential structures of further 10 TPs were proposed for the first time. Most of the identified TPs exhibited only slightly modified molecular structures featuring double bond shifts, introduction of hydroxy groups or amine demethylation. To also confirm the presence of the TPs in full-scale WWTPs, an analytical method was developed based on solid-phase extraction and LC tandem MS detection. The detection of five TPs of codeine in WWTP effluents confirmed that the results of the batch experiments are transferable to full-scale WWTPs.

Elucidation of the transformation pathways in sterile (autoclaved) and non-sterile sludge suspensions revealed for the first time that the transformation of codeine in WWTPs is characterized by a variety of chemical and biological (enzymatic) reactions. Based on the results obtained for codeine, the examination of TPs formed from structurally closely related opium alkaloids allowed for the proposal of chemical structures of 17 TPs of morphine and 2 TPs of dihydrocodeine and hydrocodon. Distinct differences in the transformation pathways of opium alkaloids could be explained by the small variations in their molecular structures. Therefore, this study is one of the first studies, which exhibited the relevance and interconnection of chemical and biological transformation of organic micropollutants during nitrification.

Zusammenfassung

Über 10.000 verschiedene organische Substanzen wie Arzneistoffe, Inhaltsstoffe von Körperpflegemitteln und Biozide werden regelmäßig im Alltag eingesetzt. Die meisten Substanzen gelangen nach ihrer Verwendung in das häusliche Abwasser. Bisherige wissenschaftliche Untersuchungen haben ergeben, dass eine konventionelle biologische Abwasserreinigung in kommunalen Kläranlagen keine ausreichende Barriere für die Emission dieser anthropogenen Stoffe in die Oberflächengewässer darstellt. Dies birgt unvorhersehbare Risiken für die aquatischen Lebensgemeinschaften und die Trinkwasserversorgung. Hinsichtlich ihres Vorkommens und Verhaltens in kommunalen Kläranlagen besteht daher insbesondere für erst kürzlich eingeführte und/oder in der Umwelt nachgewiesene Spurenstoffe (sogenannte „neuartige“ Spurenstoffe, engl.: *„emerging micropollutants“*) ein großer Forschungsbedarf.

Um ein möglichst umfassendes Bild vom Verhalten neuartiger organischer Spurenstoffe in der kommunalen Abwasserbehandlung zu erhalten, wurden im Rahmen dieser Dissertation die folgenden Substanzklassen, welche ein weites Spektrum an physiko-chemischen Eigenschaften aufweisen, untersucht: Arzneistoffe (Betablocker und psychoaktive Substanzen), UV-Filtersubstanzen, Vulkanisationsbeschleuniger (Benzothiazole), Biozide (Antischuppenmittel, Konservierungsmittel, Desinfektionsmittel) und Pestizide (Phenylharnstoff- und Triazinherbizide). Einige analytische Methoden zum Nachweis individueller Substanzengruppen in bestimmten Umweltmatrizes sind bereits literaturbekannt. Die Entwicklung neuer zuverlässiger und sensitiver Multimethoden zum simultanen Nachweis einer Vielzahl von Verbindungen in unterschiedlichen Matrizes ist, unter Berücksichtigung der stetig ansteigenden Zahl an in der Umwelt nachgewiesenen anthropogenen Spurenstoffe, jedoch unabdingbar. Erst dadurch sind weitreichende und zuverlässige Erkenntnisse über **Vorkommen**, **Verteilung** zwischen fester und flüssiger Phase (Sorption) und Abbau (**Transformation**) sowie über die **Eliminationsleistung von Kläranlagen** in Bezug auf organische Schadstoffe möglich.

Zunächst wurde eine sensitive **Multimethode** zum Nachweis zahlreicher neuartiger Schadstoffe wie Biozide, UV-Filtersubstanzen und Vulkanisationsbeschleuniger in Oberflächenwasser, Abwasser und Klärschlamm entwickelt. Die validierte Methode basiert auf einer Festphasenextraktion (SPE), gefolgt von der Detektion mittels LC Tandem MS unter Verwendung positiver und negativer Elektrospray-Ionisation (ESI). Ein besonderer Fokus lag während der Methodenentwicklung auf der Vermeidung und Kompensation von Matrix-

effekten (Ionensuppression oder Ionenverstärkung), da diese die Sensitivität und Reproduzierbarkeit von LC Tandem MS basierten analytischen Methoden erheblich beeinflussen. Durch die Verwendung isotopenmarkierter Surrogatstandards zur Kompensation der Matrixeffekte wurden für die meisten der 36 ausgewählten Substanzen relative Wiederfindungsraten zwischen 70 und 130% und Bestimmungsgrenzen im unteren ng L^{-1} Bereich (wässrige Proben) und ng g^{-1} Bereich (Klärschlamm) erzielt. Substanzen, die in den Oberflächenwasserproben detektiert wurden, waren fast ausnahmslos auch im Ablauf der beprobten Kläranlagen nachweisbar. Dies hebt sowohl das weitverbreitete **Vorkommen** dieser Substanzen als auch die Bedeutung von Kläranlagen als Punktquellen hervor.

Im Zulauf der Kläranlagen wurden für die bisher kaum untersuchten polaren UV-Filter Benzophenon-4 (BZP-4) und Phenylbenzimidazolsulfonsäure (PBSA) maximale Konzentrationen von 5,1 und 3,9 µg L^{-1} gemessen. Erstmalig wurde außerdem der vorwiegend in Antischuppenshampoos eingesetzte Wirkstoff Climbazol im Kläranlagenzulauf (maximal 1,4 µg L^{-1}) und im Klärschlamm (maximal 1,2 µg g^{-1}) nachgewiesen.

Die **Verteilung** zwischen der wässrigen und der festen Phase bestimmt maßgeblich das Verhalten von nicht-volatilen organischen Spurenstoffen in der biologischen Abwasserreinigung. Die neu entwickelte Multimethode bildete die Grundlage zur Untersuchung des Sorptionsverhaltens von 41 organischen Spurenstoffen (Biozide, UV-Filtersubstanzen sowie Triazin- und Phenylharnstoff-Herbizide) in Laborexperimenten mit Belebtschlamm. Aus den gelösten und den sorbierten Konzentrationen wurden die Schlamm-Wasser-Verteilungskoeffizienten ($K_{d,sec}$) und die Sorptionsisothermen nach Freundlich bestimmt. Die Sorptionsisothermen waren für die meisten Substanzen linear und somit war kein signifikanter Einfluss der Konzentration auf die Verteilungskoeffizienten feststellbar. Die gemessenen Verteilungskoeffizienten lagen weitestgehend unter 500 L kg^{-1}. Dies lässt auf eine vernachlässigbare Elimination (<10%) durch Sorption an Belebtschlamm in Kläranlagen schließen. Nur für die Biozide Triclocarban ($K_{d,sec}$ = 19000 ± 7000 L kg^{-1}; Eliminationsleistung: 80-95%), Triclosan (6400 ± 1400 L kg^{-1}; 55-85%), Chlorophen (2200 ± 300 L kg^{-1}; 30-60%), Imazalil (2200 ± 600 L kg^{-1}; 25-55%), Fenpropimorph (1800 ± 500 L kg^{-1}; 15-40%) und die UV-Filtersubstanz Benzophenon-3 (670 ± 220 L kg^{-1}; 5-20%) ergaben sich höhere $K_{d,sec}$-Werte und berechnete Eliminationsleistungen.

Die Ergebnisse bestätigten, dass die Sorption der, im typischen pH-Bereich der Belebung (pH 6-8), geladen vorliegenden Substanzen durch ionische Wechselwirkungen

(Coulomb-Kräfte) beeinflusst werden. Für die ungeladenen Substanzen wurde hingegen eine gute Korrelation mit den Oktanol-Wasser-Verteilungskoeffizienten (K_{OW}) beobachtet. Es ist daher davon auszugehen, dass die Sorptionsaffinität der untersuchten ungeladenen Spurenstoffe maßgeblich durch hydrophobe Wechselwirkungen mit den Bestandteilen der Schlammflocken, wie z.B. den Zellmembranen der Mikroorganismen, bestimmt wird. Die Studie führte daher zu dem Ergebnis, dass eine Abschätzung der sorptiven Elimination der ungeladen vorliegenden Spurenstoffe in Kläranlagen durch die K_{OW}-Werte möglich ist.

Die **Eliminationsleistung** der konventionellen Abwasserbehandlung wurde im Rahmen mehrerer Langzeitmesskampagnen entlang verschiedener Reinigungsstufen einer modernen Kläranlage untersucht. Betablocker und psychoaktive Substanzen wurden als Modellsubstanzen ausgewählt, da ökotoxikologische Untersuchungen auf ein potentielles Umweltrisiko durch deren Umwelteintrag hinweisen. Betablocker werden insbesondere zur Behandlung von Bluthochdruck und bei Erkrankung der Herzkranzgefäße eingesetzt, während die Gruppe der psychoaktive Substanzen Opioidanalgetika, Beruhigungsmittel und Antiepileptika beinhaltet. Da sie zu den am häufigsten verschriebenen Arzneistoffen gehören, sind sie weitverbreitet und in Konzentrationen bis in den unteren $\mu g\ L^{-1}$ Bereich in Kläranlagenzuläufen nachzuweisen.

Die Elimination von Betablockern und psycho-aktiven Substanzen war auf die zweite biologische Stufe mit einem Schlammalter von 18 d (Stufe mit Nitrifikation) beschränkt. In der ersten Stufe mit einem Schlammalter von nur 0,5 d (Stufe zur Entfernung der gelösten organischen Stoffe - DOC) war hingegen keine signifikante Reduktion der Substanzfrachten feststellbar. Allerdings lag die Effizienz der Elimination mit Ausnahme der Opiumalkaloide unter 60% und bestätigte die unzureichende Leistungsfähigkeit konventioneller Kläranlagen zur Entfernung neuartiger organischer Schadstoffe.

Durch begleitende Laborexperimente zum Abbau und zur Sorption konnte gezeigt werden, dass die Elimination in Kläranlagen hauptsächlich auf biologischen Primärabbau und nicht auf Sorption zurückzuführen ist. Der im Labor beobachtete Primärabbau der Betablocker und psychoaktiven Substanzen konnte sehr gut durch eine Kinetik pseudo-erster Ordnung beschrieben werden. Darüber hinaus ergab die Modellierung der Eliminationseffizienz der zweiten biologischen Reinigungsstufe der Kläranlage unter Verwendung der in den Laborexperimenten ermittelten biologischen Abbaukoeffizienten (k_{biol}) eine gute Übereinstimmung mit den gemessenen Werten. Die in der Kläranlage gemessenen Werte lagen für die

meisten Substanzen innerhalb der mittels Monte-Carlo-Simulation abgeschätzten 95%-Konfidenzintervale der modellierten Werte.

Die vorliegende Dissertation belegt, dass die Elimination anthropogener organischer Spurenstoffe in der Kläranlage insbesondere durch **Transformation** und nicht durch einen vollständigen Abbau im Sinne einer Mineralisierung erfolgt. Die biologische Abwasserreinigung führt daher zur Bildung von Transformationsprodukten (TPs), deren Identität und Risikopotential weitestgehend unbekannt sind. Vor diesem Hintergrund lag der Fokus weiterer Untersuchungen auf der Durchführung aerober Abbauversuche mit verdünntem Belebtschlamm zur Identifizierung bisher unbekannter TPs. Die Opiumalkaloide (Codein, Morphin, Dihydrocodein und Hydrocodon) dienten dabei als Modellsubstanzen, da diese Substanzen einen deutlichen Abbau im Rahmen der Kläranlagenmesskampagnen gezeigt hatten. Ausgehend von Codein wurden die chemischen Strukturen der TPs, nach ihrer Isolierung mittels an einen Fraktionssammler gekoppelter semi-präparativer Hochleistungsflüssigkeitschromatographie (HPLC), durch hochauflösende Massenspektrometrie (HR-MS) und Kernresonanzspektroskopie (NMR: ^{1}H-NMR, ^{13}C-NMR, ^{1}H,^{1}H-COSY, ^{1}H,^{13}C-HSQC) charakterisiert. Auf diese Weise wurden erstmalig 8 TPs von Codein eindeutig identifiziert und für 10 weitere TPs wurden Strukurvorschläge erarbeitet. Die meisten der identifizierten TPs wiesen nur geringe strukturelle Veränderungen, wie z.B. Hydroxylierungen, Demethylierung und/oder die Verschiebung einer Doppelbindung, auf. Um das Vorkommen der TPs in Kläranlagen zu bestätigen, wurde eine auf Festphasenextraktion und LC Tandem MS basierende analytische Methode entwickelt. Mit Hilfe dieser Methode wurden 5 TPs von Codein in Kläranlagenabläufen nachgewiesen und damit die Übertragbarkeit der Laborergebnisse auf die Kläranlage bestätigt.

Zur Aufklärung des Abbauweges wurden Codein und einige der isolierten TPs in sterilen (autoklavierten) und nicht sterilen Schlammsuspensionen inkubiert. Dadurch wurde erstmalig gezeigt, dass der Abbau von Codein in Kläranlagen durch die Kombinationen verschiedener chemischer und biologischer (enzymatischer) Reaktionen charakterisiert ist. Basierend auf diesen Ergebnissen war es durch die Untersuchung von weiteren strukturell ähnlichen Opiumalkaloiden möglich, Strukturvorschläge für 17 TPs von Morphin und jeweils 2 TPs von Dihydrocodein und Hydrocodon zu erarbeiten. Die teilweise deutlichen Unterschiede zwischen den Abbauwegen konnten durch die geringfügigen Abweichungen in der molekularen Struktur der Opiumalkaloide erklärt werden. Diese Studie ist damit eine der ersten, die die

Relevanz und die Verknüpfung biologischer und chemischer Abbauprozessen in der Nitrifikation belegt.

1. General Introduction

1.1 Chemical pollution of water resources

The ongoing increase of the human population and economic growth is leading to a continuous demand for clean water resources. More than one-third of the Earth's accessible renewable freshwater is already used for agricultural, industrial and domestic purposes and water withdrawals are predicted to increase by 50 percent by 2025 in developing countries, and 18 percent in developed countries (UNEP, 2007). Even in Europe, 60 percent of the cities with more than 100,000 inhabitants use groundwater in a higher quantity than it can be replenished (Stanners and Bourdeau, 1995). The water usage is often linked to a contamination with numerous synthetic and geogenic organic and inorganic compounds as well as pathogens. Hence, the protection of our water resources from water exploitation and anthropogenic pollution with chemicals and pathogens is indispensable for preserving ecologic and human health (Field et al., 2006; Kolpin et al., 2002; Schwarzenbach et al., 2006).

Modern society benefits from the high number of chemicals available for various applications in industry, agriculture, health care and sanitation as well as for household purposes. Approximately 100,000 different synthetic organic chemicals have been registered over the past 30 years for commercial use in the United States and the European Union (EU). The estimated number of chemicals marketed in the EU in volumes exceeding 1 t y^{-1} is about 30,000 (Kolpin et al., 2002; Muir and Howard, 2006). Most of these chemicals reach natural waters mainly through municipal and industrial wastewater streams, chemical spills as well as diffuse sources from agriculture. Consequently, it is expected that up to several thousand synthetic organic pollutants occur at concentration levels below the mg L^{-1} range, as so called organic micropollutants, in natural waters. However, only a few of them have been examined so far in regard to their actual occurrence, fate and ecotoxicity (Schwarzenbach et al., 2006).

During the last three to four decades numerous studies confirmed that the release of organic chemicals into the environment is a potential hazard for human and ecological health and led to an increased scientific and public awareness of this problem (Daughton and Ternes, 1999; Kolpin et al., 2002; Schwarzenbach et al., 2006). For instance, accidents at two chemical production facilities in Basel in 1986 led to the release of tons of pesticides into the river Rhine with lethal effects on aquatic organisms and contamination of the bank filtrate used for drinking water production (Kruhm-Pimpl, 1993). Another prominent example highlighting

the potential adverse effects of the continuous application of organic chemicals on aquatic ecosystems was the imposex in sea snails caused by triorganic antifouling agents leaching from ship paints (Smith, 1981).

1.2 Emission routes and occurrence of emerging organic micropollutants

Until the end of the 1990s, research regarding the impact of water pollution has focused almost exclusively on "conventional" priority substances and persistent organic pollutants (POPs), such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs) and pesticides, which are today regulated under national and international directives and conventions (Daughton and Ternes, 1999; Ternes et al., 2004a).

In recent years, so called emerging organic micropollutants received increasing scientific and public attention. Emerging organic micropollutants are generally defined as non-regulated organic trace pollutants just recently introduced or newly detected due to advanced analytical technologies (Richardson, 2007). In addition, compounds which experienced a shift in their application such as agricultural pesticides being also (or nowadays even exclusively) used in biocidal products in urban areas (see chapter 1.2.3) can be considered as emerging pollutants. Moreover, it comprises chemical and biological transformation products (TPs) and naturally occurring substances with previously unrecognized adverse effects such as algal toxins (Petrović and Barceló, 2006).

Emerging organic micropollutants often feature a relatively high polarity and/or low volatility making them not amendable to GC-MS analysis without derivatization. Thus, especially the fast development of liquid chromatography coupled with tandem mass spectrometry (LC tandem MS), which allows for the selective and sensitive detection of compounds covering a wide polarity range in various matrices, led to the detection of several contaminants never considered before (Richardson and Ternes, 2005). Examples of groups of emerging organic micropollutants detected during the last 15 years in water resources are pharmaceuticals, hormones, iodinated X-ray contrast media, personal care products such as UV-filters and preservatives, surfactants such as perfluoroalkyl substances and alkylphenole ethoxylates, disinfection by-products, brominated flame retardants and biocides such as film preservatives and antifoulings (Richardson and Ternes, 2005; Richardson, 2007, 2009; Schwarzenbach et al., 2006). In the following the occurrence and main emission routes will be discussed in detail for those classes of organic contaminants being subject of this thesis.

1.2.1 Human Pharmaceuticals

Human pharmaceuticals, including natural and synthetic hormones and iodinated X-ray contrast media (ICMs), gained increasing interest since the late 1990s (Daughton and Ternes, 1999; Ternes, 1998). The literature of these compounds is most abundant among the emerging micropollutants (Richardson, 2009). Many pharmaceuticals are regularly and widely consumed in private households, hospitals, retirement homes and radiological practices. After administration some pharmaceuticals are metabolized in the body and a mixture of the unaltered drug and its metabolites are excreted with urine and feces. Hence, these compounds reach municipal wastewater treatment plants (WWTPs) via their normal course of treatment or by improperly disposal in toilets. Since most pharmaceuticals feature a high persistency and polarity, many of them are not or only incompletely removed by conventional biological wastewater treatments and therefore are continuously discharged into the receiving water (Ternes et al., 2004a; Ternes and Joss, 2007).

Hence, a high number of more than 160 different pharmaceuticals have already been detected in low ng L^{-1} to low µg L^{-1} range in the aquatic environment (Kümmerer, 2010). However, considering that about 3,000 different pharmaceuticals are currently used in the EU and taking into account the human metabolites and the numerous mostly unknown TPs formed by chemical and biological transformation in WWTPs and environmental compartments, it can be expected that the actual number of emerging contaminants derived from pharmaceuticals is significantly higher and hardly to predict (Kümmerer, 2010; Richardson and Ternes, 2005). Among the various groups of pharmaceuticals already detected in wastewater and surface water are psycho-active drugs such as antiepileptics, antidepressives and analgesics, beta blockers, non-steroidal anti-inflammatory drugs, antibacterial drugs, lipid-regulating agents (Hummel et al., 2006; Kasprzyk-Hordern et al., 2008; Ternes et al., 1998), ICMs (Ternes and Hirsch, 2000) and antiviral drugs (Prasse et al., 2010).

1.2.2 Personal care products

In contrast to pharmaceuticals, studies about the occurrence and fate of ingredients of personal care products are rare and focused mainly on a small set of compounds such as musk fragrances, non-polar UV-filters and some biocides such as parabenes and triclosan (Nieto et al., 2009). However, personal care products, such as shampoos, soaps, sun screens, perfumes, etc., are used world-wide in huge quantities and more than thousand different ingredients can be expected. The actual annual production of PCPs has been reported to be around 800,000 t in Germany (Tolls et al., 2009). The compounds used as ingredients in PCPs comprise several

groups such as surfactants, fragrances (e.g. nitro- and polycyclic musks), UV-filters (e.g. benzophenone derivatives), preservatives (e.g. isothiazolinones) and antimicrobial agents (e.g. triclosan). The main emission source of PCPs are WWTPs. In contrast to pharmaceuticals, PCPs are not intended for ingestion but are mainly used as rinse-off products entering the wastewater after regular showering and bathing. However, PCPs such as UV-filters can also be directly introduced by swimming in recreational waters (Daughton and Ternes, 1999; Ternes et al., 2004a). PCPs have been shown to be ubiquitously present in wastewater and surface waters. Since PCPs feature quite different physico-chemical properties, they have distinct environmental fates. For instance, musk fragrances as well as many UV-filters are non-polar lipophilic compounds with octanol-water partition coefficients (K_{OW}) above 4 and thus are primarily sorbed to the solid phase and possess a high bioaccumulation potential. Hence, these compounds are partially removed in WWTPs by partition to the sludge and are therefore released into the environment via the application of digested sludge to agricultural land and/or via the effluents to surface waters (Bester, 2007; Plagellat et al., 2006). For instance, the polycyclic musks galaxolide and tonalide were found in 90% of the samples from several rivers and streams in the region Hessen in Germany with mean concentrations of 141 and 46 ng L^{-1} (Quednow and Püttmann, 2007), but also in soils after application of digested sludge at mean concentrations of 1.0 and 1.3 µg kg^{-1} (Yang and Metcalfe, 2006), respectively.

In contrast, UV-filters such as phenylbenzimidazole sulfonic acid and benzophenone-4 are very polar ($K_{OW} < 1$) and quite persistent and have just recently been detected in wastewater effluents with levels up to 1.4 and 2.7 µg L^{-1}, respectively. Benzophenone-4 was also found in drinking water in the low ng L^{-1} range (Rodil et al., 2009).

1.2.3 Biocides

Biocides regulated by the EU Biocidal Products Directive (BPD) 98/8/EC (European Commission, 1998) are generally defined as compounds used to destroy or inhibit the growth or action of certain organisms applied in products of non-agricultural use such as disinfectants for home and industrial use, preservatives for manufactured and natural products and non-agricultural pesticides for use against insects, rodents and other vertebrates. Many biocides are ingredients of PCPs or are also used for agricultural purposes. Hence, there are many borderline cases between the BPD and other directives such as the Plant Protection Products Directive (PPPD) and the Cosmetic Products Directive (CPD). Many compounds listed as biocides are also used as pesticides in agriculture. On the other hand, certain compounds with biocidal activity such as anti-dandruffs are not listed in the biocide directive, since they are

regulated exclusively in the cosmetic directive. For the sake of clarity, in this thesis all compounds with a biocidal activity in a way as defined above, which are used in non-agricultural products, are denoted as biocides.

Exposure routes bringing biocides into groundwater and surface water are more diverse than for human pharmaceuticals (Figure 1-1). Biocides used in PCPs and cleaning agents enter surface waters mainly via their discharge into municipal WWTPs. Leaching and runoff of biocides, used for examples as herbicides on lawn or as preservatives in coatings and roof sealings, lead to their infiltration into soil or their discharge into the sewer system. While in case of separate sewer systems these biocides directly reach the surface waters, combined sewer systems lead to their discharge into WWTPs from where they might be released into the receiving water (Gerecke et al., 2002; Nitschke and Schüssler, 1998). For biocides with a high sorption affinity, such as the quaternary ammonium compounds and triclosan, diffuse emission via agricultural use of contaminated sludge from WWTPs might be also of importance.

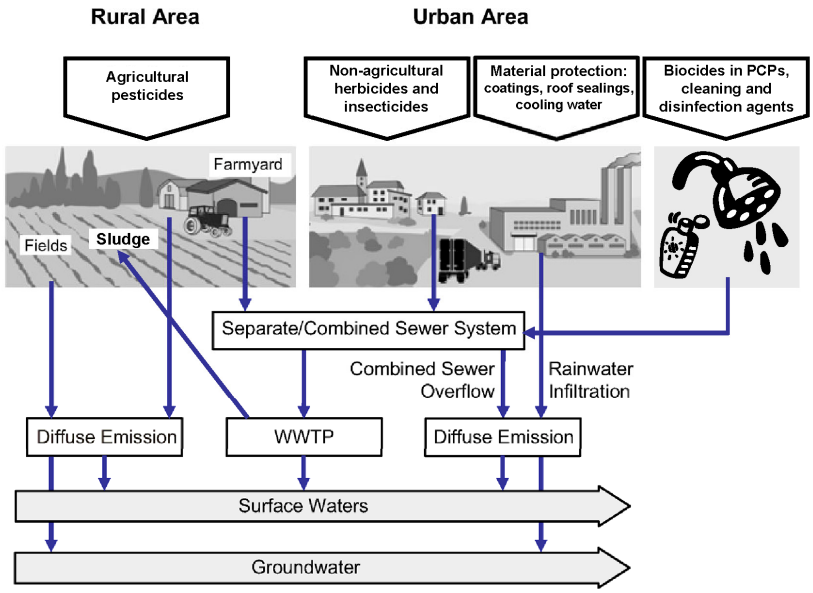


Figure 1-1. Pathways of input and distribution of biocides in the environment (adapted from Gerecke et al., 2002).

WWTPs were found to be an important point source also for biocides used both for agricultural and non-agricultural purposes. For example, a monitoring of WWTPs effluents in Switzerland revealed maximum effluent concentrations of the herbicides diuron and mecoprop and the fungicide carbendazim in the high ng L^{-1} to low µg L^{-1} range (Kupper et al., 2005). Median concentrations of about 550 ng L^{-1} of the triazine herbicide terbutryn measured in two small Hessian streams (Germany) between 2003 and 2006 were in accordance with high concentrations detected in the effluents of two adjacent WWTPs (Quednow and Püttmann, 2007).

1.3 Consequences

1.3.1 Ecotoxicological hazard

Pharmaceuticals, hormones and biocides have a designated biological activity, raising concerns regarding their potential to endanger non-target species by negatively effecting their metabolism even at low environmental concentrations. Acute or chronic effects of pharmaceuticals on aquatic and/or benthic organisms have been already confirmed by many studies, but in most cases the identified lowest observed effect concentrations (LOECs) were substantially above the measured environmental concentrations (MECs) (Fent et al., 2006). However, there are a few notable exceptions. For example, the pharmaceuticals carbamazepine, diclofenac and metoprolol have been shown to impair the general health conditions of rainbow trouts via cytological alterations of the liver, kidney and gills with lowest observed effect concentrations in the range of environmental relevant concentrations (1 µg L^{-1}) (Triebskorn et al., 2007). The synthetic hormone 17α-ethinylestradiol (EE2) used as contraceptive is a very potent estrogenic compound, which had been shown to reduce egg fertilization success and skewed sex ratios towards females of fat head minnow even at concentrations below 1 ng L^{-1} (Parrot and Blunt, 2005). The temporally breakdown of the complete fat had minnow population of a lake in Canada due to an EE2 concentration of 5-6 ng L^{-1} highlights that low environmental relevant concentrations of emerging micropollutants can have severe effects on a population level.

Biocides are a group of emerging micropollutants of particular ecotoxicological concern, since they are designated to kill organisms or at least inhibit their growth and distribution. Indeed, there are several examples confirming their ecotoxicological potential even in the ng L^{-1} range. For example, mesocosm studies have shown that the antifouling agent irgarol causes a shift in the community structure of macrophytes leading to the currently dis-

cussed environmental quality standard of only 2 ng L^{-1} (German Federal Environmental Agency, 2009; Mohr et al., 2009). Another example, is the bactericide triclosan which is ubiquitously present in rivers and streams of urban areas and has been shown to impact the metamorphosis of the North American bullfrog *Rana catesbeiana* and to cause a shift in the community structure of river algae (Veldhoen et al., 2006; Wilson et al., 2003).

Even emerging contaminants without a designated biological activity can be harmful for aquatic organisms. For instance, several UV-filters, phthalates used as plasticizers, and bisphenol A used in the manufacture of polycarbonate plastics are so called endocrine disruptive compounds which interfere with natural hormones (Fent et al., 2008; Oehlmann et al., 2006).

These few examples reveal that adverse effects cannot be ruled out, although individual concentrations of emerging pollutants are often low (usually in the ng L^{-1} range). This is especially relevant, if possible additive effects are also considered. In general, chronic effects occur at much lower concentrations, but experiments are much more elaborated and time-consuming and are often difficult to confirm (Schwarzenbach et al., 2006).

Since the TPs of most emerging micropollutants are still unknown or the availability of reference standards for toxicity testing is limited, information regarding the ecotoxicological potential of chemical and biological TPs is scarce. However, recent studies revealed that ozonation of wastewater (see chapter 1.5.4) can lead to the formation of TPs with toxicophoric structures such as aldehyde and bialdehyde moieties (Benner and Ternes, 2009) as well as to considerable developmental retardation of rainbow trouts (*Oncorhynchus mykiss)* (Stalter et al., 2010). Even biological transformation is not necessarily connected with detoxification as demonstrated for example by the biological transformation of clofibric acid (active metabolite of blood lipid regulators) to 4-chlorphenol, which is expected to have a higher toxicological potential than the parent compound (Kosjek et al., 2009).

1.3.2 Drinking water contamination

As outlined above, many emerging micropollutants are quite polar and persistent compounds continuously released into surface waters due to an incomplete removal in WWTPs. Consequently, these compounds are also prone to pass subsequent bank filtration or soil passage leading to a contamination of drinking water resources (Heberer, 2002; Stackelberg et al., 2004). Especially if drinking water treatment only consists of a single-stage process with the main objective of disinfection, these compounds can find their way into drinking water leading to indirect potable reuse (Ternes and Joss, 2007). Since most TPs have a higher envi-

ronmental mobility and persistence and often occur at a higher concentrations in surface water and groundwater than the corresponding parent compounds, they are favored to occur in drinking water resources (Kolpin et al., 2002). For instance, TPs of the ICMs iomeprole, iohexole and iopromide were detected in drinking water even after activated carbon treatment with a maximum concentration for individual compounds of 500 ng L^{-1} (Kormos et al., 2010). However, for most emerging contaminants the formation and identity of TPs and therefore their occurrence in drinking water resources and finished drinking water is currently unknown.

Even though no toxicological risks for consumers of drinking water have been identified so far, the contamination of groundwater and drinking water should be avoided due to the wide variety of unknown aspects and risk potentials especially in regard to the unknown TPs. For example, ozonation of water resources containing N,N-dimethylsulfamide, which is a biological TP of the fungicide tolylfluanide, has been recently shown to lead to the formation of carcinogenic N-nitroso-dimethylamine (NDMA) (Schmidt and Brauch, 2008).

1.4 Regulation

In the European Union several directives have been established for the approval of chemical products and their ingredients such as human pharmaceuticals, veterinary pharmaceuticals, biocides, pesticides, ingredients of cosmetics, etc. However, the occurrence and reduction of chemicals in surface waters is currently regulated only for 33 micropollutants (further 11 compounds are subject for review) by the EU legislation under the Water Framework Directive (WFD) (European Commission, 2008). One tool is the implementation of environmental quality standards (EQSs) for these micropollutants. In those cases where the EQS values of the WFD are exceeded, measures have to be taken to reduce the emission of these priority substances into the corresponding water bodies. Moreover, 13 substances are currently defined as priority hazardous substances. The emission of these substances are envisaged to be completely phased out within the next 20 years.

Up to now, emerging micropollutants are only marginally addressed and pharmaceuticals and PCPs are not included in the current list. However, this list has to be reviewed at least every four years and provisions have been made to add several hazardous emerging contaminants with EQS values based on reliable ecotoxicity data. Moreover, regarding the implementation of the WFD in German national law, a directive for the protection of surface water with additional EQS values for up to 173 additional river basin specific micropollutants including

emerging pollutants is currently in preparation (Entwurf der Verordnung zum Schutz der Oberflächengewässer vom 01.08.2010).

1.5 Wastewater treatment

State-of-the-art wastewater treatment mainly consists of a mechanical pre-treatment and a primary clarification for the removal of big and small particles, followed by a biological treatment with nitrification, denitrification and a final clarification for the removal of the soluble bulk organic load as well as nitrogen and phosphorous. These WWTPs are not designed for an efficient removal of organic micropollutants and the current knowledge of the treatment processes is very much tied to its designated scope of avoiding eutrophication in surface and groundwater. In the following, some basic concepts of biological wastewater treatment are outlined which have been shown to affect the removal efficiency of organic micropollutants. The two main removal processes for the removal of non-volatile organic micropollutants in conventional WWTPs are biological/chemical degradation and sorption onto primary and secondary sludge (Figure 1-2). However, it has to be pointed out that biological as well as chemical degradation does not necessarily result in complete degradation, i.e. mineralization to H_2O, CO_2, N_2, etc. Often compounds are only primarily degraded, i.e. transformed, into stable transformation products (TPs). Accordingly, in this doctoral thesis the term degradation is used in a general way implying both mineralization and primary degradation processes, while transformation is specifically used for primary degradation. Similarly, also the sorption of compounds onto sludge does often not lead to an elimination. If the sludge is not incinerated but used as fertilizer, the parent compounds and/or the TPs formed during sludge digestion will be released into the environment (Figure 1-2).

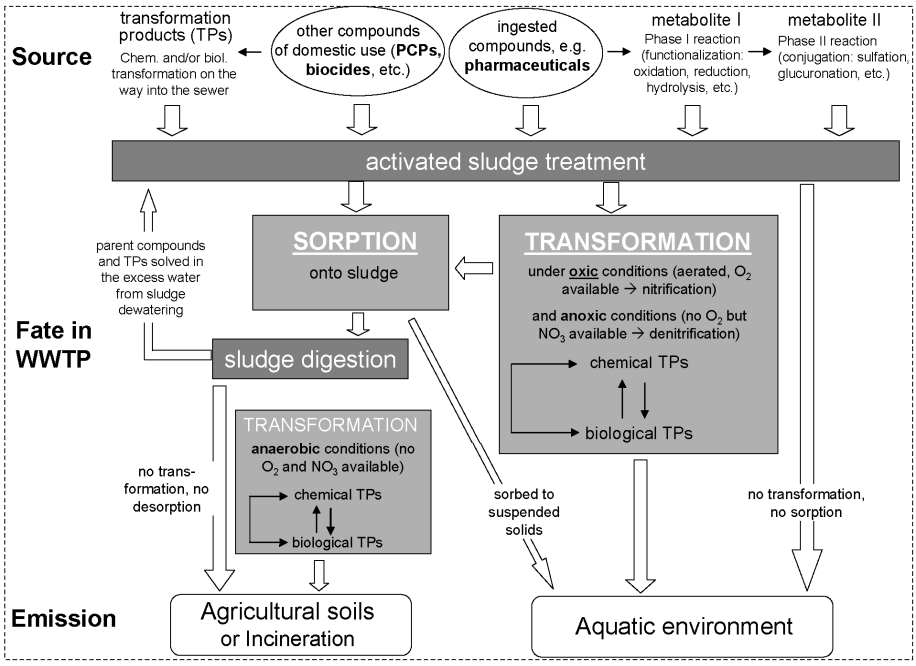


Figure 1-2. Scheme of the fate of non-volatile emerging organic micropollutants in conventional WWTPs illustrated for pharmaceuticals and their human metabolites as well as for other compounds of domestic use and their possible TPs formed on their way into the sewer. Sorption and transformation reactions in the sewer and in the primary clarifier of the WWTP are not mentioned. The processes which were examined within this thesis are underlined.

1.5.1 Biological removal – General principles

The bacterial biomass, responsible for the degradation of organics in the activated sludge reactor, is limited by the supply of nutrients and bulk organics defining the bacteria growth rates and by the dilution rate (wash-out effect). To achieve a higher biomass and therefore a higher total suspended solids concentration (TSS), settled sludge from the final clarifier is pumped back into the activated sludge reactor (sludge recycle) (Mudrack and Kunst, 1994). Hence, the biomass is unnaturally high and the growth situation is characterized by starvation. Consequently, according to the Michaelis-Menten kinetic, an increase of the substrate concentration, i.e. the sludge load defined as the five-day biological oxygen demand (BOD5) per TSS and time ($kg_{BOD5}\ kg_{TSS}^{-1}\ d^{-1}$), increases also the rate of the enzyme catalyzed degradation reactions and therefore the overall removal efficiency for bulk organics. This gives the system a certain flexibility against short-term variations of the organic load (Cypionka, 1999; Mudrack and Kunst, 1994).

To maintain a stable sludge concentration, the daily sludge production (SP) has to be withdrawn from the system as excess sludge. The sludge retention time (SRT) in the activated sludge tank is therefore defined as the ratio of TSS in the activated sludge tank and the daily

amount of sludge withdrawn by the excess sludge and the effluent (Mudrack and Kunst, 1994; Ternes and Joss, 2007):

$$SRT = \frac{V \cdot TSS}{Q \cdot TSS_{out} + Q_{ex} \cdot TSS_{ex}} \quad (1\text{-}1)$$

SRT sludge retention time [d]
V reactor volume [m^3]
TSS total suspended solids concentration in the reactor [$kg_{TSS}\ m^{-3}$]
TSS_{out} total suspended solids concentration in the effluent [$kg_{TSS}\ m^{-3}$]
TSS_{ex} total suspended solids concentration in the excess sludge [$kg_{TSS}\ m^{-3}$]
Q flow rate of the treated wastewater [$m^3\ d^{-1}$]
Q_{ex} flow rate of the excess sludge [$m^3\ d^{-1}$]

These very basic considerations have some important consequences for the removal of bulk organics and micropollutants in a completely stirred reactor (CSR): High removal rates require high sludge loads, i.e. high amounts of substrate for bacterial growth, and thus also imply unwanted elevated organic loads in the effluent of the CSR. Moreover, high sludge loads lead to an increase of the sludge production and the excess sludge and hence to a lower SRT if the suspended solids concentration is kept stable (Mudrack and Kunst, 1994). A low SRT implies a short generation time of bacteria favoring a bacterial community dominated by unspecialized heterotrophic bacteria with an enzyme pool allowing for a fast growth on bulk organics. These conditions are not favorable for the removal of generally low concentrated not rapidly biodegradable organic micropollutants (Mudrack and Kunst, 1994; Ternes and Joss, 2007). In contrast, studies on the removal of pharmaceuticals at different SRTs indicate a positive correlation of the SRT with the removal efficiency. This can be explained by an enrichment of slowly growing bacteria leading to a higher specialization level of the biocoenosis connected with increased enzymatic capabilities of the sludge (Clara et al., 2005; Saikaly et al., 2005; Ternes and Joss, 2007).

A technical solution to achieve both high removal rates for the bulk organics and micropollutants and a relatively low organic load in the effluent is the implementation of subdivided reactors (reactor cascade) characterized by successively decreasing organic loads and removal rates. Another possibility allowing also different SRTs and hence the establishment of different biocoenosis is the installation of two or more separate reactors operated with different SRTs (multi-step treatment). A first high loaded reactor with a low SRT for a first reduction of the bulk organic load (chemical oxygen demand (COD)-removing reactor) is followed by a reactor with a lower sludge load and a higher SRT for a further reduction of the

remaining organic load and nitrogen removal (nitrogen-removing reactor) (Joss et al., 2006b; Mudrack and Kunst, 1994).

Biological nitrogen removal is accomplished by the oxidation of ammonia (NH_3) to nitrate (NO_3^-) (nitrification) and the reduction of NO_3^- to nitrogen gas (N_2) (denitrification) under oxic (O_2 available) and anoxic (no O_2 but NO_3^- available) conditions, respectively. The process of nitrification can be subdivided into two steps carried out by autotrophic bacteria: First, ammonium is oxidized to hydroxylamine (NH_2OH) by the membrane bound enzyme ammonia monooxygenase (AMO), followed by a further oxidation to nitrite (NO_2^-) by the periplasmatic enzyme hydroxylamine oxidoreductase (HOA). The bacteria responsible for this nitration process all belong to the genera Nitrosomonas, Nitrosospira and Nitrosococcus (Madigan et al., 1999). The next step is the oxidation of NO_2^- to NO_3^- catalyzed by the enzyme nitrite oxidoreductase (NOR) and is carried out by bacteria from the genera Nitrobacter, Nitrospina and Nitrospira. The energy gain from the oxidation of ammonium and nitrate is relatively low leading to slow growth rates of approximately 0.8 d^{-1} for Nitrosomonas and 1.0 for Nitrobacter at 20°C. For a considerable nitrification, a sludge retention time of 2-3 times the generation time is required. Considering the influence of temperature, the minimum SRT for a year round nitrification should be at least in the range of 8-10 d (Mudrack and Kunst, 1994; Ternes and Joss, 2007). The results of some recent degradation studies indicate that nitrifyers are also involved in the transformation of micropollutants via a co-metabolic pathway or by chemical reactions of the nitrite intermediate with phenolic micropollutants (see chapter 1.5.2).

The denitrification is accomplished by heterotrophic bacteria which can use NO_3^- instead of O_2 as final electron acceptor using the same electron transport chain. The reduction of NO_3^- to N_2 proceeds via the formation of NO_2^-, nitrogen monoxide (NO) and nitrous oxide (N_2O) catalyzed by 4 different membrane-associated enzymes. Many bacteria are capable of switching to nitrate respiration in absence of oxygen. Hence, the absence of oxygen and a sufficient supply with organic substrates is prerequisite for denitrification in WWTPs (Cypionka, 1999; Madigan et al., 1999; Mudrack and Kunst, 1994). A common technical solution is the implementation of the denitrification prior to nitrification, so that the denitrifying bacteria can use the complete organic load of the influent, while being supplied with nitrate from nitrification by the sludge recycle. In some WWTPs an additional denitrification step (post-denitrification) is implemented behind the nitrification step to achieve an additional reduction of the concentration of nitrate in the effluent. Since the DOC in the effluent of the

nitrification is generally too low for an effective denitrification, an additional carbon source such as methanol is dosed into the influent of the post-denitrification (Mudrack and Kunst, 1994). The role of denitrification for the removal of micropollutants has been rarely addressed. So far, studies with estrogens and pharmaceuticals showed significantly lower removal efficiencies under anoxic denitrifying conditions compared to oxic nitrifying conditions (Joss et al., 2004; Suarez et al., 2010).

Although several tentative models have been proposed in literature to describe micropollutant removal by biological transformation, pseudo first order kinetic is the only one that has been validated at full scale under typical conditions (Joss et al., 2004). However, the availability of first order biotransformation constants (k_{biol}) usually derived from laboratory-scale batch experiments is limited and hence the model validation restricted to a few compounds (Joss et al., 2004, 2006b; Maurer et al., 2007). Moreover, the influence of the batch incubation conditions as well as the full-scale process conditions such as temperature, redox conditions and SRT on the compound specific k_{biol} values is currently not predictable.

1.5.2 Formation of transformation products

Several studies recently indicated that for the majority of organic micropollutants microbial degradation does not lead to mineralization or incorporation into the biomass (anabolism) but rather to the formation of stable TPs (Helbling et al., 2010a; Kosjek et al., 2007). The chemical structure of the TPs is often only slightly modified featuring an increased polarity due to the introduction of a hydroxyl-, carboxyl-, nitro or keto group. This makes them prone for groundwater contamination (Kormos et al., 2010; see chapter 1.3.2). According to the similarity of their molecular structure, some TPs formed are expected to possess comparable biological activities as their parent compounds raising concerns regarding their ecotoxicological hazard (Sinclair and Boxhall, 2003; Van Zelm et al., 2010; see chapter 1.3.1). Due to the low *in situ* concentrations of most organic micropollutants, it can be assumed that their transformation is mainly attributable to co-metabolic processes, i.e. transformation by enzymes produced for other primary purposes. This is supported by pseudo first-order transformation kinetics without any lag phase often observed in transformation studies with activated sludge and compound spiking levels <1 mg L^{-1} (Helbling et al., 2010b; Joss et al., 2006b).

However, a general understanding of the transformation pathways of organic micropollutants in biological wastewater treatment at environmental relevant concentrations and of the corresponding enzymes involved is still lacking. So far, well-known transformation rules

from literature, which have also been implemented in prediction tools, mainly derived from studies with pure cultures spiking the compound of concern as single carbon source. Hence, transformation pathways predicted based on these general rules might not be representative for natural environmental systems with mixed bacterial consortia and high organic loads such as WWTPs (Helbling et al., 2010b). Even though TPs have gained increasing interest as water contaminants, only a few studies have looked at the biological transformation of emerging organic micropollutants in contact to activated sludge at aerobic conditions and even less at anoxic or anaerobic conditions. One reason is the challenge of structural elucidation of TPs in natural media, especially if low concentrations of the target compound are applied. Hence, the identification of TPs and the corresponding transformation pathway is often very time-consuming and requires the use of sophisticated analytical techniques such as hybrid high-resolution mass spectrometry and nuclear magnetic resonance (NMR) (Kormos et al., 2009; Kosjek et al., 2007). Table 1-1 summarizes recent results from aerobic transformation studies with emerging organic micropollutants. If not indicated otherwise, the studies listed in table 1-1 used laboratory batch or continuous flow reactors with activated sludge and elevated concentrations of the parent compounds. Since mass balances and/or data on the occurrence of the identified TPs in WWTP effluents or natural water resources are often missing, the transferability and importance of the results with regard to full-scale plants or environmental compartments are often not confirmed. Only a few studies focused on the elucidation of the transformation pathways or on a systematic examination of the influence of certain features of the compound structure on distinct transformation reactions (Helbling et al., 2010b; Kormos et al., 2010; Schulz et al., 2008).

So far, the following enzyme-catalyzed reactions turned out to be quite commonly involved in the transformation of emerging organic micropollutants: mono- and dihydroxylation, alcohol and aldehyde oxidation, ester and amide hydrolysis, N-dealkylation and decarboxylation. Even though these basic reactions are known to be catalyzed by many different ubiquitous and constitutive enzymes with a wide substrate specificity, certain structural differences can discriminate between alternate transformation pathways (Helbling et al., 2010b). For instance, the secondary amide bezafibrate is cleaved by amide hydrolysis while other compounds featuring also secondary amide moieties such as TPs of ICMs and glibenclamide are not subject of amide hydrolysis (Table 1-1).

According to the overview given in table 1-1, the introduction of oxygen by monooxygenases is one of the most frequently observed reaction involved in the transformation of or-

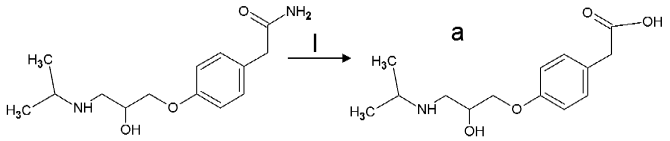
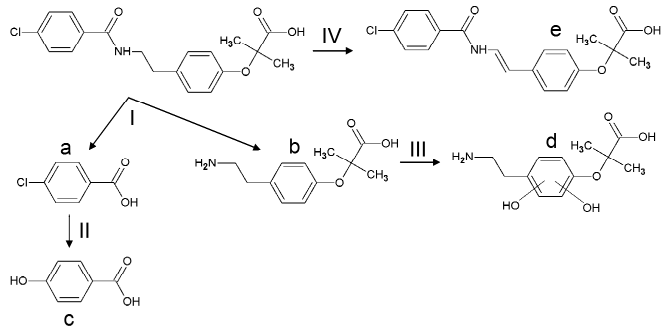
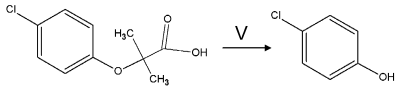
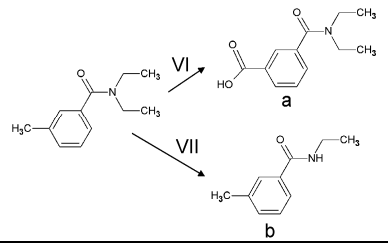
ganic micropollutants. Monooxygenases use molecular oxygen to insert one oxygen atom into the organic target molecule, while the second oxygen atom is reduced to water with electrons from the co-factors NADH or NADPH. This general reaction requires a metal center such as iron or copper, and usually additional proteins (e.g. flavoproteins) for the electron transfer from the reduced cofactors (Li et al., 2002; Neilson and Allard, 2008). In the case of ammonium monooxygenase (AMO, see chapter 1.5.1), the electron donor is cytochrome c (Madigan et al., 1999). In contrast to other reactions such as the amide hydrolysis and N-dealkylation, which are connected to a certain functional group in the molecule, the position of hydroxylation catalyzed by monooxygenases is hardly to predict. Since monooxygenases transfer the molecular oxygen into an electrophilic form, predictions can be made by considering the electron density and the nucleophilicity of the electrons of the target molecule (Schwarzenbach et al., 2003; Skotnicka-Pitak et al., 2009). However, the exact positions of the oxygen often remains unclear without using nuclear magnetic resonance experiments (NMR) for structural elucidation.

One important group of monooxygenases are those belonging to the cytochrome P450 superfamily, which are known to be ubiquitously present in bacteria, fungi and yeasts and to mediate many hydroxylations (Neilson and Allard, 2008). In regard to biological wastewater treatment, especially the role of AMO in the transformation of micropollutants has been discussed, since several studies revealed a link between nitrification and the transformation efficiency of micropollutants such as EE2, iopromide and trimethoprim (Batt et al., 2006; Vader et al., 2000). However, while some authors brought forward a co-metabolic transformation of EE2 by AMO (Forrez et al., 2009; Skotnicka-Pitak et al., 2009; Yi and Harper, 2007), others suggested that EE2 removal by enriched communities or axenic cultures of ammonia-oxidizing bacteria is mediated by abiotic nitration due to a high nitrite levels resulting from nitrification (Gaulke et al., 2008).

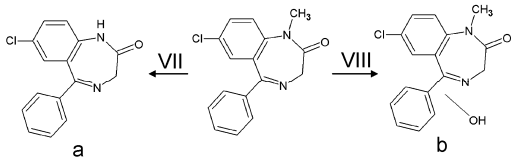
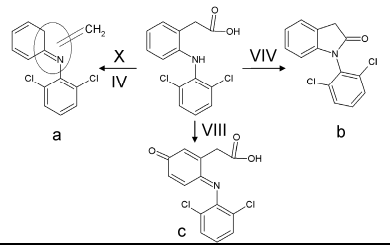
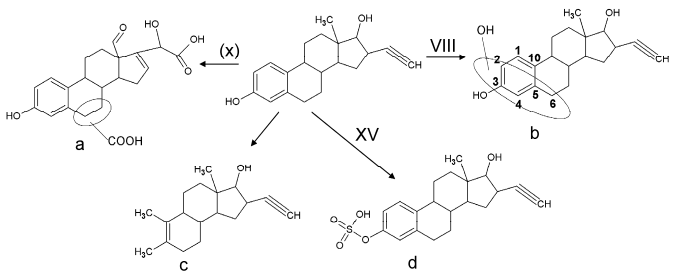
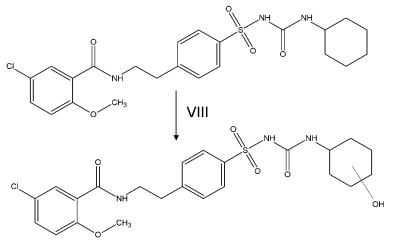
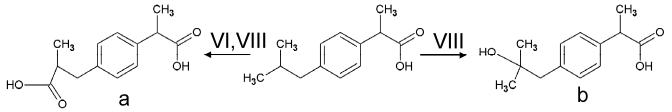
Abiotic reactions and especially their interaction with biotic reactions occurring during activated sludge treatment have been fairly addressed. Nitrification of phenolic moieties in presence of nitrite has not only been reported for EE2, but also for the analgesic acetaminophen. In contrast to the finding that nitration is an artifact of elevated ammonium concentrations used in laboratory experiments, the detection of nitrophenolic TPs of acetaminophen in samples from full-scale WWTPs demonstrated that these reactions might be also of importance in biological wastewater treatment (Chiron et al., 2010). The examination of the fate of diclofenac in a fixed-bed column reactor filled with natural river sediment revealed to be de-

termined by a coupling of biotic with abiotic processes: (i) enzyme catalyzed oxidation of diclofenac to 5-hydroxydiclofenac, (ii) chemical autoxidation to the p-benzoquinone imine of 5-hydroxydiclofenac and (iii) further abiotic chemical reactions with the sediment matrix (Gröning et al., 2007). Since both p-benzoquinone imines as well as nitrophenolic compounds are of toxicological concern, these examples indicate that the role of abiotic reactions in the transformation of micropollutants deserves further attention.

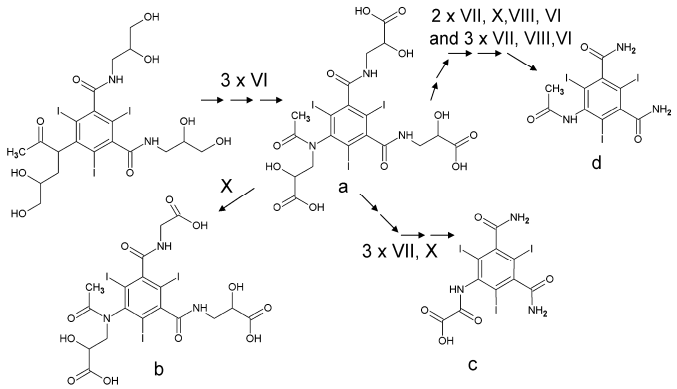
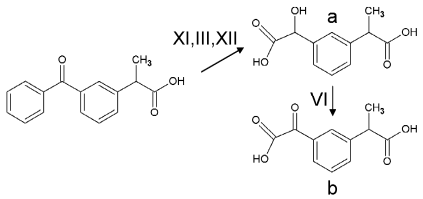
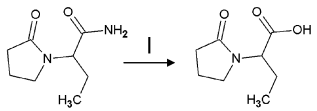
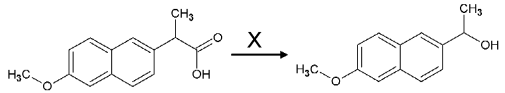
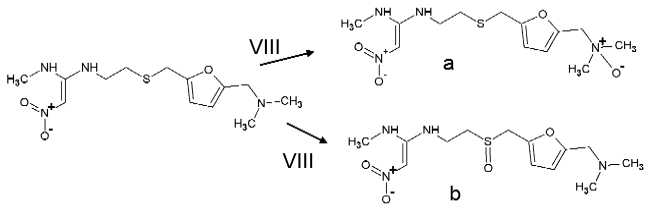
Table 1-1. Biological transformation of emerging micropollutants under aerobic conditions: Chemical structures of parent compounds, transformation products (TPs), proposed enzymatic reactions, identification method and confirmed occurrence in full-scale WWTPs as reported in recent studies. Transient TPs which can be expected to be formed based on the proposed transformation pathway but which were not detected are not listed. More than one arrow indicates that transient TPs were detected and identified but were not included in this summary.

Parent compound	Proposed transformation pathway	Proposed enzymatic reactions	Analytical techniques	WWTP detection	Ref. and comments
Atenolol beta blocker		I: amide hydrolysis	LC-Qq-LIT-MS LC-Qq-TOF-MS	yes	Radjenović et al., 2008; Helbling et al., 2010a; Kern et al., 2010
Bezafibrate lipid-regulator		I: amide hydrolysis II: hydrolytic dehalogenation III: dioxygenation IV: dehydrogenation	LC-QqQ-MS (TP a) LC-LTQ-Orbitrap-MS (all TPs)	partly (TP a)	Quintana et al., 2005 (TP a) Helbling et al., 2010a Kern et al., 2010 (all TPs)
Clofibric acid active metabolite of lipid-regulators		V: ether cleavage	LC-Qq-TOF-MS	no	Kosjec et al., 2009
N,N-Diethyl-meta-toluamide (DEET) insect repellent		VI: consecutive alcohol and aldehyde oxidation VII: N-dealkylation	LC-LTQ-Orbitrap-MS	no	Helbling et al., 2010a

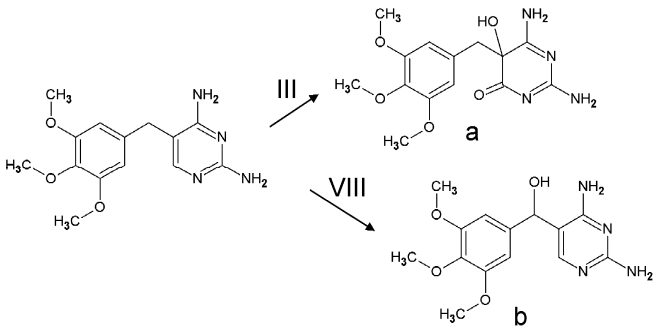
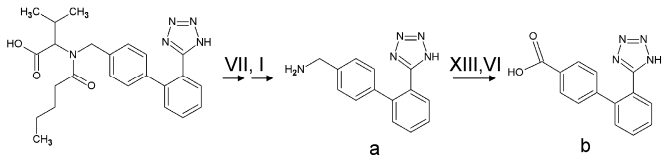
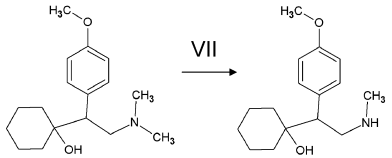
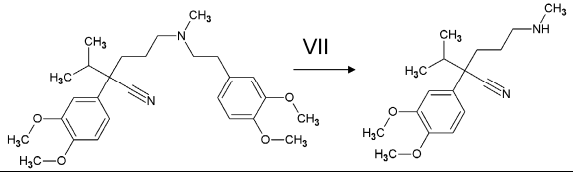
continues

Diazepam tranquilizer		VII: N-dealkylation VIII: monooxygenation	LC-LTQ-Orbitrap-MS	no	Helbling et al., 2010a
Diclofenac non-steroidal anti-inflammatory drug		VIV: amide bond formation X: decarboxylation IV: dehydrogenation VIII: monooxygenation (followed by autoxidation)	LC-Qq-TOF-MS (TP a and b) LC-Q-TOF-MS ^{1}H-NMR (TP c)	no	Kosjec et al., 2009 (TP a and b) Gröning et al., 2007 (TP c, river sediment)
17α-Ethinylestradiol (EE2) semisynthetic steroidal estrogen (contraceptive)		VIII: monooxygenation (x) processes assumed to be involved include formation of acetylene epoxide (unstable), hydrolysis and alcohol and aldehyde oxidation XV: sulfonation	Skotnicka-Pitak et al., 2009: LC-ITMS ^{1}H-NMR (only for a) Yi and Harper, 2007: TLC, ^{1}H-NMR	no	Skotnicka-Pitak et al., 2009 (batch (TP a and b) and chemostat cultures (TP b) of *Nitrosomonas europaea,* OH position of TP b at C2, C4 or C6) Yi and Harper, 2007 (nitrifying continuous flow bioreactor, TP b, c and d, OH position of TP b at C2)
Glibenclamide hypoglycaemic agent		VIII: monooxygenation	LC-Qq-LIT-MS Qq-TOF-MS	no	Radjenović et al., 2008
Ibuprofen non-steroidal anti-inflammatory drug		VIII: monooxygenation VI: consecutive alcohol and aldehyde oxidation	GC-IT-MS (TP a and b) LC-QqQ-MS (TP b)	no	Zwiener et al., 2002 (TP a and b) Quintana et al., 2005 (TP b)

continues

Iodinated X-ray contrast media (ICMs) example: iohexol (in total 46 TPs from 4 ICMs)		VI: consecutive alcohol and aldehyde oxidation VII: N-dealkylation VIII: monooxygenation X: decarboxylation	LC-Qq-LIT-MS ^{1}H-NMR, ^{13}C-NMR	yes	Kormos et al., 2010 (sediment-water batch reactors for identification) Schulz et al., 2008
Ketoprofen non-steroidal anti-inflammatory drug		XI: reduction (keto group) III: dioxygenation XII: Ring-fission-dioxygenation + hydrolysis VI: alcohol oxidation	LC-QqQ-MS	no	Quintana et al., 2005
Levitiracetam antiepileptica		I: amide hydrolysis	LC-LTQ-Orbitrap-MS	no	Helbling et al., 2010a
Naproxen non-steroidal anti-inflammatory drug		X: decarboxylation	GC-MS	no	Kosjek et al., 2007
Ranitidine antihistamine		VIII: monooxygenation a → N-oxide b → S-oxide	LC-LTQ-Orbitrap-MS	yes	Helbling et al., 2010b; Kern et al., 2010

continues

Trimethoprim antibiotic		III: dioxygenation VIII: monooxygenation	LC-IT-MS (H/D-exchange experiments) LC-Qq-TOF-MS	no	Eichhorn et al., 2005
Valsartan angiotensin receptor blocker (antihypertensive)		VII: N-dealkylation I: amide hydrolysis XIII: amine oxidation to aldehydes VI: aldehyde oxidation	LC-LTQ-Orbitrap-MS	yes	Helbling et al., 2010a; Kern et al., 2010
Venlafaxine antidepressant		VII: N-dealkylation	LC-LTQ-Orbitrap-MS	yes	Kern et al., 2010
Verapamil calcium channel blocker (antihypertensive)		VII: N-dealkylation	LC-IT-MS	no	Trautwein et al., 2008

1.5.3 Sorption

Sorption onto particulate matter can be an important removal process for organic micropollutants with a high affinity to primary or secondary sludge. Assuming a sorption equilibrium, the ratio of the sorbed compound concentration per amount of sludge dry matter C_s (µg g_{TSS}^{-1}) and the soluble compound concentration C_w (µg L^{-1}) is defined as the sludge-water distribution coefficient $K_{d,sludge}$ (L g_{TSS}^{-1}):

$$K_{d,sludge} = \frac{C_s}{C_w} \tag{1-2}$$

The full-scale compound removal by sorption onto activated sludge (secondary sludge) can be predicted by the ratio of the sorbed amount withdrawn from the system by the excess sludge (depending on the sludge production) and the influent load being the sum of the soluble and sorbed amount (non-settled primary sludge particles) (Ternes and Joss, 2007):

$$\frac{M_{SP}}{M_{in}} = \frac{C_{s,out} \cdot SP \cdot K_{d,sec}}{C_{s,in} \cdot \left(1 + TSS_{in} \cdot K_{d,prim}\right)} \tag{1-3}$$

M_{SP}	compound load withdrawn with the excess sludge [µg L^{-1}]
M_{in}	influent load [µg L^{-1}]
$C_{s,out}$	soluble concentration in the effluent [µg L^{-1}]
$C_{s,in}$	soluble concentration the influent of the activated sludge reactor [µg L^{-1}]
SP	sludge production [g_{TSS} L^{-1}]
$K_{d,prim}$	sludge-water distribution coefficient for primary sludge [L g_{TSS}^{-1}]
$K_{d,sec}$	sludge-water distribution coefficient for secondary sludge [L g_{TSS}^{-1}]
$TSS_{,in}$	total suspended solids concentration in the influent of the activated sludge reactor [g_{TSS} L^{-1}]

However, this formula is only valid assuming equilibrium conditions which can be expected for compounds being degraded at a rate < 2.5 L g_{TSS}^{-1} d^{-1} (Joss et al., 2006b). For those compounds not being significantly degraded, the influent load M_{in} can be expressed as the sum of the soluble concentration in the effluent $C_{s,out}$ and the compound load withdrawn with the excess sludge M_{SP} and then eq. 1-3 can be simplified to:

$$\frac{M_{SP}}{M_{in}} = \frac{SP \cdot K_{d,sec}}{1 + SP \cdot K_{d,sec}} \tag{1-4}$$

Since the sludge production in a conventional municipal WWTP is typically in the range of 0.1 to 0.4 g_{TSS} L^{-1}, a significant removal (> 10%) by sorption onto activated sludge can only be expected for compounds with $K_{d,sec}$ values > 300 L kg_{TSS}^{-1} (Ternes and Joss, 2007).

These considerations show that the determination of sludge-water distribution coefficients is the crucial point for predicting the removal by sorption in full-scale plants. Since the

knowledge about the physico-chemical interactions between the micropollutant and the sludge matrix is still too limited for an accurate prediction of distribution coefficients, they have to be determined by laboratory batch experiments with sludge amended with the corresponding compounds. However, reliable experimental data about the sludge-water distribution of emerging organic contaminants is still scarce hindering the modeling of their fate in waste-water treatment (Kim et al., 2009).

Regarding the experimental design, several factors such as the knowledge of the time necessary to reach sorption equilibrium, the background concentrations of compounds, the water content of the sludge, biological transformation of the compound as well as the concentration dependency of the sludge-water distribution coefficients have to be considered for reliable data (Clara et al., 2004; Stein et al., 2008; Ternes et al., 2004b).

The sludge-water distribution is expected to be mainly affected by (i) hydrophobic interactions (van-der-Waals interactions) of non-polar moieties such as aliphatic and aromatic groups, (ii) hydrogen bonding and electrostatic interaction of partial charges of polar moieties such as alcohols and ketones and (iii) ionic interactions of charged moieties such as carboxyl groups and amines (Flemming and Wingender, 2010). So far, studies revealed that a positive compound charge significantly increases its sorption affinity to secondary sludge (Golet et al., 2003; Kim et al., 2009; Ternes et al., 2004b). This can be explained by an ionic attraction between the mainly negatively charged sites of the extracellular polymeric substances (EPS) and the bacterial surface and the positive compound charge. Consequently, negatively charged compounds typically have low $K_{d,sec}$ values due to ionic repulsion. Hence, for compounds with a pKa in the typical pH range of secondary sludge (pH 6-8), the $K_{d,sec}$ values can be expected to be strongly dependent on the ambient pH. This has to be considered when predicting the full-scale removal of those compounds using $K_{d,sec}$ values determined at a certain pH (Ternes et al., 2004a,b; Ternes and Joss, 2007). On the other hand, also the quantity and quality of the organic matter of the sludge are expected to have an influence on the sludge-water distribution. For instance, the significantly higher sorption affinity of the antibiotics ciprofloxacin and norfloxacin observed for secondary sludge compared to primary sludge was attributed to an increased bacterial density of the secondary sludge and the ionic attraction of the positively charged secondary amine moieties of the antibiotics and the negatively charged bacterial surface (Golet et al., 2003; Ternes and Joss, 2007).

1.5.4 Advanced physico-chemical treatment processes

Due to the increasing awareness about the potential threat of aquatic ecosystems and drinking water resources as well as their impending regulation, an upgrade of conventional biological WWTPs is discussed (Joss et al., 2008). Several different advanced physical-chemical treatments such as tight membrane filtration (nanofiltration and reversed osmosis), activated carbon treatment, ozonation and advanced oxidation processes (AOP) have been proposed as end of pipe techniques. However, among those especially the oxidation with ozone and the sorption onto powdered activated carbon have been shown to be promising techniques for an efficient and economic feasible removal of micropollutants from secondary effluents municipal WWTPs (Ternes and Joss, 2007).

Several studies (Benner et al., 2008; Huber et al., 2005; Hollender et al., 2009) have confirmed that ozone treatment can be very efficient in the oxidation of a wide range of micropollutants featuring electron-rich moieties such as activated aromatic rings, amine functions and double bonds (e.g. beta blockers, antibiotics, estrogens). Even for a medium ozone dose of 0.6 g O_3 g^{-1} DOC, removal rates >85% have been observed for many organic micropollutants with different functional groups in a municipal WWTP upgraded with a full-scale post-ozonation (Hollender et al., 2009). In addition, ozonation has been found to reduce or to eliminate the pharmacological and biological effects of micropollutants (Escher et al., 2009). However, ozonation has the potential to produce toxic oxidation by-products such as N-nitrosodimethylamine (NDMA) and compounds with bialdehyde functional groups (Benner and Ternes, 2009; Krauss et al., 2010). This is in accordance with recent results from chronic *in vivo* toxicity tests indicating an enhanced toxicity and genotoxicity of wastewater after ozonation (Stalter et al., 2010). However, the observed toxic effects were reported to be removed by subsequent biological active sand filtration indicating a detoxification by a further biological transformation of the oxidation by-products.

Sorption to powdered activated carbon has also shown to have a high efficiency for micropollutant removal at a economically feasible PAC dosage of 10 to 20 mg L^{-1} (Nowotny et al., 2007; Snyder et al., 2007). The main advantage of PAC treatment compared to ozonation is the complete elimination of micropollutants by sorption and subsequent incineration avoiding the risk of producing toxic by-products. However, depending on the plant size and the DOC content the total costs of PAC treatment of wastewater has been expected to be 0.08 to 0.20 Euro·m^{-3} and therefore slightly higher than for ozonation (0.05 – 0.15 Euro·m^{-3}). From

an economically point of view, both techniques seem to be feasible regarding the expected costs of 5 to 20 Euros per person and year (Joss et al., 2008).

1.6 Objectives

The overall objective of this thesis was to elucidate the fate (sorption and transformation) of a wide spectrum of emerging organic micropollutants in biological wastewater treatment and the importance of WWTP effluents for the contamination of rivers and streams.

Due to the lack of appropriate analytical methods, the first important aspect of this thesis was the development, validation and application of a **multi-residue method (*Chapter 2*)** for the simultaneous determination of biocides, UV-filters and benzothiazoles, whose occurrence and fate in wastewater have not or rarely been addressed so far. The challenge was to analyze various compounds with different physico-chemical properties in complex aqueous matrices such as wastewater and activated sludge down to the low ng L^{-1} and ng g^{-1} range, respectively. A special focus was placed on the susceptibility of different interfaces (ESI versus APCI) to matrix effects and the possibility of their compensation by use of stable isotope-labeled surrogate standards. Furthermore, the validated method was applied to investigate the **occurrence** of the selected target compounds in WWTPs as well as rivers and streams.

The fate of emerging organic micropollutants in biological wastewater treatment was investigated by a) studying the sorption behavior and b) monitoring the overall removal along the treatment lane of a state-of-the-art WWTP. Therefore, one task was to use the newly developed analytical method to study the **partitioning** ***(Chapter 3)*** of several micropollutants under sorption equilibrium conditions in laboratory batch experiments. Besides the determination of sludge-water distribution coefficients, which are a prerequisite for predicting the full-scale removal by sorption, it was expected that these experiments with a large number of different compounds deliver new insights into the relevant processes responsible for the sorption of emerging micropollutants onto activated sludge.

Another aim was to determine the capability of different biological treatment units of state-of-the-art WWTPs for the **full-scale removal** ***(Chapter 4)*** of beta blockers and psychoactive drugs. One key question was whether the information gained from laboratory batch experiments, such as biotransformation kinetic constants and sludge-water distribution coefficients, are appropriate for modeling the observed removal in full-scale WWTPs.

The final task was the elucidation of the **transformation** ***(Chapter 5)*** of codeine and further opium alkaloids during biological wastewater treatment on a molecular level. The aim was to identify their transformation products and transformation pathways in order to achieve a better understanding of abiotic and biotic reactions of organic micropollutants during activated sludge treatment.

1.7 Outline

The outline of this thesis is a follows:

Development and application of a multi-residue analytical method

Chapter 2 describes the development, validation and application of a multi-residue method for the analysis of biocides, UV-filters and benzothiazoles in surface water, wastewater and activated sludge with a special focus on matrix effects and evaluating the performance of two different interfaces (ESI and APCI).

Partition to activated sludge

Chapter 3 reports about the sorption of biocides, UV-filters as well as triazine and phenylurea herbicides onto activated sludge. Freundlich sorption isotherms have been determined and sludge-water distribution coefficients are compared with water-octanol partition coefficients and used to predict the full-scale removal of the target compounds by sorption.

Overall removal in full-scale WWTPs

Chapter 4 is about the removal of beta blockers and psycho-active drugs along the treatment units of a state-of-the-art WWTP. The removal efficiencies were measured by long-term measurement campaigns and compared to those predicted with a model using information from laboratory batch experiments.

Transformation processes in biological wastewater treatment

Chapter 5 deals with the identification of TPs of codeine formed under aerobic conditions in contact to activated sludge. Furthermore, the elucidation of the transformation pathway of codeine and the transferability of the results to full-scale WWTPs as well as to other structurally related opium alkaloids is the main topic of this chapter.

Final Conclusions

Chapter 6 discusses the main outcomes of the studies mentioned above, draws some final conclusions and outlines some further research needs.

2. Comparison of electrospray ionization and atmospheric pressure chemical ionization for multi-residue analysis of biocides, UV-filters and benzothiazoles in aqueous matrices and activated sludge by liquid chromatography-tandem mass spectrometry

Arne Wick, Guido Fink, Thomas A. Ternes

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Abstract

This chapter describes the development of a multi-residue method for the determination of 36 emerging organic pollutants (26 biocides, 5 UV filters and 5 benzothiazoles) in raw and treated wastewater, activated sludge and surface water using liquid-chromatography – tandem mass spectrometry (LC-MS/MS). The target analytes were enriched from water samples adjusted to pH 6 by solid-phase extraction (SPE) on Oasis HLB 200 mg cartridges and eluted with a mixture of methanol and acetone (60/40, v/v). Extraction of freeze-dried sludge samples was accomplished by pressurized liquid extraction (PLE) using a mixture of methanol and water (50/50, v/v) as extraction solvent followed by SPE. LC-tandem MS detection was compared using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in positive and negative ionization mode. ESI exhibited strong ion suppression for most target analytes, while APCI was generally less susceptible to ion suppression but partially leading to ion enhancement of up to a factor of 10. In general, matrix effects could be compensated using stable isotope-labeled surrogate standards, indicated by relative recoveries ranging from 70 to 130%.

In wastewater, activated sludge and surface water up to 33 analytes were detected. Maximum concentrations up to 5.1 and 3.9 µg L^{-1} were found in raw wastewater for the water-soluble UV filters benzophenone-4 (BZP-4) and phenylbenzimidazole sulfonic acid (PBSA), respectively. For the first time, the anti-dandruff climbazole was detected in raw wastewater and in activated sludge with concentrations as high as 1.4 µg L^{-1} and 1.2 µg g_{TSS}^{-1}, respectively.

Activated sludge is obviously a sink for four benzothiazoles and two isothiazolones, as concentrations were detected in activated sludge between 120 ng g_{TSS}^{-1} (2-n-octyl-4-isothiazolin-3-one, OIT) to 330 ng g_{TSS}^{-1} (benzothiazole-2-sulfonic acid, BTSA).

2.1 Introduction

In recent years, biocides and UV-filters have gained increasing interest as so called emerging contaminants since they are ingredients of various products used in every day life such as personal care products (PCPs), cleaning agents and paints and coatings (Bester et al., 2008; Richardson, 2009). Another crucial class are benzothiazoles which are mainly used as vulcanization accelerators and are present in all kinds of rubber made products (De Wever et al., 2001). As used mainly in rinse-off products, biocidal ingredients of PCPs and cleaning agents such as the preservative 1,2-benzisothiazolin-3-one (BIT) as well as water-soluble UV-filters such as benzophenone-4 (BZP-4) are discharged into municipal wastewater treatment plants (WWTPs) (Rafoth et al., 2007; Rodil et al., 2009). Biocides used as film-preservatives in paintings, coatings and roof sealings such as carbendazim and mecoprop reach WWTPs by leaching, washing of equipment and the disposal of unused products. In case of an incomplete removal all these contaminants are further discharged into the receiving waters (Gerecke et al., 2002; Nitschke and Schüssler, 1998).

Since biocides are biological active compounds applied to destroy or to inhibit the growth or action of organisms (European Commission, 1998), even low environmental concentrations might have negative impacts on the aquatic environment. For example, triclosan has been shown to induce changes in the thyroid hormone-mediated process of metamorphosis of the North American bullfrog *Rana catesbeiana* and to cause a significant shift in the community structure of a natural river algae community at environmental relevant concentrations as low as 30 and 15 ng L^{-1}, respectively (Veldhoen et al., 2000; Wilson et al., 2003). Carbendazim seriously effected the macroinvertebrate community in a freshwater microcosm in the low µg L^{-1} range (Cuppen et al., 2000) and the antifouling irgarol was found to effect the community of macrophytes in mesocosms with EC_{50} values down to 0.2 µg L^{-1} for the species *Myriophyllum verticillatum* (Mohr et al., 2009). UV filters, such as benzophenone-1 (BZP-1) and benzophenone-2 (BZP-2), have been reported to show estrogenic activity (Kawamura et al., 2003; Kunz et al., 2006).

Most of the currently available analytical methods for the determination of biocides and UV-filters in wastewater and activated sludge are based on GC-MS using a variety of derivatization techniques (Balmer et al., 2005; Bester, 2003; Gerecke et al., 2002; Kupper et al., 2006; Nagtegaal et al., 1997; Öllers et al., 2001; Plagellat et al., 2004, 2006). For isothiazolones, conazoles or water-soluble UV-filters analytical methods for identification and quan-

tification in wastewater or activated sludge are hardly available. A GC-MS method for the measurement of isothiazolones in aqueous matrices was developed by Rafoth et al. (2007). The anti-dandruff ketoconazole was quantified together with other conazoles in surface water and wastewater using LC-MS/MS (Van De Steene et al., 2006), but to our knowledge no analytical methods for the analysis of the anti-dandruff climbazole in any environmental matrices have been published so far. UPLC-ESI/MS/MS was used recently to determine benzophenonic UV-filters in surface water, wastewater (Kasprzyk-Hordern et al., 2008) and activated sludge (Nieto et al., 2009). The UV-filter PBSA was measured together with BZP-3 and BZP-4 in Spanish tap water, surface water and wastewater by Rodil et al.(2009). BZP-4 was found to occur in the μg L^{-1} range in raw and treated wastewater and was also detected in 4 of 5 tap water samples at a mean concentration of 12 ng L^{-1}.

Nowadays, analytical methods based on LC-MS/MS offer a tool to identify and quantify compounds of medium to high polarity in all kinds of water bodies and solid matrices (Reemtsma and Quintana, 2006; Richardson and Ternes, 2005). However, a serious drawback of LC-MS/MS methods is their susceptibility to matrix effects, e.g. the signal suppression or enhancement by matrix compounds entering the ion source at the same time. Matrix effects can strongly vary with the environmental matrix and result in poor analytical accuracy and reproducibility (Niessen et al., 2006; Srinivas, 2009; Van De Steene, 2006). It has been reported that atmospheric pressure chemical ionization (APCI) is generally less sensitive to matrix effects than the more commonly used electrospray ionization (ESI) (Matuszewski et al., 2003; Matuszewski 2006; Schlüsener and Bester, 2005; Vanderford et al., 2003; Zuehlke et al., 2004), but only few studies focused on matrix effects using APCI in direct comparison to ESI for different compound groups. Since especially for emerging contaminants stable isotope-labeled surrogate standards are often not available and only compensate but not reduce matrix effects, APCI was evaluated as an alternative ionization interface. Other measures, which have been successfully used to reduce matrix effects such as changing the composition of the mobile phases, additional clean-ups and post-column switching (Benijts et al., 2004; Dijkman et al., 2001; Kloepfer et al., 2005b; Van De Steene, 2006), are often accompanied by compound losses if applied to multi-residue methods with a broad compound spectrum.

The objective of the current study was the development of a sensitive multi-residue method for the determination of biocides, UV-filters and benzothiazoles in surface waters, wastewater and activated sludge using LC-MS/MS. The challenge was to analyze the structurally diverse analytes from the same sample using a single extraction procedure. Matrix

effects in the ESI and APCI interface were assessed for each analyte using post-extraction spikes. The suitability of the use of deuterated and ^{13}C-labeled surrogate standards for compensation of matrix effects was evaluated for both interfaces. The selected analytes are listed in Table 2-1.

Table 2-1. Selected target analytes, abbreviations and properties. TP: transformation product

Name	Abbreviation	Application	CAS no	Formula	log K_{OW}[a]	pKa[a]
Biocides						
Diuron		Herbicide	330-54-1	$C_9H_{10}Cl_2N_2O$	2.85	
Isoproturon		Herbicide	34123-59-6	$C_{12}H_{18}N_2O$	2.50	
Mecoprop		Herbicide	7085-19-0	$C_{10}H_{11}ClO_3$	0.1	3.74
Propiconazole		Fungicide	60207-90-1	$C_{15}H_{17}Cl_2N_3O_3$	3.72	
Tebuconazole		Fungicide	107534-96-3	$C_{16}H_{22}ClN_3O$	3.70	
Imazalil		Fungicide	35554-44-0	$C_{14}H_{14}Cl_2N_2O$	3.82	6.5
Climbazole		Fungicide (anti-dandruff)	38083-17-9	$C_{15}H_{17}ClN_2O_2$	3.33[b]	7.5[b]
Ketoconazole		Fungicide (anti-dandruff)	65277-42-1	$C_{26}H_{28}Cl_2N_4O_4$	4.30[b]	6.6[b]
Carbendazim		Fungicide	10605-21-7	$C_9H_9N_3O_2$	1.51	4.2
Thiabendazole		Fungicide	148-79-8	$C_{10}H_7N_3S$	2.47[b]	4.70[b]
Terbuthylazine		Herbicide	5915-41-3	$C_9H_{16}ClN_5$	3.04	2.0
Terbutryn		Herbicide	886-50-0	$C_{10}H_{19}N_5S_2$	3.65	4.3
irgarol		Herbicide /Algicide	28159-98-0	$C_{11}H_{19}N_5S$	3.72	4.1
2-Methylthio-4-tert-butylamino-6-amino-s-triazine	M1	TP of irgarol	30125-65-6	$C_8H_{15}N_5S$		
Dimethomorph		Fungicide	110488-70-5	$C_{21}H_{22}ClNO_4$	2.63	1.0
Fenpropimorph		Fungicide	67564-91-4	$C_{20}H_{33}NO$	4.40	7.0
Tridemorph		Fungicide	24602-86-6	$C_{19}H_{39}NO$	6.99[b]	7.4[b]
1,2-Benzisothiazolin-3-one	BIT	Microbicide	2634-33-5	C_7H_5NOS	1.24[b]	
2-n-Octyl-4-isothiazolin-3-one	OIT	Microbicide	26530-20-1	$C_{11}H_{19}NOS$	2.45	
4,5-Dichloro-2-n-octyl-4-isothiazolin-3-one	DCOIT	Microbicide	64359-81-5	$C_{11}H_{17}Cl_2NOS$	4.77[b]	
N,N-Dimethyl-N'-p-tolylsulfamide	DMST	TP of the fungicide tolylfluanide	66840-71-9	$C_9H_{14}N_2O_2S$		
N,N-Dimethyl-N'-phenylsulfamide	DMSA	TP of the fungicide dichlofluanide	4710-17-2	$C_8H_{12}N_2O_2S$		
3-Iodo-2-propynylbutyl-carbamate	IPBC	Fungicide	55406-53-6	$C_8H_{12}INO_2$	2.81[c]	
Triclosan		Microbicide	3380-34-5	$C_{12}H_7Cl_3O_2$	4.76[b]	8.0[b]
Triclocarban		Microbicide	101-20-2	$C_{13}H_9Cl_3N_2O$	5.10[b]	
Chlorophene		Microbicide	120-32-1	$C_{13}H_{11}ClO$	4.14[b]	9.6[b]
UV-filter						
Benzophenone-1	BZP-1	UV-filter	131-56-6	$C_{13}H_{10}O_3$	2.92[b]	8.0[b]
Benzophenone-2	BZP-2	UV-filter	131-55-5	$C_{13}H_{10}O_5$	2.08[b]	8.0[b]
Benzophenone-3	BZP-3	UV-filter	131-57-7	$C_{14}H_{12}O_3$	3.79[b]	8.0[b]
Benzophenone-4	BZP-4	UV-filter	4065-45-6	$C_{14}H_{12}O_6S$	0.39[b]	0.7[b]
Phenylbenzimidazole sulfonic acid	PBSA	UV-filter	27503-81-7	$C_{13}H_{10}N_2O_3S$	1.03[b]	4.9,0.7[b]
Benzothiazoles						
Benzothiazole	BT	Vulcanization	95-16-9	C_7H_5NS	2.01[b]	1.2[b]
2-Methylthiobenzothiazole	MTBT	Vulcanization	615-22-5	$C_8H_7NS_2$	3.15[b]	2.5[b]
Benzothiazole-2-sulfonic acid	BTSA	Vulcanization	941-57-1	$C_7H_5NO_3S_2$	-0.39[b]	2.4,-1.0[b]
2-Hydroxybenzothiazole	OHBT	Vulcanization	934-34-9	C_7H_5NOS	2.12[b]	6.2[b]
2-(4-Morpholinyl) benzothiazole	Morpholinyl-BT	Vulcanization	4225-26-7	$C_{11}H_{12}N_2OS$	2.59[b]	4.5[b]

[a] C.D.S. Tomlin (ed.), The Pesticide Manual, British Crop Protection Council (BCPC), Farnham, UK, 10th ed., 1994.
[b] ALOGPS 2.1, Virtual Computational Chemistry Laboratory, 2005: http://www.vcclab.org/lab/alogps/.
[c] MRI. 1990. Analysis of Polyphase P-100 octanol/water partition coefficient (63-11). Final report. MRI Project No. 9555-F(01). Prepared for Troy Chemical Corporation, Newark, NJ. MRI, Kansas City, MO.

2.2 Experimental Methods

2.2.1 Chemicals

The following compounds were analyzed: dimethomorph, fenpropimorph, tridemorph, 2-n-octyl-4-isothiazolin-3-one (OIT), triclosan (purchased from Fluka, Buchs, Switzerland); imazalil, carbendazim, irgarol (purchased from Riedel-de Haen, Seelze, Germany); 1,2-benzisothiazolin-3-one (BIT), 3-iodo-2-propynyl-N-butylcarbamate (IPBC), triclocarban, benzothiazole (BT), benzothiazole-2-sulfonic acid (BTSA), 2-methylthiobenzothiazole (MTBT), 2-hydroxybenzothiazole (OHBT), 2-(4-morpholinyl)benzothiazole (morpholinyl-BT) (purchased from Sigma-Aldrich, Schnelldorf, Germany); thiabendazole, propiconazole, tebuconazole, climbazole, ketoconazole, diuron, isoproturon, mecoprop, terbutryn, terbuthylazine, N,N-dimethyl-N'-phenylsulfamide (DMSA, transformation product of dichlofluanide), N,N-dimethyl-N'-p-tolylsulfamide (DMST, transformation product of tolylfluanide), chlorophene (purchased from Dr. Ehrenstorfer, Augsburg, Germany); 2-methylthio-4-tert-butylamino-6-amino-s-triazine (M1, transformation product of irgarol) (purchased from Ciba Speciality Chemicals); 4,5-dichloro-2-n-octyl-4-isothiazolin-3-one (DCOIT) (purchased from Chemos, Regenstauf, Germany); phenylbenzimidazole sulfonic acid (PBSA), benzophenone-1 (BZP-1), benzophenone-2 (BZP-2), benzophenone-3 (BZP-3), benzophenone-4 (BZP-4) (kindly provided by Prof. Dr. J. Oehlmann, University Frankfurt).

The surrogate standards carbendazim-d_4, thiabendazole-d_6, propiconazole-d_5, tebuconazole-d_6, imazalil-d_5, terbutryn-d_5, terbuthylazine-d_5, diuron-d_6, isoproturon-d_6, mecoprop-d_3 were obtained from Dr. Ehrenstorfer (Augsburg, Germany), triclosan-$^{13}C_{12}$, triclocarban-$^{13}C_6$ from Cambridge Isotope Laboratories (Andover, MA, USA) and ketoconazole-d_8 from Campro Scientific (Berlin, Germany).

Methanol (picograde) and acetonitrile (HPLC gradient grade) were purchased from LGC Promochem (Wesel, Germany). Formic acid and sulfuric acid (both p.a.) were purchased from Merck (Darmstadt, Germany) and ammonium formate (purum grade) from Sigma-Aldrich (Schnelldorf, Germany). Pure water was obtained from a Milli-Q system (Integral 3/5/10/15, Millipore, Billericia, MA, USA).

Separate standard solutions of all analytes and surrogate standards were prepared in methanol at a concentration of 10 µg mL^{-1} and 1 µg mL^{-1}, respectively and stored in the dark at 4°C.

2.2.2 Sampling of wastewater, activated sludge, surface water and groundwater

The wastewater samples used for method validation derived from WWTP 1 serving approximately 320,000 population equivalents (PE). WWTP 1 consists of a mechanical treatment (screen, grit removal and primary clarifier), a trickling filter followed by an activated sludge treatment with nitrification and denitrification, phosphate removal and a final clarification. Grab samples were taken from the influent (after primary clarification) and from the final effluent on 11th March 2008. Samples from surface water were taken from the river Rhine in Koblenz (Germany) at river kilometer 590.3 on the same day as the wastewater samples. The sludge samples were taken from the activated sludge tank (nitrification zone) of WWTP 1 on 26th November 2008.

Additional wastewater samples were obtained from WWTP 2 serving approximately 307,000 PE with a treatment lane comparable to WWTP 1 but without a trickling filter prior to the activated sludge treatment. Grab samples were taken after grit removal prior to the primary clarifier and from the final effluent on 2nd July 2009. Furthermore, grab samples were taken on 1st September 2009 close to the mouth of two small tributaries of the river Main. The sampling point of stream 1 (Schwarzbach) and stream 2 (Wickerbach) was located about 3 km and 0.1 km downstream the last discharge of a WWTP, respectively. All samples were taken in solvent rinsed amber glass bottles and immediately cooled down to 4°C until further sample preparation (within 1-2 days).

The groundwater used in this study was collected from a well in Koblenz-Arenberg (Germany). Measurements of groundwater blank samples subjected to the entire preparation and analysis procedure were included in every series of analysis and showed that the groundwater was pristine and free of all targeted analytes.

2.2.3 Sample preparation and extraction

Aqueous samples

Solid-phase extraction (SPE) was used for compound extraction and enrichment from aqueous samples. Different types of adsorbents (Bakerbond C18, 500 mg, Mallinckrodt Baker, Phillipsburg, USA; Strata-X, 200 mg, 33 µm, Phenomenex, Aschaffenburg, Germany; Isolute ENV+, 200 mg, 90 µm, Biotage, Uppsala, Sweden; Oasis HLB, 200 mg, 30 µm, Waters, Milfort, USA; Oasis MCX, 60 mg, 30 µm, Waters, Milfort, USA; Strata-X-C, 200 mg, 33 µm, Phenomenex, Aschaffenburg, Germany), pH values of the samples and elution solvents were tested using 1000 mL of groundwater spiked with 100 ng L^{-1} of each analyte. Based on these results, the following optimal SPE procedure has been established:

Water samples were filtered through glass fiber filters (GF 6, Whatman). For the solid-phase extraction 100 mL of raw wastewater, 200 mL of treated wastewater, and 1 L of surface water were adjusted to pH 6 with 3.5 M sulfuric acid and spiked with 200 ng of each surrogate standard. Oasis HLB cartridges (200 mg, 30 µm, Waters, Milfort, USA) were washed and conditioned with 1 x 2 mL heptane, followed by 1 x 2 mL acetone, 3 x 2 mL methanol and 4 x 2 mL groundwater (adjusted to pH 6 with 3.5 M sulphuric acid). The water samples were then passed through the pre-conditioned cartridges at a flow rate of approximately 5 mL min^{-1}. The solid-phase material was dried by a continuous nitrogen stream for approximately 1 h. Elution was accomplished with 4 x 2 mL of a mixture of methanol and acetone (60/40, v/v). The extracts were evaporated to 500 µL under a gentle stream of nitrogen and filled up to a final volume of 1 mL with 0.1% formic acid.

Sludge samples

Extraction of sludge samples were conducted by pressurized liquid extraction (PLE). The solid part of the activated sludge was separated from the aqueous phase by centrifugation for 15 min at 4000 rpm. Subsequently, the sludge was freeze-dried and ground with a pestle. Approximately 200 mg of the dry sludge was weighed into 22 mL stainless steel extraction cells filled to one half with baked out sea sand before the internal standard mixture was added (1 µg g_{TSS}^{-1}). After the solvent was completely evaporated, the cell was filled up with baked out sea sand (Riedel-de Haen, Seelze, Germany). The extraction was accomplished with a Dionex ASE 200 instrument (Sunnyvale, CA, USA). A variety of extraction solvents (water/methanol (50/50%, v/v), 100% methanol and 100% acetone) as well as extraction temperatures (80°C, 100°C, 120°C) were tested to optimize the extraction efficiencies. The final PLE conditions were as follows: prefill method; solvent, water/methanol (50/50%, v/v); equilibration, 5 min; static time, 10 min; flush volume, 120%; purge time, 60 s; static cycles, 4; temperature, 80°C. The PLE extracts (~ 30 mL) were diluted with groundwater to a volume of 800 mL, adjusted to pH 6 with 3.5 M sulfuric acid and a SPE was performed as described above for the aqueous samples.

2.2.4 LC-MS/MS analysis

An Agilent 1200 Series (Agilent Technologies, Waldbronn, Germany) liquid chromatographic system consisting of a membrane degasser, binary high-pressure gradient pump, autosampler, and a column thermostat was used. Chromatographic separation was carried out

on a Synergi Fusion-RP 80 Å column (150 x 3 mm, 4 µm) equipped with a SecurityGuard pre-column (4 x 3 mm) (Phenomenex, Aschaffenburg, Germany).

Two different LC-MS/MS methods (method 1 and 2) were developed for the analysis of target analytes. For method 1, mobile phase A consisted of 10 mM ammonium formate buffer adjusted to pH 3.2 with formic acid, and acetonitrile with 0.1% formic acid served as mobile phase B. The applied gradient elution was as follows: start of the run with 0% B, kept isocratic for 1 min, increase to 30% B within 1 min, further increase to 80% B within 17 min, kept isocratic for 6 min, return to the initial conditions within 2 min which were hold for the last 5 min.

Using method 2, mobile phase A consisted of 0.1% formic acid, and acetonitrile served as mobile phase B. The applied gradient elution was as follows: start of the run with 0% B, kept isocratic for 1 min, increase to 40% B within 1 min, further increase to 80% B within 17 min, kept isocratic for 7 min, return to the initial conditions within 2 min which were hold for the last 5 min. For both methods, the flow rate was kept constant at 0.4 mL min^{-1} and the sample volume injected was 25 µL.

The HPLC was coupled to a tandem mass spectrometer (API 4000, Applied Biosystems, Foster City, CA, USA) operated in the positive (method 1) and in the negative ion mode (method 2) using multiple reaction monitoring (MRM). Electrospray ionization (ESI) as well as atmospheric pressure chemical ionization (APCI) were applied to compare sensitivity, recoveries and ion suppression of both ionization interfaces.

The ESI source conditions were adjusted as follows (values for negative ion mode (method 2) are given in parenthesis): collision gas, medium (medium); curtain gas, 15 psi (15 psi); ion source gas 1 and ion source gas 2, both 35 psi (40 psi); source temperature, 600°C (550°C); entrance potential, 10 V (-10 V); ion spray voltage 5.5 kV (-2.0 kV). The corresponding APCI source conditions were adjusted as follows (values for negative ion mode (method 2) are given in parenthesis): collision gas, medium (medium); curtain gas, 10 psi (15 psi); nebuliser current, 3 µA (-3 µA); ion source gas 1, 30 psi (30 psi), ion source gas 2, 35 psi (40 psi); source temperature, 450°C (450°C); entrance potential, 10 V (-10 V).

Two MRM transitions for each compound were monitored for quantification (transition 1) and confirmation (transition 2) of all target compounds. The compound specific parameters such as declustering potential, collision energy, and the cell exit potential were optimized individually for each compound in continuous flow mode via direct injection of standard solutions (200 ng mL^{-1}) solved in acetonitrile/water (90:10) at a flow rate of 10 µL min^{-1}

and are listed together with the retention times, MRM transitions, transition intensity ratios and dwell times in Table 2-2. Only the data for ESI are shown, since for APCI the same transitions were selected and the compound specific parameters did not differ significantly. For most analytes dwell times were set to 25 ms. Higher dwell times of 100 ms were chosen for selected analytes for which relatively low sensitivities with ESI were observed.

2.2.5 Method validation

Recoveries

Determination of recoveries was assessed for the different matrices (groundwater, surface water, wastewater and activated sludge) at different concentration levels. Groundwater and surface water were spiked at a concentration level of 0.1 µg L^{-1}. WWTP effluent, influent and freeze-dried activated sludge were spiked at two concentration levels of 0.5 and 2 µg L^{-1}, 1 and 4 µg L^{-1} and 0.5 and 2 µg g_{TSS}^{-1}, respectively. Due to the impossibility to obtain WWTP samples and surface water samples free of analytes, the background concentrations were determined in non-spiked samples (n = 4) and subtracted from the concentrations measured in the spiked samples. The relative recoveries describing the accuracy of the entire analytical procedure were calculated as the ratio of the spiked concentrations and the quantified concentrations. Deviations from the mean values are given as 95% confidence intervals (n = 4) . The instrumental precision, determined as relative standard deviation (%RSD), was obtained from the repeated injection of a spiked groundwater extract during the same day (intra-day precision, n = 5) and on three different days (inter-day precision, n = 3).

Calibration curves and quantification limits

Calibration curves with 14 different calibration points ranging from 0.2 to 2000 ng L^{-1} were obtained by spiking 1000 mL of pristine groundwater. A constant amount of surrogate standards (200 ng) was added. Samples were then subjected to the SPE as described above. The linearity range was between 0.2 and 200 ng L^{-1}. A quadratic fitting ($y = ax^2 + bx + c$) with a weighing factor of 1/x was used from 200 ng L^{-1} to 2000 ng L^{-1}. The limit of quantification (LOQ) was defined as the second lowest calibration point in the regression as long as the calculated signal to noise ratio (S/N) of the compounds in the native sample extracts was > 10 for the first transition (t1) used for quantification and > 3 for the second transition (t2) used for confirmation. Taking into account the different sample volumes used for SPE and the sludge amount used for PLE, the LOQs for the influent, effluent and sludge samples were calculated by multiplying the LOQ achieved for the extraction of groundwater by a factor of

10, 5 and 5, respectively. Still the criteria of a S/N ratio > 10 for quantification and > 3 for confirmation had to be fulfilled. For confirmation, the S/N ratios of both transitions were determined using non-spiked extracts for analytes with background concentrations above the LOQs, whereas for analytes with background concentrations < LOQ spiked sample extracts were used (Table SI 2-5). For most analytes the S/N ratios were in accordance with the LOQs calculated from the enrichment factors. In a few cases the LOQs were individually adapted based on the determined S/N ratios.

Matrix effects

Ion suppression or enhancement was assessed using post-extraction spikes according to Matuszewski et al. (2003). Briefly, final sample extracts from groundwater, surface water and WWTP influent and effluent were divided into two aliquots of 200 µL. While one aliquot served as blank sample and was only supplemented with 50 µL of methanol, the other one was spiked with 50 µl of a 1 µg mL^{-1} compound standard solution resulting in a final spike concentration of 200 ng mL^{-1}. Similar to the calculation of the absolute recovery, the matrix effect (ME) was calculated as the percental ratio of the analyte peak area in the spiked sample ($PA_{post\text{-}spike}$) subtracted by the peak area in the non-spiked blank sample (PA_{blank}) to the peak area in a non-enriched external standard (PA_{EXT}):

$$ME = \left(\frac{PA_{post-spike} - PA_{blank}}{PA_{EXT}} \right) \cdot 100 \qquad (2\text{-}1)$$

ME values less than 100% indicate signal suppression, while values above 100% indicate signal enhancement due to the influence of matrix in the sample extracts in contrast to the non-enriched external standard prepared in 0.1% formic acid.

Table 2-2. Precursor, product ions, and retention times in LC-MS/MS detection (ESI, positive and negative ionization mode). (*) Analytes determined in negative ionization mode.

Recovery [%]	Retention time [min]	Transition 1[a] (t1) [m/z]	Transition 2[a] (t2) [m/z]	[t1]/[t2] (%RSD)	Dwell time [ms]	DP[b] (t1/t2) [V]	CE[b] (t1/t2) [eV]	CXP[b] (t1/t2) [V]
Biocides								
Diuron	12.1	235.0/72.1	233.0/72.1	0.8 (2)	25	50/50	30/30	12/11
Isoproturon	11.9	207.1/72.1	207.1/143.0	13.4 (12)	25	65/65	35/33	10/10
Mecoprop(*)	10.6	213.0/140.8	213.0/71.0	1.9 (3)	25	-50/-50	-18/-16	-11/-1
Propiconazole	16.1	342.1/159.1	344.1/161.1	1.6 (3)	25	76/76	45/37	14/12
Tebuconazole	15.1	308.1/70.0	310.1/70.0	2.3 (4)	25	66/81	49/45	6/4
Imazalil	11.3	297.1/159.0	297.1/201.0	1.8 (2)	25	76/76	31/25	12/12
Climbazole	12.1	293.1/69.0	295.1/199.0	1.7 (8)	25	50/60	37/23	10/10
Ketoconazole	12.3	533.1/491.1	531.1/244.1	1.2 (8)	25	125/110	46/47	10/10
Carbendazim	7.4	192.1/160.1	192.1/132.1	5.1 (14)	25	61/61	25/41	12/10
Thiabendazole	7.8	202.1/175.1	202.1/131.1	1.6 (4)	25	91/91	37/47	14/10
Terbuthylazine	13.3	230.1/174.1	230.1/104.1	7.7 (7)	25	61/61	25/45	14/6
Terbutryn	13.1	242.1/186.1	242.1/91.0	6.6 (7)	25	50/50	25/38	15/5
Irgarol	13.5	254.1/198.1	254.1/83.0	4.3 (6)	25	70/70	26/41	6/6
M1	10.3	214.1/68.0	214.1/110.1	1.9 (5)	25	50/50	53/37	3/10
Dimethomorph	13.5	388.1/301.1	388.1/165.1	4 (19)	25	105/105	28/43	16/15
Fenpropimorph	13.2	304.3/147.2	304.3/117.1	1.6 (6)	25	81/81	41/77	10/10
Tridemorph	16.3	298.4/130.2	298.4/98.2	1.5 (6)	25	86/86	35/41	10/8
BIT	7.9	152.0/105.0	152.0/108.8	0.8 (4)	100	71/71	33/31	7/8
OIT	15.0	214.1/102.0	214.1/84.0	37 (6)	25	70/70	21/56	6/10
DCOIT	18.9	282.0/170.0	284.0/172.0	1.4 (8)	25	60/60	22/22	12/12
DMST	11.2	215.1/106.1	215.1/79.1	2.6 (6)	25	43/43	20/39	6/6
DMSA(*)	8.7	199.0/90.9	199.0/154.9	1.5 (9)	100	-45/-45	-30/-21	-4/-8
IPBC	12.8	282.0/57.2	282.0/164.9	0.6 (3)	100	56/56	23/23	2/12
Triclosan(*)	15.2	287.0/35.0	289.0/35.0	1.5 (2)	100	-45/-45	-30/-32	-3/-3
Triclocarban(*)	15.1	313.0/159.9	315.0/161.9	1.7 (3)	25	-65/-65	-22/-18	-9/-15
Chlorophene(*)	13.2	217.0/35.0	217.0/180.9	1.1 (2)	100	-70/-70	-46/-26	-3/-10
UV-filter								
BZP-1(*)	10.4	213.1/135.0	213.1/91.0	1.0 (2)	25	-70/-70	-28/-36	-11/-5
BZP-2(*)	8.3	245.1/135.0	245.1/109.0	1.4 (3)	25	-50/-50	-22/-28	-9/-7
BZP-3	15.7	229.1/151.1	229.1/105.1	1.5 (2)	25	66/66	27/27	10/8
BZP-4(*)	9.3	307.0/211.0	307.0/227.0	0.5 (4)	100	-90/-90	-46/-32	-15/-19
PBSA	7.0	275.0/194.0	275.0/166.0	2.8 (7)	25	100/100	43/67	14/12
Benzothiazoles								
Benzothiazole	10.0	136.0/109.0	136.0/64.8	2.3 (6)	100	61/61	33/49	8/6
MTBT	13.5	182.0/167.0	182.0/109.0	5.7 (4)	100	70/70	29/48	11/6
BTSA	7.2	216.0/134.0	216.0/90.1	2.9 (4)	25	66/66	33/53	10/8
OHBT(*)	8.1	150.0/42.0	150.0/121.8	3.1 (8)	100	-70/-70	-50/-26	-5/-1
Morpholinyl-BT	11.3	221.1/177.1	221.1/109.0	1.2 (2)	25	66/66	33/51	16/8
Surrogates								
Diuron-d_6	12.0	239.1/78.2	239.1/160.0	17.7 (4)	25	70/70	42/39	12/14
Diuron-d_6(*)	10.2	236.9/185.9	239.1/188.1	2.0 (4)	25	-70/-70	-25/-25	-9/-9
Isoproturon-d_6	11.8	213.2/78.1	213.2/171.2	3.9 (7)	25	65/65	30/22	12/14
Mecoprop-d_3(*)	10.6	216.1/71.0	216.1/143.9	26.6 (3)	25	-50/-50	-16/-20	-1/-11
Propiconazole-d_5	16.0	347.2/158.9	349.2/161.1	1.6 (2)	25	80/83	34/51	10/13
Tebuconazole-d_6	15.0	314.2/72.1	316.2/72.1	3.0 (3)	25	84/71	59/46	10/4
Imazalil-d_5	11.2	302.1/159.1	302.1/203.1	2.1 (3)	25	70/70	28/27	11/12
Ketoconazole-d_8	12.2	539.1/497.1	539.1/244.1	2.0 (5)	25	75/75	43/49	12/12
Carbendazim-d_4	7.3	196.2/164.2	196.2/136.2	5.7 (12)	25	70/70	26/42	12/11
Thiabendazole-d_6	7.7	208.2/136.2	208.2/181.2	1.3 (3)	25	90/90	47/37	8/16
Terbuthylazine-d_5	13.3	235.2/179.1	235.2/101.0	6.3 (5)	25	61/61	25/39	14/6
Terbutryn-d_5	13.1	247.1/191.1	247.1/91.1	4.0 (10)	25	50/50	24/41	12/5
Triclosan-$^{13}C_{12}$(*)	15.1	298.9.0/35.1	301.0/37.1	3.5 (4)	100	-45/-60	-26/-31	-3/-1
Triclocarban-$^{13}C_6$(*)	15.1	318.9/159.9	321.0/161.8	1.2 (3)	25	-60/-65	-20/-21	-9/-13

[a] precursor ion/product ion, [b] DP = declustering potential, CE = collision energy, CXP = collision exit potential

2.3 Results and Discussion

2.3.1 Method development

Aqueous matrices

Highest absolute recoveries and reproducibility were achieved using the HLB material (Table SI 2-1) and was therefore chosen for further optimization of the SPE conditions: The effect of pH on the extraction efficiency was tested in a range of pH 5-8 (Figure 2-1(a)). Due to diversity of target analytes with a broad range of pKa values, no optimal pH value for all analytes could be found. For most acidic analytes such as OHBT (pKa 6.2) and benzophenone-1 (pKa 8.0) the recoveries increased by up to 20% when increasing the pH from 5 to 8, whereas for some basic analytes, such as the morpholines fenpropimorph and tridemorph and for some neutral analytes such as the isothiazolones OIT and DCOIT, the recoveries decreased by a maximum of up to 30%. However, for most analytes no effect was observed or was statistically insignificant. Nevertheless, to ensure reproducible recoveries, the pH was adjusted to 6 as a compromise for the selected analytes.

Most analytes could be eluted efficiently with methanol (Figure 2-1(b)). Elution with acetone led to a slight increase of the elution efficiency for less polar compounds such as fenpropimorph and DCOIT. In contrast, the recovery of other analytes such as BIT and BZP-1 strongly decreased down to less than 10% when pure acetone was used. Thus, a mixture of methanol and acetone (60/40, v/v) was chosen, since it did not show the negative effect on the recovery of BIT and benzophenone-1 but at least slightly increased the recovery of compounds such as DCOIT and terbutryn.

Activated sludge

The PLE of the activated sludge was conducted using a mixture of methanol and water (50/50, v/v), 100% methanol and 100% acetone as extraction solvents. With 100% acetone as extraction solvent certain analytes such as BIT and mecoprop could not be recovered at all. Highest recoveries were achieved with the mixture of methanol and water, which was therefore chosen for further analyses and method validation (Figure 2-2(a)). No significant increase of recoveries were obtained with an increase in PLE temperature from 80 to 120°C (Figure 2-2(b)). Slightly higher recoveries were measured for example for carbendazim and BIT but could also be achieved by increasing the number of extraction cycles from 3 to 4. Since for some compounds such as diuron and propiconazole the recoveries even slightly de-

creased with higher temperatures, further extraction with PLE was conducted using 4 extraction cycles and an extraction temperature of 80°C. Information regarding the results of the PLE tests for the examined target analytes not included in Figure 2-2(a) and (b) are shown in Table SI 2-2.

The analytes DCOIT and IPBC were recovered from activated sludge with efficiencies of < 5%. These analytes could not be quantitatively extracted from activated sludge with the tested methods and were therefore excluded from further analyses.

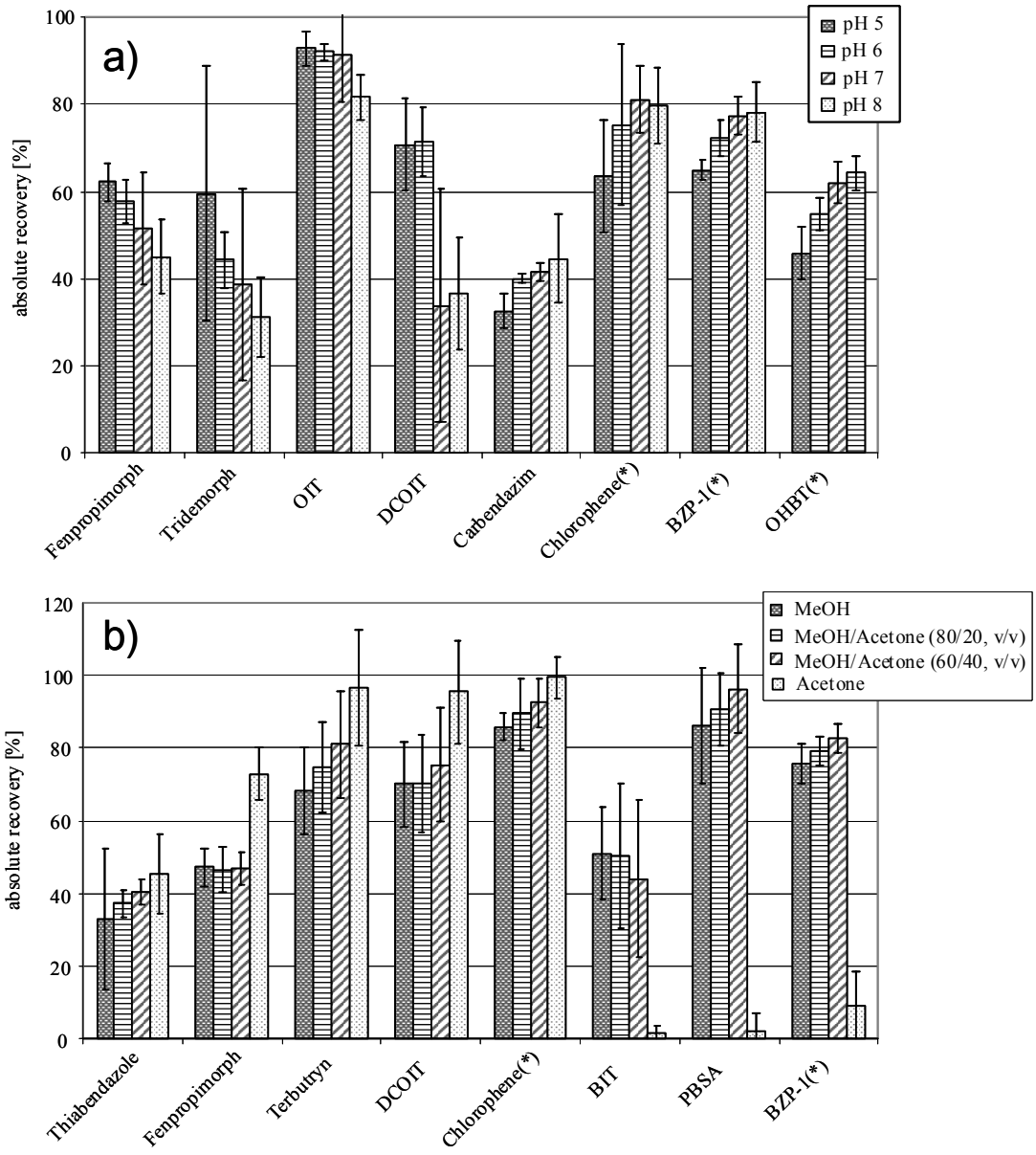


Figure 2-1. Optimization of SPE conditions: influence of matrix pH (a) and the elution solvent (b) on the absolute recoveries of selected target analytes. The error bars indicate the 95% confidence intervals (n=4). (*) Analytes determined in negative ionization mode.

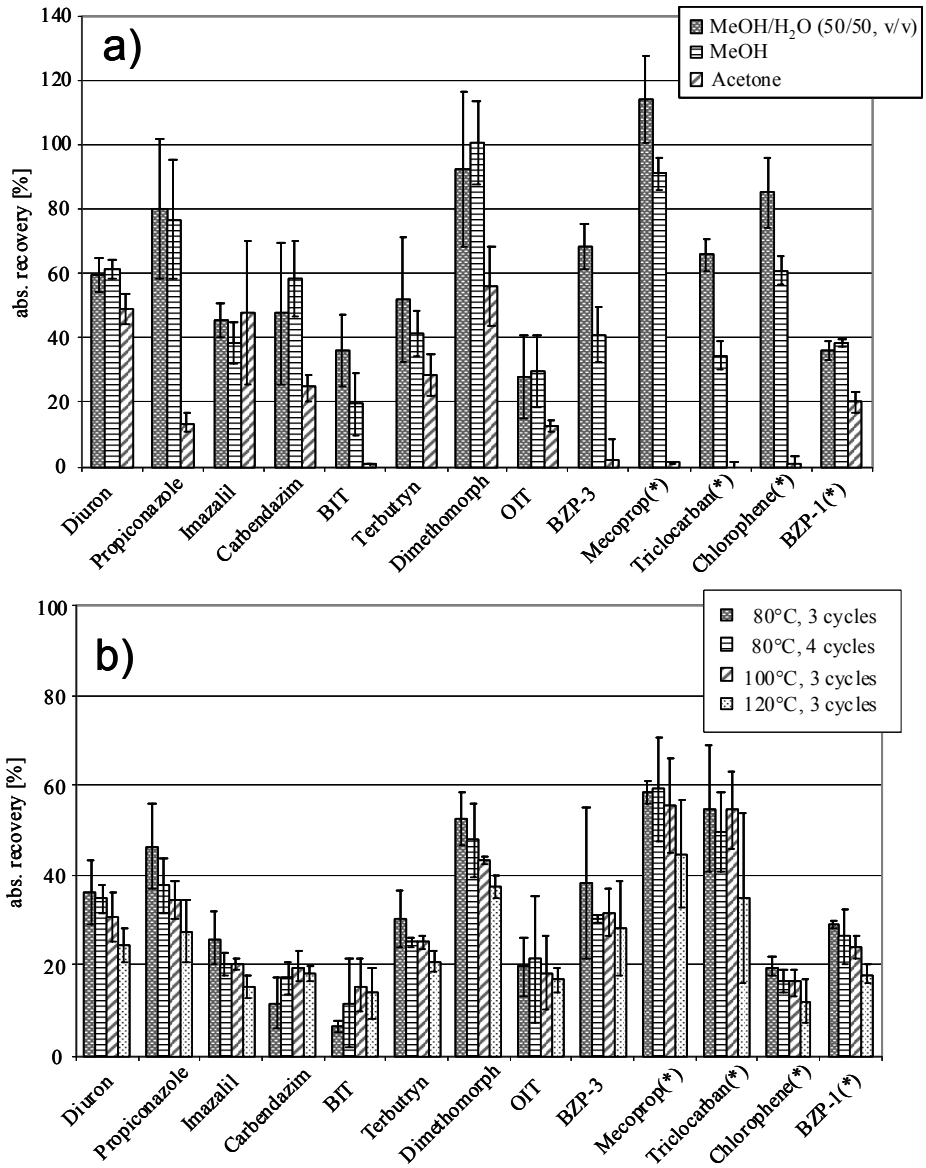


Figure 2-2. Optimization of the PLE: influence of different extraction solvents (a) and extraction temperatures (b) on the absolute recoveries of selected target analytes. The error bars represent the 95% confidence intervals (n=4). (*) Analytes determined in negative ionization mode.

2.3.2 Method validation

Aqueous matrices

In general, selected biocides, benzothiazoles and UV-filters can be analyzed with an acceptable accuracy in groundwater, surface water and raw and treated wastewater with ESI as well as APCI (Table 2-3). The relative recoveries were in an acceptable range of 70 to 130% and the 95% confidence intervals were less than 25% for most target analytes.

ESI: Absolute recoveries were mainly below 70% in surface water and raw and treated wastewater and revealed significant ion suppression by natural matrix components in negative and positive ionization mode. For instance, absolute recoveries of carbendazim in Rhine water, treated wastewater and raw wastewater were as low as 18, 8 and 10%, respectively. The strong ion suppression in surface water can be explained by the larger extraction volume of 1 L instead of 100 mL for raw wastewater. Nevertheless, using appropriate surrogate standards to compensate for the signal reduction by ion suppression, the relative recoveries of the target analytes were mainly between 69% (DCOIT in treated wastewater) and 130% (BZP-3 in surface water) for all selected matrices. Lower relative recoveries and high confidence intervals of 66 ± 32% and 42 ± 57% determined in treated wastewater for PBSA and BTSA, respectively, can be attributed to the high original background concentration in comparison to the spiked analyte concentration. Using a higher spike concentration of 4 µg L^{-1} yielded acceptable relative recoveries of 80 ± 13% (PBSA) and 78 ± 12% (BTSA) (Table SI 2-3).

However, for DCOIT, BZP-1 and BZP-2 the standard addition method had to be used for quantification in some matrices, since the matrix effects could not be sufficiently compensated in every tested matrix by stable isotope-labeled surrogate standards. Whereas the use of imazalil-d_5 for DCOIT and diuron-d_6 for BZP-1 led to significantly elevated relative recoveries of 198 ± 32% in surface water and 180 ± 14% in raw wastewater, respectively, the relative recovery of BZP-2 in surface water was too low (53 ± 7%) using triclocarban-$^{13}C_6$ for compensation.

APCI: Using APCI, three analytes (carbendazim, IPBC and BTSA) could not be quantified due to insufficient ionization efficiency. In all selected matrices the absolute recoveries were higher than 70% for most analytes measured with APCI (Table 2-3). Thus, in most cases the matrix did not suppress the ionization using APCI in contrast to ESI. However, for certain analytes measured in the positive ionization mode such as for BZP-3 and MTBT, significantly elevated absolute recoveries above 100% were determined. The UV-filter PBSA could not be quantified, since the absolute recoveries were even higher than 1000% in wastewater and

Rhine water. Similarly, absolute recoveries up to 300% were obtained with negative ionization for triclosan, triclocarban and BZP-2. These elevated recoveries for some of the analytes indicated that the sample matrix can lead to a significant ion enhancement using the APCI interface. However, in most cases the use of appropriate surrogate standards and the internal standard calibration led to acceptable relative recoveries in the range of 70% (DCOIT in surface water) to 135% (BZP-2 in surface water).

Only for DCOIT and BZP-3 no appropriate surrogate standards could be assigned to compensate for the ion enhancement in raw and treated wastewater when using APCI, and thus standard addition had to be applied. Since for DCOIT the absolute recoveries were even higher in groundwater, the internal calibration without use of surrogate standards led to low relative recoveries of 58 ± 22 and 28 ± 4% for DCOIT in raw and treated wastewater, respectively. BZP-3 was detected with relative recoveries of 62 ± 17% and 59 ± 28% in raw and treated wastewater, respectively.

For both ionization interfaces, the method validation already indicated that the matrix significantly affected the absolute recoveries of most of the selected analytes. Since the matrix content and composition is variable, it is crucial to determine the individual relative recoveries in complex matrices at least for all those analytes for which labeled surrogate standards are not available. In those cases where no surrogate can sufficiently compensate for the matrix effects, the standard addition method has to be used.

Table 2-3 Recoveries of biocides, UV-filters and benzothiazoles measured with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in different aqueous matrices with 95% confidence intervals (n = 4). Groundwater and Rhine water was spiked with 0.1 µg L^{-1}, whereas raw and treated wastewater was spiked with 1 and 0.5 µg L^{-1}, respectively. (*) Analytes determined in negative ionization mode. ND: not determined

	ESI								APCI							
	Groundwater		Rhine water		WWTP effluent		WWTP influent		Groundwater		Rhine water		WWTP effluent		WWTP influent	
Recovery [%]	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery
Biocides																
Diuron[a/a]	91 ± 4	105 ± 2	43 ± 3	104 ± 5	26 ± 1	100 ± 3	28 ± 1	99 ± 4	100 ± 9	95 ± 19	113 ± 9	106 ± 20	112 ± 5	89 ± 4	111 ± 7	96 ± 9
Isoproturon[b/b]	94 ± 2	105 ± 4	45 ± 4	101 ± 2	27 ± 1	96 ± 2	29 ± 1	96 ± 2	107 ± 4	103 ± 6	122 ± 5	98 ± 5	124 ± 5	97 ± 11	118 ± 9	95 ± 5
Mecoprop(*)[k/k]	94 ± 1	106 ± 4	51 ± 3	106 ± 6	32 ± 1	110 ± 17	51 ± 2	106 ± 7	103 ± 2	100 ± 8	122 ± 4	101 ± 15	116 ± 6	93 ± 10	110 ± 10	93 ± 10
Propiconazole[c/c]	105 ± 4	98 ± 1	96 ± 9	96 ± 3	61 ± 2	91 ± 5	49 ± 2	93 ± 7	103 ± 7	101 ± 6	120 ± 7	100 ± 10	121 ± 4	95 ± 4	120 ± 4	96 ± 4
Tebuconazole[d/i]	107 ± 6	104 ± 4	98 ± 9	103 ± 3	63 ± 2	97 ± 3	53 ± 2	91 ± 4	103 ± 7	96 ± 13	116 ± 4	95 ± 8	115 ± 7	100 ± 5	110 ± 10	100 ± 9
Imazalil[e/e]	81 ± 2	101 ± 3	40 ± 3	101 ± 2	22 ± 1	92 ± 4	22 ± 1	89 ± 3	96 ± 4	103 ± 3	132 ± 7	102 ± 3	139 ± 11	94 ± 7	104 ± 6	90 ± 7
Climbazole[j/ND]	86 ± 6	91 ± 5	ND	ND	61 ± 6	95 ± 4	57 ± 19	95 ± 16	ND	ND	ND	ND	ND	ND	ND	ND
Ketoconazole[f/ND]	71 ± 10	103 ± 11	ND	ND	55 ± 4	97 ± 8	48 ± 8	93 ± 10	ND	ND	ND	ND	ND	ND	ND	ND
Carbendazim[g/ND]	64 ± 2	107 ± 8	18 ± 1	116 ± 4	8 ± 1	117 ± 10	10 ± 1	122 ± 5	ND	ND	ND	ND	ND	ND	ND	ND
Thiabendazole[h/h]	66 ± 6	100 ± 2	21 ± 1	101 ± 4	10 ± 1	95 ± 7	16 ± 1	90 ± 7	97 ± 10	107 ± 10	95 ± 6	92 ± 9	86 ± 7	88 ± 8	95 ± 9	95 ± 13
Terbuthylazine[i/i]	99 ± 4	108 ± 2	64 ± 4	94 ± 2	39 ± 1	86 ± 1	34 ± 1	89 ± 3	102 ± 7	103 ± 10	117 ± 10	105 ± 8	109 ± 10	103 ± 8	99 ± 3	98 ± 5
Terbutryn[e/b]	95 ± 5	91 ± 6	51 ± 7	100 ± 17	29 ± 5	91 ± 17	23 ± 2	71 ± 7	100 ± 5	96 ± 5	140 ± 5	113 ± 7	142 ± 5	111 ± 14	120 ± 8	96 ± 10
Irgarol[e/b]	99 ± 3	98 ± 6	54 ± 4	108 ± 7	31 ± 2	101 ± 10	26 ± 1	82 ± 4	100 ± 5	92 ± 3	130 ± 4	101 ± 6	139 ± 6	104 ± 8	128 ± 16	98 ± 16
M1[e/b]	73 ± 1	101 ± 6	30 ± 3	84 ± 3	19 ± 1	85 ± 3	24 ± 1	108 ± 1	105 ± 6	101 ± 3	134 ± 3	107 ± 5	143 ± 7	111 ± 12	128 ± 6	102 ± 8
Dimethomorph[i/b]	108 ± 9	92 ± 5	96 ± 11	109 ± 12	57 ± 4	98 ± 7	62 ± 3	124 ± 6	107 ± 4	92 ± 4	147 ± 5	108 ± 5	149 ± 3	106 ± 10	132 ± 5	96 ± 3
Fenpropimorph[b/n]	78 ± 3	106 ± 3	44 ± 4	118 ± 4	23 ± 3	98 ± 9	23 ± 2	94 ± 10	93 ± 6	96 ± 6	110 ± 14	114 ± 15	90 ± 6	93 ± 6	82 ± 8	85 ± 9
Tridemorph[e/d]	35 ± 10	104 ± 28	21 ± 9	125 ± 39	11 ± 2	104 ± 21	9 ± 2	86 ± 16	39 ± 10	100 ± 20	43 ± 15	119 ± 70	38 ± 9	97 ± 40	61± 12	128 ± 27
BIT[e/n]	67 ± 8	99 ± 10	30 ± 3	90 ± 8	15 ± 1	73 ± 7	23 ± 1	110 ± 6	89 ± 17	103 ± 19	92 ± 13	107 ± 14	79 ± 15	92 ± 17	80 ± 14	93 ± 16
OIT[b/n]	93 ± 3	104 ± 3	66 ± 5	129 ± 5	35 ± 1	103 ± 3	26 ± 1	79 ± 1	105 ± 4	102 ± 4	109 ± 11	105 ± 11	95 ± 6	91 ± 5	103 ± 5	99 ± 5
DCOIT[e/n]	68 ± 21	83 ± 23	84 ± 11	198 ± 32	17 ± 3	69 ± 13	20 ± 4	78 ± 19	332 ± 174	70 ± 42	478 ± 24	107 ± 6	149 ± 25	28 ± 4	280 ± 96	58 ± 22
DMST[a/n]	89 ± 6	101 ± 6	39 ± 3	94 ± 5	24 ± 1	90 ± 4	27 ± 3	92 ± 9	88 ± 14	112 ± 11	77 ± 22	95 ± 18	73 ± 14	85 ± 7	81 ± 10	93 ± 15
DMSA(*)[k/n]	62 ± 1	100 ± 3	25 ± 1	76 ± 2	19 ± 2	93 ± 20	28 ± 1	83 ± 5	91 ± 16	97 ± 18	93 ± 16	99 ± 16	85 ± 7	90 ± 8	85 ± 14	90 ± 15
IPBC[j/ND]	93 ± 1	95 ± 3	63 ± 4	97 ± 4	40 ± 2	93 ± 4	40 ± 1	101 ± 1	ND	ND	ND	ND	ND	ND	ND	ND
Triclosan[l/l]	75 ± 31	102 ± 3	75 ± 8	102 ± 2	37 ± 2	98 ± 7	47 ± 10	95 ± 3	210 ± 127	107 ± 6	304 ± 45	106 ± 7	213 ± 38	99 ± 16	263 ± 31	93 ± 7
Triclocarban[m/m]	60 ± 33	98 ± 6	47 ± 10	105 ± 5	23 ± 2	100 ± 5	29 ± 3	102 ± 6	113 ± 20	113 ± 20	183 ± 54	111 ± 5	177 ± 44	107 ± 7	248 ± 38	110 ± 5
Chlorophene[a/n]	84 ± 20	104 ± 24	33 ± 2	120 ± 14	13 ± 2	90 ± 16	15 ± 4	108 ± 35	107 ± 24	110 ± 25	125 ± 15	128 ± 15	112 ± 14	112 ± 14	98 ± 14	99 ± 14
UV-filters																
BZP-1(*)[a/a]	69 ± 5	114 ± 7	21 ± 1	98 ±11	11 ± 1	93 ± 8	19 ± 1	180 ± 14	105 ± 4	99 ± 15	119 ± 10	102 ± 19	107 ± 4	83 ± 7	106 ± 9	93 ± 5
BZP-2(*)[m/k]	23 ± 4	142 ± 71	6 ± 1	53 ± 7	6 ± 1	93 ± 15	9 ± 1	111 ± 6	163 ± 15	101 ± 16	257 ± 21	135 ± 25	230 ± 18	117 ± 12	198 ± 23	107 ± 18
BZP-3[j/n]	105 ± 20	95 ± 21	93 ± 25	130 ± 31	46 ± 6	102 ± 17	42 ± 5	100 ± 12	263 ± 134	84 ± 43	257 ± 62	82 ± 20	196 ± 48	62 ± 17	183 ± 86	59 ± 28
BZP-4(*)[n/ND]	92 ± 3	107 ± 3	69 ± 6	81 ± 7	89 ± 9	105 ± 11	89 ± 3	105 ± 3	ND	ND	ND	ND	ND	ND	ND	ND
PBSA[a/-]	106 ± 3	101 ± 3	57 ± 1	100 ± 10	26 ± 11	66 ± 32	34 ± 6	96 ± 19	180 ± 60	ND	> 1000	ND	> 1000	ND	> 1000	ND
Benzothiazoles																
Benzothiazole[d/n]	98 ± 8	95 ± 12	89 ± 6	97 ± 6	68 ± 7	109 ± 13	67 ± 12	124 ± 21	165 ± 34	102 ± 21	155 ± 41	96 ± 27	115 ± 75	72 ± 47	109 ± 38	70 ± 25
MTBT[n/n]	88 ± 11	91 ± 12	93 ± 8	98 ± 8	80 ± 10	86 ± 11	83 ± 9	87 ± 10	281 ± 31	104 ± 12	295 ± 59	110 ± 22	254±109	96 ± 41	253 ± 56	94 ± 21
BTSA[a/ND]	108 ± 4	101 ± 3	35 ± 9	62 ± 9	24 ± 22	42 ± 57	46 ± 2	98 ± 12	ND	ND	ND	ND	ND	ND	ND	ND
OHBT(*)[k/n]	34 ± 2	99 ± 6	13 ± 1	70 ± 5	13 ± 3	102 ± 42	16 ± 1	84 ± 12	96 ± 15	115 ± 17	88 ± 5	105 ± 6	81 ± 33	93 ± 38	71 ± 33	83 ± 37
Morpholinyl-BT[e/n]	73 ± 2	94 ± 4	32 ± 1	84 ± 5	21 ± 1	89 ± 6	22 ± 1	94 ± 5	105 ± 3	106 ± 3	103 ± 5	104 ± 5	98 ± 7	99 ± 7	95 ± 3	97 ± 3

Indices (a-n) indicate the surrogate standards used for calculation of the analyte concentration by internal standard calibration for the measurement with ESI (first index) and APCI (second index). [a] diuron-d_6, [b] isoproturon-d_6, [c] propiconazole-d_5, [d] tebuconazole-d_6, [e] imazalil-d_5, [f] ketoconazole-d_8, [g] carbendazim-d_4, [h] thiabendazole-d_6, [i] terbuthylazine-d_5, [j] terbutryn-d_5, [k] mecoprop-d_3, [l] triclosan-$^{13}C_{12}$, [m] triclocarban-$^{13}C_6$, [n] no surrogate.

Activated sludge

ESI: Except for DCOIT and IPBC the selected biocides, benzothiazoles and UV-filters could be analyzed with an acceptable accuracy in activated sludge taken from the nitrification tank of a conventional WWTP using ESI in the positive and negative ionization mode (Table 2-4). Consistent with the results from the aqueous matrices, relatively low absolute recoveries as low as 9% for thiabendazole were determined in activated sludge. Using appropriate surrogate standards to compensate for the underestimation presumably caused mainly by ion suppression, for most analytes relative recoveries between 74% (BZP-1) and 119% (ketoconazole) could be achieved. Only for climbazole a slightly elevated recovery of 136 ± 28% was determined. The precision given by the 95% confidence intervals were mainly less than 25%. The high confidence interval of ± 57% determined for triclosan can be explained by the high background concentration of approximately 2.7 µg g_{TSS}^{-1}. For the higher spiking level of 2 µg g_{TSS}^{-1} an acceptable relative recovery of 102 ± 21% was obtained (Table SI 2-4).

APCI: The use of APCI revealed an ion enhancement for many analytes leading to absolute recoveries significantly higher than 100% (Table 2-4). Nevertheless, for most compounds the relative recoveries were within the range of 86% (BZP-3 and fenpropimorph) to 128% (BZP-2) due to the use of surrogate standards. A slightly elevated value of 138 ± 25% was only determined for BZP-2, whereas the relative recoveries were too low for BT (67 ± 49%) and OIT (43 ± 14%). Thus, the used surrogate standard isoproturon-d_6 could not sufficiently compensate for the low absolute recoveries of BZP-2 and BT. The relatively high 95% confidence intervals revealed a lower precision of the APCI measurement in comparison to ESI.

Table 2-4. Recoveries of biocides, UV-filters and benzothiazoles measured with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in activated sludge with 95% confidence intervals (n = 4). The activated sludge was spiked with 0.5 µg g_{TSS}^{-1} and extracted using PLE with MeOH/H_2O (50/50, v/v). (*) Analytes determined in negative ionization mode. ND: not determined.

Recovery [%]	ESI		APCI	
	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery
Biocides				
Diuron[a/a]	28 ± 2	88 ± 9	115 ± 21	99 ± 13
Isoproturon[b/b]	34 ± 3	111 ± 11	134 ± 23	114 ± 12
Mecoprop(*)[k/k]	114 ± 14	112 ± 13	118 ± 26	113 ± 4
Propiconazole[c/c]	84 ± 4	105 ± 12	150 ± 17	127 ± 11
Tebuconazole[d/d]	84 ± 6	115 ± 14	143 ± 9	119 ± 26
Imazalil[e/e]	27 ± 3	106 ± 14	127 ± 39	95 ± 21
Climbazole[e/c]	36 ± 8	136 ± 28	259 ± 176	138 ± 25
Ketoconazole[e/b]	28 ± 6	119 ± 11	101 ± 54	97 ± 48
Carbendazim[g/ND]	18 ± 2	114 ± 23	ND	ND
Thiabendazole[h/h]	9 ± 1	118 ± 19	90 ± 8	88 ± 25
Terbuthylazine[i/i]	48 ± 3	107 ± 12	150 ± 37	114 ± 25
Terbutryn[j/j]	29 ± 7	104 ± 31	142 ± 62	100 ± 43
Irgarol[j/j]	25 ± 1	101 ± 8	150 ± 14	116 ± 20
M1[j/j]	17 ± 3	84 ± 16	169 ± 20	115 ± 9
Dimethomorph[n/n]	99 ± 5	90 ± 5	195 ± 21	119 ± 13
Fenpropimorph[e/i]	23 ± 2	101 ± 8	114 ± 12	86 ± 6
Tridemorph[e/d]	13 ± 1	87 ± 5	64 ± 16	98 ± 22
BIT[g/b]	17 ± 3	96 ± 28	62 ± 24	92 ± 20
OIT[h/b]	16 ± 3	110 ± 33	41 ± 15	43 ± 14
DCOIT[e/n]	< 2	ND	< 2	ND
DMST[d/n]	49 ± 4	84 ± 10	100 ± 11	103 ± 12
DMSA(*)[n/a]	83 ± 9	91 ± 10	117 ± 37	107 ± 28
IPBC[j/ND]	< 2	ND	ND	ND
Triclosan(*)[l/l]	18 ± 35	101 ± 57	36 ± 111	116 ± 11
Triclocarban(*)[m/m]	66 ± 5	108 ± 11	53 ± 10	107 ± 11
Chlorophene(*)[l/l]	85 ± 11	96 ± 18	75 ± 36	112 ± 28
UV-filters				
BZP-1(*)[m/k]	36 ± 3	74 ± 9	109 ± 27	105 ± 7
BZP-2(*)[m/n]	18 ± 1	99 ± 11	236 ± 70	128 ± 36
BZP-3[i/i]	49 ± 3	104 ± 14	95 ± 18	86 ± 10
BZP-4(*)[l/ND]	93 ± 15	114 ± 28	ND	ND
PBSA[i/ND]	67 ± 8	118 ± 19	ND	ND
Benzothiazoles				
Benzothiazole[e/b]	68 ± 16	89 ± 29	60 ± 50	67 ± 49
MTBT[e/b]	80 ± 11	90 ± 5	84 ± 22	90 ± 20
BTSA[h/ND]	17 ± 7	99 ± 25	ND	ND
OHBT(*)[l/k]	53 ± 6	105 ± 24	94 ± 23	104 ± 15
Morpholinyl-BT[i/b]	46 ± 6	92 ± 16	113 ± 16	102 ± 12

Indices (a-n) indicate the surrogate standards used for calculation of the analyte concentration by internal standard calibration for the measurement with ESI (first index) and APCI (second index). [a] diuron-d_6, [b] isoproturon-d_6, [c] propiconazole-d_5, [d] tebuconazole-d_6, [e] imazalil-d_5, [f] ketoconazole-d_8, [g] carbendazim-d_4, [h] thiabendazole-d_6, [i] terbuthylazine-d_5, [j] terbutryn-d_5, [k] mecoprop-d_3, [l] triclosan-$^{13}C_{12}$, [m] triclocarban-$^{13}C_6$, [n] no surrogate.

2.3.3 Matrix effects (ME)

Aqueous matrices

In order to reduce matrix effects, different enrichment volumes of 1 L, 0.2 L and 0.1 L were chosen for groundwater and surface water, treated wastewater and raw wastewater, respectively. Nevertheless, for most of the selected compounds ion suppression was still significantly higher in raw and treated wastewater in comparison to water from the river Rhine. In this study, the influence of matrix effects on the absolute recovery was evaluated independently from any other factor by spiking the analytes into final extracts of groundwater, Rhine water and raw and treated wastewater. The ME determined in the different aqueous matrices are shown for all analytes which could be analyzed with ESI and APCI in Figure 2-3 (positive ionization mode) and Figure 2-4 (negative ionization mode).

It can be seen that for most analytes the ME values determined with ESI for groundwater were not significantly different from 100%. However, for a few compounds such as thiabendazole, DCOIT and BZP-2 relatively low ME values of less than 60% indicate that even groundwater with a very low DOC of ~ 0.6 mg L^{-1} cannot a priori be regarded as being free of any interference from matrix components. As expected in regard to lower absolute recoveries in aqueous matrices with higher matrix loads, ME values were significantly decreased in Rhine water and even more in raw and treated wastewater. ME values of less than 40% were determined for 10 out of 21 analytes measured in the positive ionization mode and for all analytes measured in the negative ionization mode in at least one of the wastewater samples. Strongest ion suppression with ME values of 10 to 15% were observed for thiabendazole, tebuconazole and BZP-2. Consistent with these results, strong matrix effects of down to 15% were also observed by Marín et al. (2009) in diluted leachates for carbendazim, thiabendazole, imazalil, diuron, isoproturon, terbuthylazine and terbutryn using UPLC-ESI/MS/MS .

In Figure 2-3 the ME values of the target analytes are shown in order of their retention time. In general, for analytes with a retention time below 12 min ME values were lower than for compounds eluting afterwards. Thus, a correlation between ME and retention time can be assumed. Consistent with these results, Dijkman et al. (2001) showed that the salinity caused strong ion suppression of early eluting acidic pesticides measured with ESI, while the DOC hardly effected their absolute recoveries. However, since the correlation of ME and retention time cannot explain the relatively high ME values for benzothiazole and the low values for

DCOIT, the influence of matrix components cannot be exclusively related to the retention time of the target analytes.

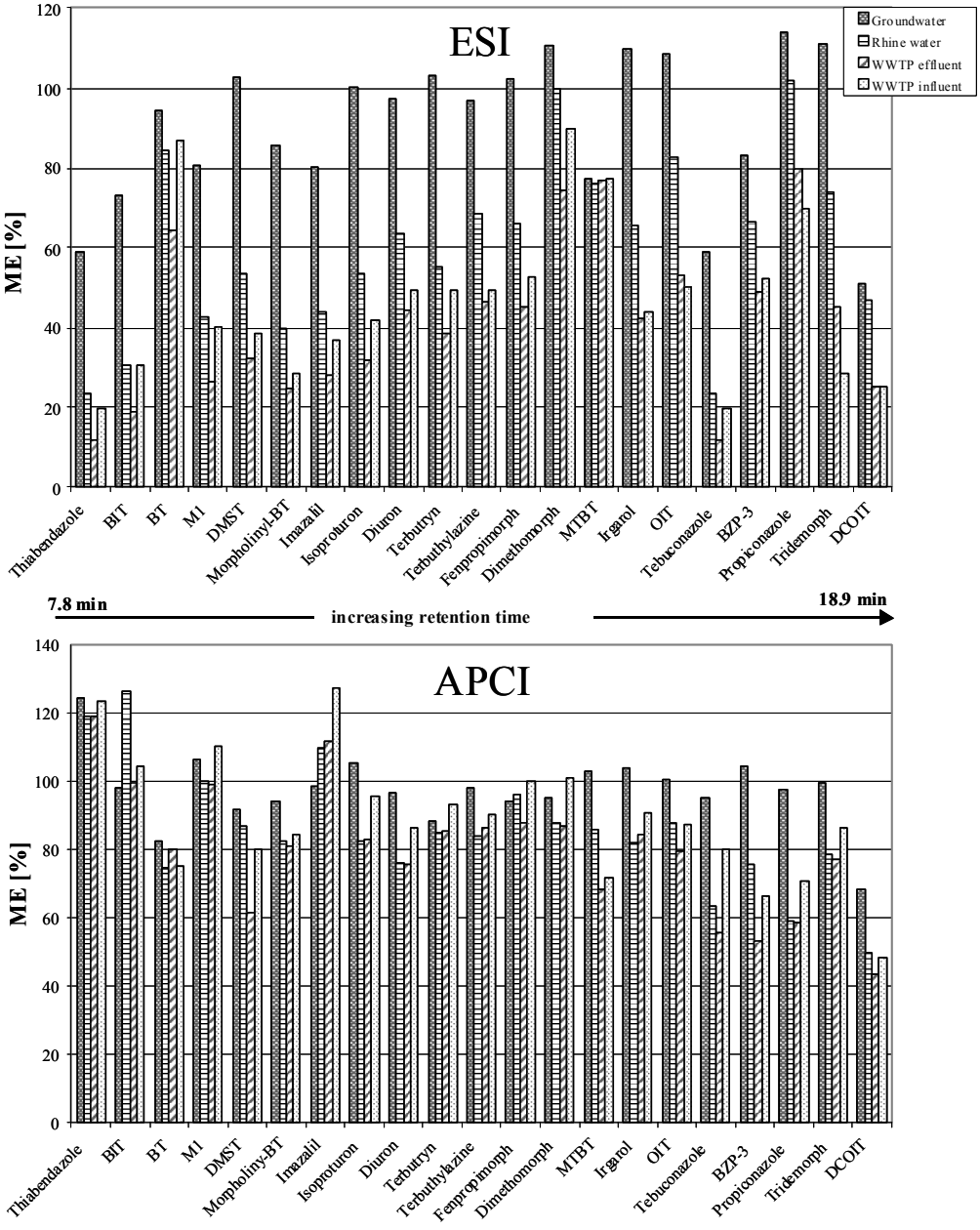


Figure 2-3. Matrix effects (ME) determined for biocides, UV-filters and benzothiazoles spiked into extracts of groundwater, Rhine water and raw and treated wastewater at a concentration of 200 ng mL^{-1} using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in the positive ionization mode. The analytes are ordered from left to right according to their increasing chromatographic retention time. Results for the analytes PBSA, BTSA, carbendazim and IPBC are not shown since they could not be analyzed using APCI.

Using APCI, all ME values were above 40% and for 15 from 21 analytes values between 74% (benzothiazole) and 127% (imazalil) were determined (Figure 2-3). This confirmed that APCI is quite less sensitive to matrix effects than ESI for the target compounds

measured in the positive ionization mode. These results consist with other studies reporting APCI being the favorable ionization source regarding the reduction of ion suppression (Matuszewski et al., 2003; Matuszewski, 2006; Schlüsener and Bester, 2005). However, for triclosan and BZP-2 measured in the negative ionization mode (Figure 2-4), highly elevated ME values of 204 and 344% were found, respectively. These results illustrate that significant ion enhancement can occur with APCI for certain analytes, even with groundwater. Zhao and Metcalfe (2008) reported an ion enhancement measuring neutral pharmaceuticals in wastewater using APCI. This differential behavior of the ionization interfaces under identical conditions was also reported by Liang et al. (2003), who examined the influence of stable isotope-labeled surrogate standards on the response of the target analytes and vice versa.

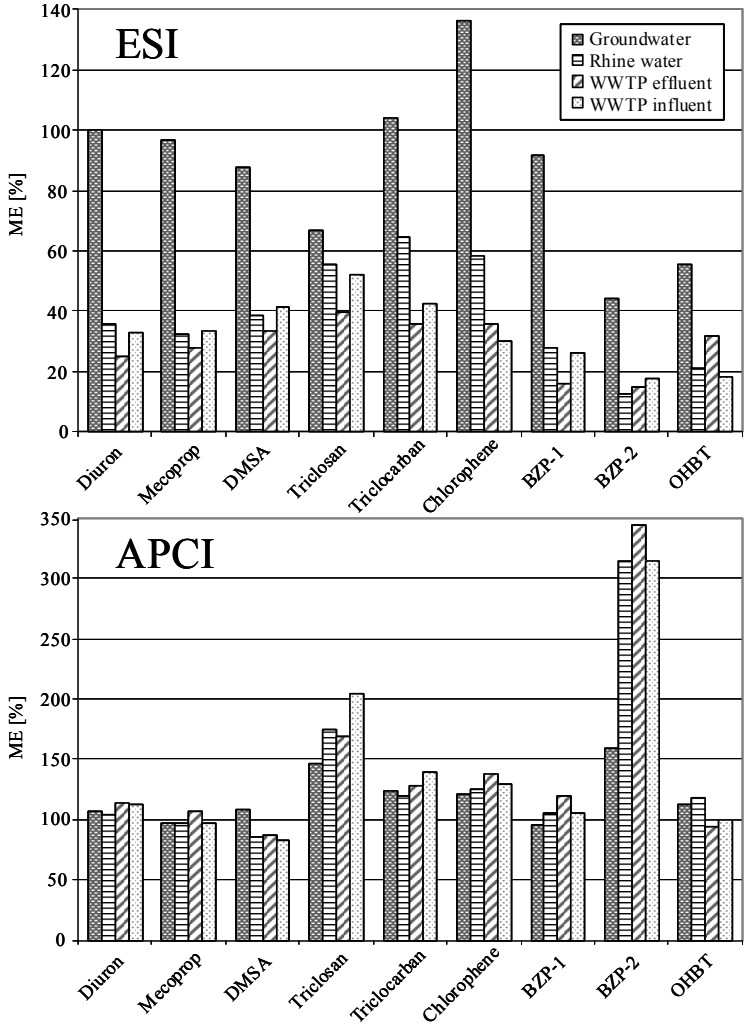


Figure 2-4. Matrix effects (ME) determined for biocides, UV-filters and benzothiazoles spiked into extracts of groundwater, Rhine water and raw and treated wastewater at a concentration of 200 ng mL^{-1} using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in the negative ionization mode.

Activated sludge

In Figure 2-5, ME values for activated sludge given as the absolute recoveries in post-extraction spikes are compared with the absolute recoveries determined in samples spiked prior to PLE (pre-extraction spikes) to assess both matrix effects and the extraction efficiency of the PLE. Consistent with the results for wastewater, ME values down to 20% (thiabendazole) showed that the measurement of sludge extracts with ESI was also strongly influenced by ion suppression. However, in extracts of activated sludge ME values below 40% were only determined for a lower number of 6 analytes indicating a slightly lower ion suppression compared to the tested wastewater matrices. In contrast, when using APCI as ionization interface ion enhancement was even more pronounced for activated sludge than for wastewater. ME values above 150% were observed for 17 analytes with a maximum ME value of 200% (climbazole). Higher 95% confidence intervals of the post- and pre-extraction spikes indicated a lower precision of the APCI measurement for the sludge extracts.

The comparison of the ME values (absolute recovery in post-extraction spikes) with the absolute recoveries in the pre-extraction spikes revealed that for most analytes absolute recoveries can be attributed mainly to matrix effects. However, for thiabendazole, fenpropimorph, tridemorph, BIT and OIT significantly higher absolute recoveries determined in the post-extraction spikes in comparison to the pre-extraction spikes with both ionization interfaces indicated losses during sample preparation, presumably by an incomplete sludge extraction.

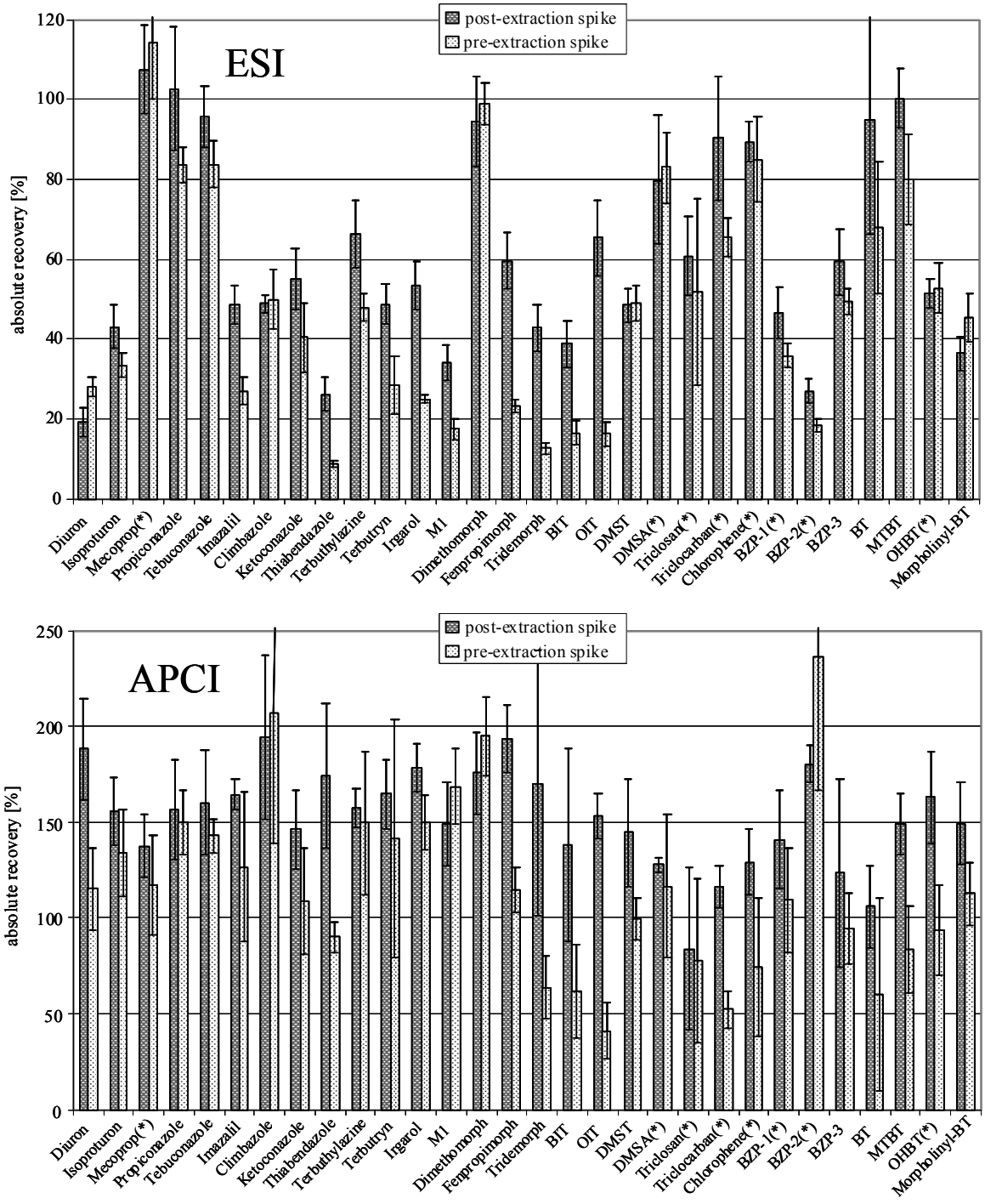


Figure 2-5. Absolute recoveries determined for biocides, UV-filters and benzothiazoles spiked into freeze-dried secondary sludge before PLE (pre-extraction spikes) and prior to LC-MS/MS analysis (post-extraction spikes) using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in the positive and negative ionization mode. The absolute recoveries determined in the pre-extraction spikes refer to activated sludge spiked with 2 µg g_{TSS}^{-1} for triclosan, climbazole and ketoconazole (due to their high background concentrations) and to activated sludge spiked with 0.5 µg g_{TSS}^{-1} for all other target compounds. The absolute recoveries determined in the post-extraction spikes refer to sample extracts spiked at a concentration of 200 ng mL^{-1} for all analytes. The error bars represent the 95% confidence intervals (n=4). (*) Analytes determined in negative ionization mode.

2.3.4 ESI *versus* APCI

The ratio of the signal intensity ($R_I = I_{APCI}/I_{ESI}$) measured in an external standard was compared with the ME ratio ($R_{ME} = ME_{APCI}/ME_{ESI}$) measured in an influent sample (Table 2-5) to decide which ionization source should be preferred for the target analytes. If the product of these ratios (R_I x R_{ME}) is significantly higher than 1, APCI leads to higher signal intensities in the samples and consequently to lower LOQs as long as the background noise is not increasing.

Table 2-5. Comparison of the ratio of matrix effects (R_{ME}) determined in an extract of WWTP influent and activated sludge using APCI (ME_{APCI}) and ESI (ME_{ESI}) with the ratio of the signal intensities (R_I) determined in an external standard using APCI (I_{APCI}) and ESI (I_{ESI}) together with respective LOQ values. (*) Analytes determined in negative ionization mode. ND: not determined.

		WWTP influent						Activated sludge					
	R_I	ME_{ESI} [%]	ME_{APCI} [%]	R_{ME}	R_{ME} x R_I	LOQ_{ESI} [ng L^{-1}]	LOQ_{APCI} [ng L^{-1}]	ME_{ESI} [%]	ME_{APCI} [%]	R_{ME}	R_{ME} x R_I	LOQ_{ESI} [ng L^{-1}]	LOQ_{APCI} [ng L^{-1}]
Biocides													
Diuron	0.31	42	86	2.1	0.64	5	20	19	188	9.9	3.1	2.5	25
Isoproturon	0.15	44	96	2.2	0.33	10	50	43	156	3.6	0.54	5	5
Mecoprop(*)	0.44	33	97	2.9	1.3	20	20	108	138	1.3	0.57	10	10
Propiconazole	0.47	70	71	1.0	0.48	10	20	103	157	1.5	0.71	5	10
Tebuconazole	0.78	83	80	1.0	0.75	5	20	96	160	1.7	1.3	5	5
Imazalil	0.10	37	127	3.4	0.33	20	50	49	165	3.4	0.34	5	50
Climbazole	0.10	ND	ND	ND	ND	10	20	49	194	4.0	0.4	5	10
Ketoconazole	0.12	ND	ND	ND	ND	50	50	55	146	2.7	0.32	25	25
Carbendazim	ND	16	ND	ND	ND	5	ND	32	ND	ND	ND	5	ND
Thiabendazole	0.32	20	123	6.2	2.0	5	10	26	174	6.7	2.1	2.5	5
Terbuthylazine	0.57	49	90	1.8	1.0	5	10	66	157	2.4	1.4	2.5	2.5
Terbutryn	0.48	49	93	1.9	0.90	5	10	49	165	3.4	1.6	2.5	5
Irgarol	0.08	44	91	2.1	0.17	5	20	54	178	3.3	0.26	2.5	10
M1	0.11	34	110	3.2	0.36	5	10	34	149	4.4	0.48	2.5	5
Dimethomorph	0.67	90	101	1.1	0.76	10	10	94	176	1.9	1.3	5	5
Fenpropimorph	0.12	53	100	1.9	0.23	5	10	60	193	3.2	0.38	2.5	5
Tridemorph	0.05	28	86	3.0	0.14	20	100	43	170	4.0	0.2	25	100
BIT	0.89	31	104	3.4	3.0	100	200	39	138	3.5	3.1	50	100
OIT	0.29	50	87	1.8	0.51	10	20	66	153	2.3	0.67	10	25
DCOIT	0.52	25	48	1.9	1.0	10	10	31	160	ND	ND	ND	ND
DMST	0.12	39	80	2.1	0.25	20	200	49	145	3.0	0.36	10	100
DMSA(*)	13	41	82	2.0	26	50	200	80	128	1.6	21	25	50
IPBC	ND	49	ND	ND	ND	50	ND	49	ND	ND	ND	ND	ND
Triclosan(*)	0.13	52	204	3.3	0.44	20	50	61	84	1.4	0.18	10	100
Triclocarban(*)	0.04	42	140	4.3	0.18	5	5	90	116	1.3	0.05	2.5	5
Chlorophene(*)	0.18	30	130	4.3	0.78	10	50	89	129	1.4	0.25	10	50
UV-filters													
BZP-1(*)	0.19	26	106	4.1	0.77	5	50	47	141	3.0	0.57	2.5	5
BZP-2(*)	0.08	17	315	18.1	1.5	5	5	27	180	6.7	0.54	2.5	5
BZP-3	1.4	52	66	1.3	1.8	50	50	59	124	2.1	2.9	25	50
BZP-4(*)	ND	26	ND	ND	ND	10	ND	81	ND	ND	ND	5	ND
PBSA	ND	42	ND	ND	ND	10	ND	85	ND	ND	ND	5	ND
Benzothiazoles													
Benzothiazole	19	87	75	0.86	17	200	50	95	106	1.1	21	100	25
MTBT	18	77	72	0.93	17	50	10	100	149	1.5	27	25	5
BTSA	ND	44	ND	ND	ND	20	ND	65	ND	ND	ND	10	ND
OHBT(*)	3.2	18	100	5.6	18	200	200	51	163	2.8	9.0	100	100
Morpholinyl-BT	0.77	28	85	3.0	2.3	10	20	37	149	4.0	3.1	2.5	10

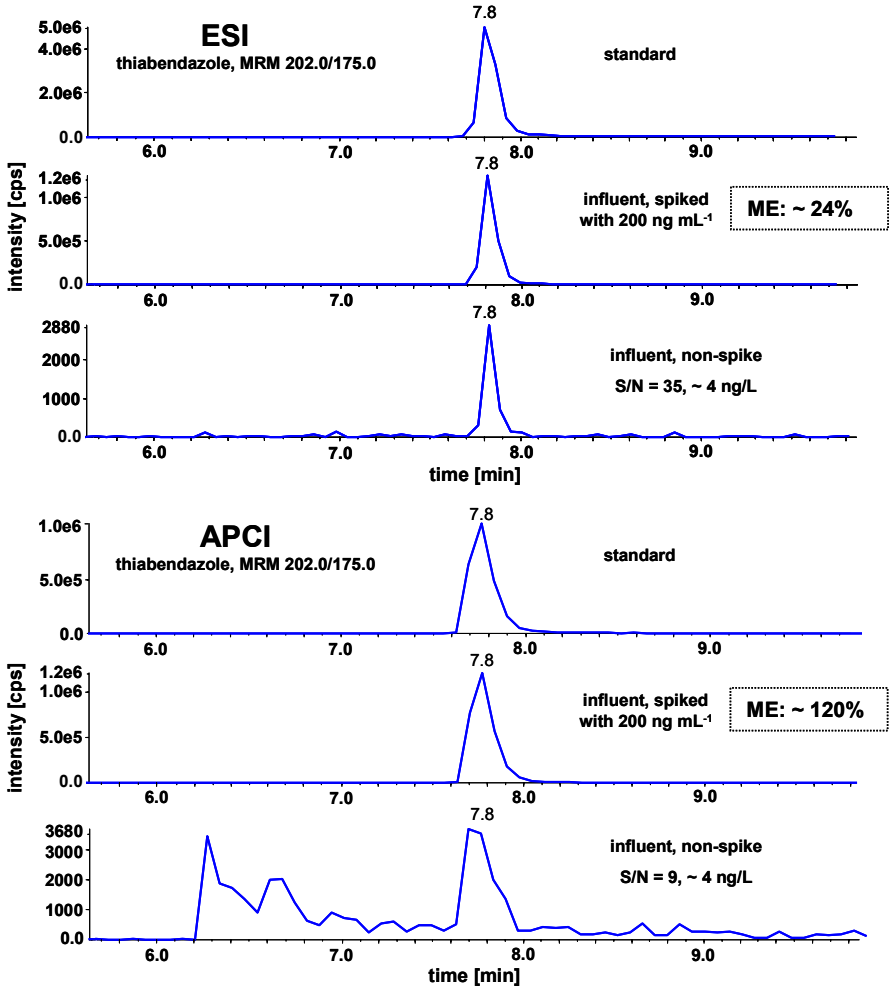


Figure 2-6. ESI and APCI MRM chromatograms of thiabendazole in a standard solution (200 ng mL^{-1}) in comparison to non-spiked and spiked (200 ng mL^{-1}) extracts of WWTP influent. Matrix effects (ME) are calculated by dividing the peak height for thiabendazole of the spiked sample extracts by the peak height for thiabendazole of the standard solution according to eq. 2-1.

Table 2-5 shows that BT, MTBT and OHBT were measured with approximately 20 times higher signal intensities in the tested influent samples using APCI due to a higher sensitivity and with respect to OHBT also due to avoided matrix effects. For the transformation product DMSA three times lower ion suppression and 13 times higher sensitivity led to a 26-fold signal increase. For all the other analytes the sensitivities of the APCI measurement were similar or significantly lower compared to ESI and only for thiabendazole, BIT, morpholinyl-BT, mecoprop and BZP-2 the positive effect of lower ion suppression slightly overweighed the negative effect of a decreased sensitivity.

However, for many analytes such as thiabendazole and BIT the background noise was significantly higher in the APCI chromatograms and thus higher analyte signals did not result in lower LOQs. This is particularly obvious for thiabendazole in Figure 2-6. Looking at the

peak heights of one transition of thiabendazole, the chromatograms of a standard solution show five times higher signals using ESI in comparison to APCI. The comparable peak heights in the chromatograms of the raw wastewater extracts show that the lower sensitivity was compensated by an approximately five times lower matrix effect using APCI. But the chromatograms of a non-spike sample extract show that the S/N ratio was approximately 4 times lower with APCI due to a higher background noise. Accordingly, the comparison of the S/N ratios calculated for all analytes and matrices using either the background concentrations in non-spiked sample extracts or different spiked amounts (Table SI 2-5) revealed similar or lower S/N ratios using APCI except for benzothiazole and MTBT. For these analytes the LOQs were assessed from the S/N ratios to be 4 times lower for influent and sludge samples using APCI, whereas for all other analytes up to 10 times higher LOQs were determined (Table 2-5).

Higher values of the product of ME ratio and sensitivity ratio were observed for many analytes when measuring activated sludge samples with APCI due to a strong ion enhancement. However, in comparison to the influent samples no further product values higher than 1 were determined except for diuron and BZP-3. Moreover, the stable isotope-labeled surrogates could not completely compensate for ion enhancement by the APCI interface and the precision was lower compared to the ESI measurement (Table 2-4, Figure 2-5).

2.3.5 Method application

Since the comparison of the interfaces revealed a better performance of ESI with regard to lower LOQs and the susceptibility of APCI to ion enhancement influencing accuracy and precision, ESI was chosen as the preferred ionization source for quantifying the target analytes in the different matrices. A summary of the method validation data is given in Table SI 2-6.

Occurrence in wastewater and surface water

The most prominent biocides in the influents of both sampled WWTPs were the anti-dandruff climbazole and the bacteriostatics chlorophene and triclosan with maximum influent concentrations of 1350 ± 70 ng L^{-1}, 664 ± 55 ng L^{-1} and 841 ± 31 ng L^{-1}, respectively (Table 2-6). Climbazole was also the biocide found at the highest concentrations in both WWTP effluents and in stream 2 (Wickerbach) with maximum concentrations of 443 ± 11 ng L^{-1} (WWTP 2) and 530 ± 70 ng L^{-1} (Wickerbach), respectively. This emphasizes the importance of this biocide as a biological active micropollutant emitted by WWTPs, which was up to now

not considered in other studies. The concentrations of the antifouling agent irgarol ranged from 6 to 22 ng L^{-1} in both sampled WWTP effluents and streams and were therefore significantly above the environmental quality value of 2 ng L^{-1} proposed by the German Working Group on water issues of the Federal States and the Federal Government (LAWA) (German Federal Environmental Agency, 2009).

All selected water-soluble UV-filters were detected in the influents of both WWTPs. Highest concentrations were determined for the sulfonic acids PBSA and BZP-4. In WWTP 2 sampled during summer time, BZP-4 and PBSA were detected in the influent at concentrations as high as 5130 ± 140 ng L^{-1} and 3890 ± 170 ng L^{-1}, respectively. Maximum effluent concentrations of 572 ± 15 (WWTP 1) and 1820 ± 240 ng L^{-1} (WWTP 2) for BZP-4 and PBSA, respectively, show the importance of WWTPs for the emission of these water-soluble UV-filters into the receiving water, even at least BZP-4 seems to be significantly removed by the treatment processes. Similar concentrations for BZP-4 were also reported by Kasprzyk-Hordern et al. (2008) and Rodil (2009) in wastewater from WWTPs in Wales and Spain, respectively. Consistent with the high effluent concentrations, BZP-4 and PBSA were also the dominant analytes detected in all surface water samples. In the Wickerbach sampled close downstream (~ 100 m) of a discharging WWTP, stream concentrations were as high as 1980 ± 130 ng L^{-1} (BZP-4) and 3240 ± 140 ng L^{-1} (PBSA).

Consistent with the study by Kloepfer et al. (2005a) the selected benzothiazoles were detected in the high ng L^{-1} to the low µg L^{-1} range in influents and effluents with BTSA being the most prominent analyte.

Occurrence in activated sludge

The dominant target analytes found in activated sludge were the bacteriostatics triclosan (2730 ± 90 ng g_{TSS}^{-1}) and chlorophene (322 ± 17 ng g_{TSS}^{-1}) as well as the anti-dandruffs climbazole (1160 ± 80 ng g_{TSS}^{-1}) and ketoconazole (328 ± 45 ng g_{TSS}^{-1}) (Table 2-6). The preservatives BIT and OIT, which were both below the LOQ in the wastewater samples, were found at concentrations of 179 ± 64 and 120 ± 85 ng g_{TSS}^{-1}, respectively. Concentrations of the selected polar UV-filters were quite low ranging from < LOQ (PBSA) to 132 ± 23 ng g_{TSS}^{-1} (BZP-3), while the polar benzothiazoles with K_{OW} values < 3 could be detected in activated sludge at considerable concentrations ranging from 157 ± 62 ng g_{TSS}^{-1} (MTBT) to 326 ± 147 ng g_{TSS}^{-1} (BTSA). To our knowledge, this is the first time that the anti-

dandruffs climbazole and ketoconazole, the isothiazolinones BIT and OIT as well as the benzothiazoles BT, MTBT, OHBT and BTSA were measured and detected in activated sludge.

Table 2-6. Concentrations of biocides, UV-filters and benzothiazoles determined in grab samples of activated sludge, wastewater (influent and effluent) and surface water. Sludge samples were taken from WWTP 1 on the 26th November 2008 (n = 4), wastewater samples from WWTP 1 on 11th February (n = 4) and from WWTP 2 on 2nd July 2009 (n = 3). Surface water samples were obtained from the river Rhine on 11th March 2008 (n = 4) and from two streams on 1st September 2009 (n = 3). Samples were measured with LC-MS/MS using ESI in the positive and negative ionization mode. The range indicates the 95% confidence interval. (*) Analytes measured in negative ionization mode. ND: not determined.

	Sludge [ng g_{TSS}^{-1}]		WWTP influent [ng L^{-1}]			WWTP effluent [ng L^{-1}]			surface water [ng L^{-1}]			
	LOQ	WWTP 1	LOQ	WWTP 1	WWTP 2	LOQ	WWTP 1	WWTP 2	LOQ	Rhine	stream 1	stream 2
Biocides												
Diuron	2.5	24 ± 18	5	23 ± 5	68 ± 7	2.5	25 ± 4	182 ± 15	0.5	9.9 ± 0.8	32 ± 9	24 ± 4
Isoproturon	5	<LOQ	10	39 ± 3	6.6 ± 1.3	5	58 ± 5	50 ± 2	1	18 ± 1	7.9 ± 0.6	113 ± 2
Mecoprop(*)	10	<LOQ	20	252 ± 18	37 ± 6	10	203 ± 16	72 ± 14	2	10 ± 1	126 ± 21	14 ± 3
Propiconazole	5	12 ± 2	10	16 ± 4	<LOQ	2.5	14 ± 1	10 ± 2	0.5	5.1 ± 0.5	5.6 ± 1.4	6.0 ± 0.6
Tebuconazole	5	<LOQ	5	<LOQ	8.9 ± 2.8	2.5	3.6 ± 0.3	6.4 ± 1.6	0.5	2.4 ± 0.2	5.9 ± 1.2	11 ± 1
Imazalil	5	23 ± 7	20	<LOQ	<LOQ	5	<LOQ	6.0 ± 0.9	1	<LOQ	2.6 ± 0.3	6.6 ± 0.4
Climbazole	5	1160 ± 80	10	475 ± 44	1350 ± 70	5	312 ± 12	443 ± 11	1	ND	47 ± 4	530 ± 70
Ketoconazole	25	328 ± 45	50	<LOQ	90 ± 15	25	<LOQ	<LOQ	5	ND	<LOQ	<LOQ
Carbendazim	5	8.5 ± 0.8	5	41 ± 6	143 ± 26	2.5	48 ± 4	88 ± 14	0.5	18 ± 1	94 ± 22	84 ± 4
Thiabendazole	2.5	6.7 ± 4.0	5	<LOQ	13 ± 2	2.5	4.7 ± 0.6	13 ± 1	0.5	0.7 ± 0.1	18 ± 3	5.4 ± 2.0
Terbuthylazine	2.5	<LOQ	5	<LOQ	18 ± 2	2.5	<LOQ	33 ± 1	0.5	2.4 ± 0.1	13 ± 1	2.9 ± 0.3
Terbutryn	2.5	59 ± 55	5	26 ± 3	116 ± 10	2.5	28 ± 4	123 ± 7	0.5	5.6 ± 0.3	51 ± 4	169 ± 12
Irgarol	2.5	3.7 ± 1.0	5	21 ± 3	<LOQ	2.5	22 ± 2	6.3 ± 0.8	0.5	1.0 ± 0.1	11 ± 1	6.8 ± 0.8
M1	2.5	<LOQ	5	9.1 ± 3.2	<LOQ	2.5	9.2 ± 1.2	10 ± 1	0.5	0.7 ± 0.1	12 ± 2	3.1 ± 1.0
Dimethomorph	5	<LOQ	10	<LOQ	18 ± 2	5	<LOQ	8.9 ± 1.0	1	<LOQ	<LOQ	2.5 ± 0.7
Fenpropimorph	2.5	<LOQ	5	<LOQ	<LOQ	2.5	<LOQ	<LOQ	0.5	<LOQ	<LOQ	<LOQ
Tridemorph	25	<LOQ	20	<LOQ	<LOQ	10	<LOQ	<LOQ	2	<LOQ	<LOQ	<LOQ
BIT	50	179 ± 64	100	<LOQ	<LOQ	50	<LOQ	<LOQ	10	<LOQ	<LOQ	37 ± 17
OIT	10	120 ± 85	10	11 ± 1	<LOQ	5	<LOQ	<LOQ	1	<LOQ	<LOQ	<LOQ
DCOIT		ND	10	<LOQ	<LOQ	5	<LOQ	<LOQ	1	<LOQ	<LOQ	<LOQ
DMST	10	<LOQ	20	<LOQ	<LOQ	10	<LOQ	16 ± 2	2	<LOQ	4.7 ± 1.5	6.3 ± 1.9
DMSA(*)	25	<LOQ	50	<LOQ	<LOQ	25	<LOQ	48 ± 14	5	5.7 ± 0.9	22 ± 11	31 ± 3
IPBC		ND	50	<LOQ	<LOQ	25	<LOQ	<LOQ	5	<LOQ	<LOQ	<LOQ
Triclosan(*)	10	2730 ± 90	20	372 ± 10	841 ± 31	10	162 ± 25	12 ± 5	2	3.3 ± 0.6	18 ± 1	268 ± 7
Triclocarban(*)	2.5	116 ± 10	5	<LOQ	12 ± 1	2.5	<LOQ	<LOQ	0.5	<LOQ	3.5 ± 0.5	3.5 ± 0.6
Chlorophene(*)	10	322 ± 17	10	664 ± 55	216 ± 13	5	181 ± 14	<LOQ	2	<LOQ	3.4 ± 1.4	4.8 ± 1.8
UV-filters												
BZP-1(*)	2.5	5.1 ± 1.5	5	43 ± 4	488 ± 19	2.5	12 ± 1	<LOQ	0.5	0.9 ± 0.3	2.2 ± 0.7	29 ± 2
BZP-2(*)	2.5	11 ± 2	5	35 ± 6	93 ± 10	2.5	14 ± 3	<LOQ	0.5	<LOQ	1.8 ± 1.9	6.7 ± 2.4
BZP-3	25	132 ± 23	50	195 ± 31	518 ± 55	25	96 ± 12	<LOQ	5	<LOQ	<LOQ	47 ± 29
BZP-4(*)	5	29 ± 7	10	2120 ± 220	5130 ± 140	5	572 ± 15	105 ± 11	1	51 ± 5	332 ± 11	1980 ± 130
PBSA	5	<LOQ	10	275 ± 27	3890 ± 170	5	316 ± 25	1820 ± 240	1	48 ± 3	1310 ± 200	3240 ± 140
Benzothiazoles												
Benzothiazole	100	265 ± 67	200	1120 ± 150	394 ± 75	100	313 ± 30	<LOQ	20	<LOQ	158 ± 6	560 ± 82
MTBT	25	157 ± 62	50	170 ± 24	379 ± 25	25	453 ± 27	261 ± 34	5	13 ± 1	119 ± 15	838 ± 25
BTSA	10	326 ± 147	20	1490 ± 220	1280 ± 90	10	2040 ± 90	393 ± 23	2	71 ± 8	1640 ± 240	2800 ± 490
OHBT(*)	100	307 ± 17	200	806 ± 26	619 ± 61	100	512 ± 68	<LOQ	20	<LOQ	199 ± 17	671 ± 55
Morpholinyl-BT	2.5	5.3 ± 1.2	10	20 ± 5	10 ± 2	2.5	19 ± 1	9.0 ± 1.5	0.5	0.8 ± 0.1	5.9 ± 2.4	5.9 ± 0.8

2.4 Conclusions

A multi-residue method for the determination of 26 biocides, 5 water-soluble UV-filters and 5 benzothiazoles in activated sludge, raw and treated wastewater, and surface water has been developed using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in the positive and negative ionization mode. Special emphasis has been made in this work to study the matrix effects in the different sample matrices using ESI and APCI. Ion suppression using ESI was identified to significantly reduce absolute recoveries of most target analytes, and thus making the use of appropriate labeled surrogate standards crucial to achieve acceptable relative recoveries in the range of 75 to 125%. Even APCI was shown to be less susceptible to ion suppression, the use of surrogate standards was needed for some of the target analytes to compensate for significant ion enhancement. The advantage of higher absolute recoveries when using APCI as ionization source was overweighed for most analytes by lower sensitivities and partly by higher background noise leading to higher LOQs. However, for benzothiazole and MTBT 4 times lower LOQs were determined in matrix containing samples using APCI. It can be concluded that the choice of ionization source depends on the target analytes and the matrices. In case ion suppression is significantly lower in comparison to ESI and no significant increase of the background noise occurs, APCI should be preferred to ESI if

(i) sensitivity is comparable or higher to ESI or less sensitivity does not equal higher responses due to less ion suppression and/or

ii) no appropriate surrogates (e.g. stable isotope-labeled analytes) are available and ion enhancement does not occur or can be compensated by an internal calibration due to similar relative matrix effects.

For certain groups of analytes, APCI could be definitely more suitable than the still more commonly used ESI and should be evaluated in regards to matrix effects even a measurement of non-enriched external standards reveals less sensitivity. However, this study indicates that for multi-residue methods including a broad spectrum of analyte groups applied to different complex matrices ESI is favorable. If stable isotope-labeled surrogate standards are not available for every analyte, the matrix effects have to be determined for every analyte/matrix combination to assure the appropriate compensation of the matrix effects.

A first application of the ESI method revealed that, besides the benzothiazoles, the analytes used in ingredients of PCPs such as the biocides climbazole and triclosan and the

UV-filters BZP-4 and PBSA were the dominant analytes in the analyzed wastewater samples from urban WWTPs. These data indicate that, in addition to the more prominent analytes such as triclosan, the parabenes or the musk fragrances, high amounts of ingredients of PCPs are emitted by WWTPs which are still not included in monitoring programs such as the water-soluble UV-filters or the anti-dandruff climbazole. The proposed environmental quality value of 2 ng L^{-1} for irgarol proposed by LAWA was found to be exceeded by a factor of 10 in a WWTP effluent indicating that WWTPs have to be considered as important point sources in regard to this quality norm.

The benzothiazoles BT, MTBT, OHBT and BTSA as well as the isothiazolinones BIT and OIT were detected for the first time in activated sludge in the mid ng g_{TSS}^{-1} range. This shows that analytical methods for water and sludge phase are crucial to correctly assess the fate (sorption and biotransformation) in WWTPs even for these relatively polar analytes with log K_{OW} values < 3.

2.5 Acknowledgement

The authors thank Manoj Schulz and Carsten Prasse for the supply of samples and Michael Schlüsener for reviewing the manuscript (all BfG). Financial support by EU commission for the EU-project NEPTUNE (Project no. 036845) is gratefully acknowledged.

3. Sorption of biocides, triazine and phenylurea herbicides, and UV-filters onto secondary sludge

Arne Wick, Olivian Marincas, Zaharie Moldovan, Thomas A. Ternes

Water Research ***2011****, 45, 3638-3652*

Abstract

The sludge-water distribution of a total of 41 organic micropollutants (9 phenylurea herbicides, 11 triazines, 16 biocides and 5 UV-filters) was investigated by laboratory batch experiments with fresh secondary sludge taken from a municipal WWTP. Sorption kinetics as well as sorption isotherms were examined by analyzing the compound concentration in the aqueous and solid phase for mass balance control and quality assurance. The sorption kinetic experiments revealed a sorption equilibrium time of <2 h and adverse effects of sodium azide on the sludge-water distribution of several compounds. Sorption isotherms were constructed for 6 different spiking levels spanning 3 orders of magnitude (100 ng L^{-1} – 30000 ng L^{-1}) and were well described by the Freundlich model. For some compounds non-linear sorption with Freundlich exponents n < 1 revealed a decreased sorption affinity to the sludge flocs with increasing aqueous phase concentration. Therefore, sludge-water distribution coefficients ($K_{d,sec}$) were calculated from the isotherm data for a constant concentration level of 1 µg L^{-1}. Based on the sludge dry weight (dw), the $K_{d,sec}$ values of phenylurea herbicides ranged from 9 L $kg_{dw\ sludge}^{-1}$ (isoproturon) to 320 L $kg_{dw\ sludge}^{-1}$ (neburon), those of triazines from 5 L $kg_{dw\ sludge}^{-1}$ (atrazine) to 190 L $kg_{dw\ sludge}^{-1}$ (terbutryn), those of biocides from 10 L $kg_{dw\ sludge}^{-1}$ (N,N-dimethyl-N'-p-tolylsulfamide) to 40000 L $kg_{dw\ sludge}^{-1}$ (triclocarban) and those of UV-filters from 9 L $kg_{dw\ sludge}^{-1}$ (phenylbenzimidazole sulfonic acid) to 720 L $kg_{dw\ sludge}^{-1}$ (benzophenone-3). For most compounds $K_{d,sec}$ values were below 500 L $kg_{dw\ sludge}^{-1}$ and thus removal in WWTPs by the withdrawal of excess sludge is expected to be negligible (<10%), except for the biocides triclocarban (80 - 95%), triclosan (55 - 85%), chlorophene (30 - 60%), imazalil (25 - 55%) and fenpropimorph (15 - 40%) as well as the UV-filter benzophenone-3 (5 - 20%). A simple linear free energy relationship (LFER) approach using the logarithmized octanol-water partition coefficient $\log K_{OW}$ as single descriptor is discussed for a rough classification of nonionic compounds regarding their potential removal in WWTPs by sorption.

3.1 Introduction

For many emerging organic micropollutants of ecotoxicological concern such as pharmaceuticals, biocides, UV-filter or perfluorinated flame retardants, WWTPs are the main source for their release into the environment (Kupper et al., 2006; Richardson, 2009). Sorption on activated sludge is one of the key factors controlling the removal of organic micropollutants in conventional wastewater treatment plants (WWTPs) (Ternes and Joss, 2007). Thus, information on sludge-water distribution is crucial to predict the influence of sorption on their fate in WWTPs and the quantity released into the aquatic environment.

However, especially for the often moderately polar emerging micropollutants systematic studies on sludge-water distribution are rare (Carballa et al., 2008; Maurer et al., 2007; Ternes et al., 2004b). Many previous studies relied on single point concentrations rather than sorption isotherms, thus assuming sorption being independent from the actual concentration. However, for compounds featuring high hydrophobicity or specific polar and ionic moieties leading to specific interactions with the sludge surface, the linear approach might not be valid due to a limited sorption capacity (Clara et al., 2004; Langford et al., 2005; Thomas et al., 2009).

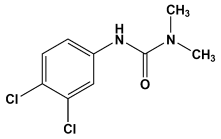
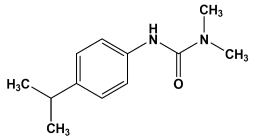
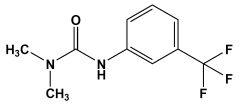
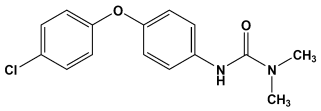
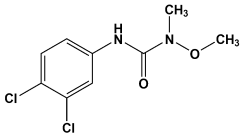
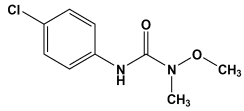
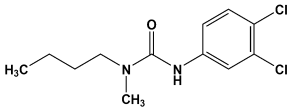
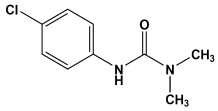
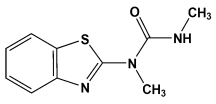
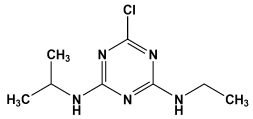
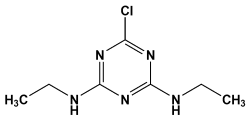
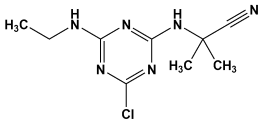
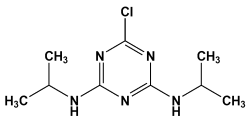
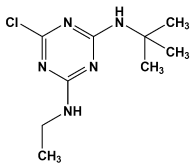
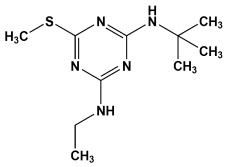
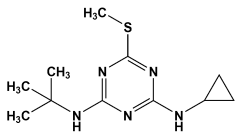
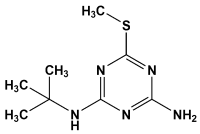
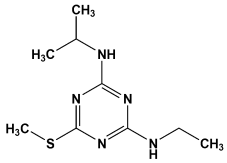
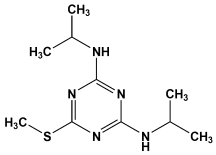
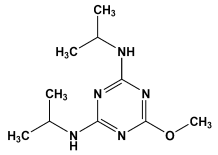
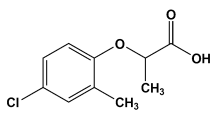
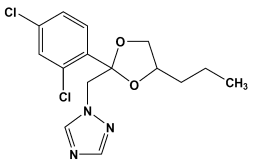
So far, the results suggest that simple empirical model approaches based on the correlation of the effective octanol-water partitioning coefficient (K_{OW}) with the organic carbon based sludge-water distribution coefficient (K_{OC}) are inappropriate for predicting the sludge sorption affinity of polar and ionic compounds (Carballa et al., 2008; Ternes et al., 2004b). The sorption behavior can depend on different physico-chemical properties of the micropollutant (hydrophobicity, hydrogen bonds, electrostatic interactions, etc.) and the sorbent, e.g. different sludge types (primary, secondary and digested sludge) and sludge retention times (SRTs) (Barret et al., 2010; Langford et al., 2005; Ochoa-Herrera and Sierra-Alvarez, 2008).

In case of rapid biotransformation of the target compounds ($k_{biol} > 0.1 \times k_{sorb}$), sorption equilibrium might not be achievable and hence, reliable sorption coefficients cannot be determined without inhibiting the microbial activity (Ternes and Joss, 2007). Even if biotransformation kinetics are slow compared to sorption kinetics and a sorption equilibrium can be assumed, calculation of the sorbed quantity from the soluble quantity as proposed by the OECD guideline 106 can lead to an overestimation of sludge-water distribution coefficients (Ramil et al., 2010; Stein et al., 2008; Wick et al., 2009).

Several methods including the addition of inhibitors of microbial activity such as sodium azide (NaN_3) (Dobbs et al., 1995), mercury chloride ($HgCl_2$) (Maurer et al., 2007) and mercury sulfate ($HgSO_4$) (Clara et al., 2004) as well as autoclaving (Dobbs et al., 1995; Zhao et al., 2008) and freeze-drying followed by heating (Andersen et al., 2005) have been applied for sludge deactivation. However, freeze-drying and autoclaving alters the texture of the sludge flocs and thus the sorbent structure (Barret et al., 2010; Berns et al., 2008). Chemical deactivation of sludge might also influence the sorption processes due to inactivation of cells and consecutive cell lysis, or by chemical interaction and reaction with the target compounds (Chefetz et al., 2006; Gaillardon, 1996).

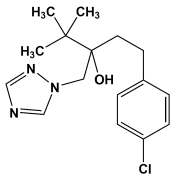
The objective of the current study was to elucidate the sorption behavior of several structurally diverse emerging organic micropollutants including biocides, UV-filters as well as triazines and phenylurea herbicides as for most of these compounds this data is still lacking. A special focus was set on (i) the sorption kinetics with and without using NaN_3 for inhibition of the microbial activity, (ii) the concentration dependency of sorption (sorption isotherms) and (iii) the correlation of the $\log K_{OW}$ as a measure of hydrophobicity with sludge-water distribution coefficients. The selected analytes are listed in Table 3-1.

Table 3-1. Chemical structures of the investigated phenylurea and triazine herbicides, biocides, and UV-filters.

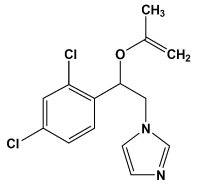
Analyte Application CAS number	Chemical structure	Analyte Application CAS number	Chemical structure
Diuron Herbicide/Biocide CAS: 33-54-1		Isoproturon Herbicide/Biocide CAS: 34123-59-6	
Fluometuron Herbicide CAS: 2164-17-2		Chloroxuron Herbicide CAS: 1982-47-4	
Linuron Herbicide CAS: 330-55-2		Monolinuron Herbicide CAS: 1746-81-2	
Neburon Herbicide CAS: 555-37-3		Monuron Herbicide CAS: 150-68-5	
Methabenzthiazuron Herbicide CAS: 18691-97-9		Atrazine Herbicide CAS: 1912-24-9	
Simazine Herbicide/Algicide CAS: 122-34-9		Cyanazine Herbicide CAS: 21725-46-2	
Propazine Herbicide CAS: 139-40-2		Terbuthylazine Herbicide/Biocide CAS: 5915-41-3	
Terbutryn Herbicide/Algicide/ Biocide (Antifouling) CAS: 886-50-0		Irgarol Herbicide/Algicide/ Biocide (Antifouling) CAS: 28159-98-0	
M1 Transformation product of irgarol CAS: 30125-65-6		Ametryn Herbicide CAS: 834-12-8	
Prometryn Herbicide CAS: 7287-19-6		Prometon Herbicide CAS: 1610-18-0	
Mecoprop Herbicide/Biocide CAS: 7085-19-0		Propiconazole Fungicide/Biocide CAS: 60207-90-1	

continues

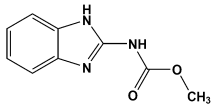
Tebuconazole
Fungicide/Biocide
CAS: 107534-96-3



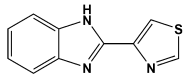
Imazalil
Fungicide/Biocide
CAS: 35554-44-0



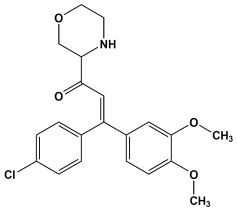
Carbendazim
Fungicide/Biocide
CAS: 10605-21-7



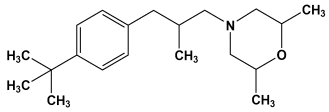
Thiabendazole
Fungicide/Biocide
CAS: 107534-96-3



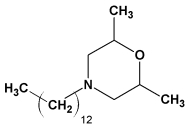
Dimethomorph
Fungicide
CAS: 110488-70-5



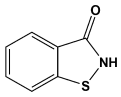
Fenpropimorph
Fungicide
CAS: 67564-91-4



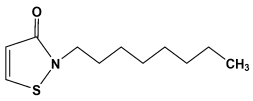
Tridemorph
Fungicide
CAS: 24602-86-6



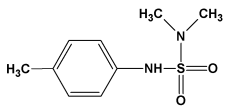
1,2-Benzisothiazolin-3-one (BIT)
Microbicide/Biocide
CAS: 2634-33-5



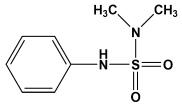
2-n-Octyl-4-isothiazolin-3-one (OIT)
Microbicide/Biocide
CAS: 26530-20-1



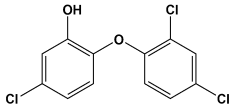
N,N-Dimethyl-N'-p-tolylsulfamide (DMST)
Transformation product of tolylfluanide
CAS: 668-40-71-9



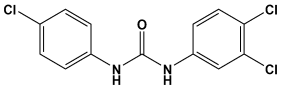
N,N-Dimethyl-N'-phenylsulfamide (DMSA)
(Transformation product of dichlofluanide
CAS: 4710-17-2



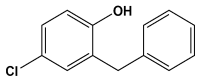
Triclosan
Microbicide/Biocide
CAS: 3380-34-5



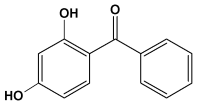
Triclocarban
Microbicide/Biocide
CAS: 101-20-2



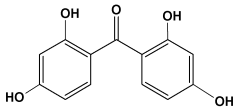
Chlorophene
Microbicide/Biocide
CAS: 120-32-1



Benzophenone-1 (BZP-1)
UV-Filter
CAS: 131-56-6



Benzophenone-2 (BZP-2)
UV-Filter
CAS: 131-55-5



Benzophenone-3 (BZP-3)
UV-Filter
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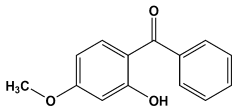

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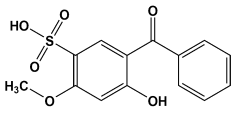
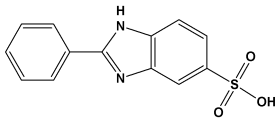


Phenylbenzimidazole sulfonic acid (PBSA)
UV-Filter
CAS: 27503-81-7



3.2 Experimental Methods

3.2.1 Chemicals

Triazines and phenylurea herbicides were purchased from Dr. Ehrenstorfer (Augsburg, Germany). All other reference standards were received from the same suppliers as described in Wick et al. (2010). Methanol (picograde) and acetonitrile (HPLC gradient grade) were purchased from LGC Promochem (Wesel, Germany). Formic acid and sulfuric acid (both p.a.) were purchased from Merck (Darmstadt, Germany) and ammonium formate (purum grade) from Sigma-Aldrich (Schnelldorf, Germany). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Separate standard solutions of all analytes and surrogate standards were prepared in methanol at a concentration of 10 and 1 μg mL^{-1}, respectively, and stored at 4°C in the dark.

3.2.2 Sampling

The sludge for the laboratory batch experiments was taken in solvent-rinsed amber glass bottles from the nitrifying zone of the activated sludge treatment of a German municipal WWTP serving 300,000 population equivalents (PE). The activated sludge system is operated with a hydraulic retention time (HRT) and sludge retention time (SRT) of 12 h and 10 - 12 d, respectively. Details of the complete treatment train can be found elsewhere (Wick et al., 2010). Fresh sludge (concentration of total suspended solids (TSS): 4 g_{TSS} L^{-1}, total organic carbon (TOC) per gram dry weight of sludge: 0.29 g_{OC} $g_{dw\ sludge}^{-1}$, pH 6.8) was directly transported to the lab and sorption batch experiments were initiated within 2 h.

3.2.3 Sorption kinetics: Sorption equilibrium and influence of sodium azide

A homogenous suspension (190 mL) of the activated sludge was filled into 500 mL centrifuge bottles (polypropylene). For each sampling time point (0, 0.75, 1.5, 3.2, 6 and 24 h) three samples with 0, 0.2 and 1% NaN_3 concentrations were prepared by adding 10 mL of Milli Q water, 4% or 20 % NaN_3 solution, respectively. Target compounds were spiked to a concentration of 10 μg L^{-1} and the samples were placed on a horizontal shaker for the different incubation times. The background concentration of the target compounds in the sludge was assessed in non-spiked controls for the first sampling point (t = 0 h). The ambient pH of the sludge was stable (6.8 ± 0.2) throughout the incubation period. After the defined exposure time, the sludge was centrifuged (15 min, 3640 g) and the supernatant was stored at 4°C for

solid-phase extraction (SPE). The solid sludge particles were freeze-dried for micropollutant analysis and the water content f_{water} (L $g_{dw\ sludge}^{-1}$) was determined by the difference of the sludge wet and dry weight before and after freeze-drying, respectively.

3.2.4 Freundlich sorption isotherms

The experimental set-up and sludge characteristics were similar to the equilibrium experiments described above. The Freundlich isotherms were determined by spiking the target compounds into 200 mL of secondary sludge to a concentration of 0.1, 0.3, 1, 3, 10 and 30 µg L^{-1}. All samples were prepared in duplicate and incubated for 1.5 h to ensure sorption equilibrium. Non-spiked samples were analyzed at the beginning and at the end of the incubation period to assess the natural background concentration. Filtered (Whatman, GF6) samples of the supernatant of centrifuged sludge were spiked to a concentration of 10 µg L^{-1} and served as controls for any compound losses due to sorption to the glass vessels.

3.2.5 Analytical methods

The analytical method used for extraction and detection of the target analytes has been described in detail in Wick et al. (2010) (chapter 2). Briefly, the supernatant was filtered through glass-fiber filters (Whatman, GF6) and 200 mL were adjusted to pH 6 with 3.5 M H_2SO_4. After addition of 200 ng of an internal standard mix (1 µg mL^{-1} stock solution), the analytes were enriched by SPE using 200 mg Oasis HLB cartridges (Waters, Milfort, USA) and eluted with 4 x 2 mL of a mixture of methanol and acetone (60/40, v/v). The detection was performed using reversed-phase liquid chromatography (Synergi Fusion-RP, 150 x 3 mm, 4 µm, Phenomenex, Aschaffenburg, Germany) coupled to electrospray mass spectrometry operated in the positive and negative ionization mode (API 4000 tandem MS, Applied Biosystems, Foster City, USA).

Extraction of the freeze-dried sludge samples was conducted by pressurized liquid extraction (PLE) (ASE 200 Accelerated Solvent extractor, Dionex, Idstein, Germany). Approximately 0.2 g of the freeze-dried sludge was filled into 22 mL cells and 200 ng of the internal standard mix was added. PLE was accomplished with a mixture of water and methanol (50/50, v/v) at 80°C and 100 bar (4 cycles). The extract was diluted with groundwater to a final volume of 800 mL and further preparation was performed as described for the aqueous samples.

All target analytes were quantified using an internal standard calibration and the limit of quantification (LOQ) was defined as the second lowest calibration point in the regression

as long as the calculated signal to noise ratio (S/N) of the analytes in the native sample extracts was >10 for the first transition (MRM 1) used for quantification and >3 for the second transition (MRM 2) used for confirmation. The retention time, MRM transitions and corresponding MS parameters of triazines and phenylurea herbicides not included in the study of Wick et al. (2010) (chapter 2), are provided in the Supplementary Information (Table SI 3-1).

3.2.6 Method validation

The analytical method was entirely validated within the study of Wick et al. (2010) (Chapter 2). Since the assignment of stable-isotope-labeled standards was adapted for some analytes and additional triazines and phenylurea herbicides were included, relative recoveries were examined to confirm the reliability of the method for all analytes within the current study. The relative recoveries were determined as the ratio of the spiked concentrations and the quantified concentrations at a spiking level of 2 µg L^{-1} and 4 µg $g_{dw\ sludge}^{-1}$ in the aqueous phase and freeze-dried sludge, respectively (dublicate measurements). The results are reported in the Supplementary Information (Table SI 3-2).

3.2.7 Calculations

Sorption isotherms were fitted with the Freundlich model which is a commonly applied empirical model to describe environmental sorption processes (Schwarzenbach et al., 2003). According to the Freundlich model, the sorbed concentration C_s (µg $kg_{dw\ sludge}^{-1}$) is related to the soluble concentration C_w (µg L^{-1}) at sorption equilibrium based on the following equation:

$$C_s = K_f * C_w^n \qquad (3\text{-}1)$$

where K_f (µg^{1-n} L^n $kg_{dw\ sludge}^{-1}$) is the Freundlich sorption coefficient and n (dimensionless) the Freundlich affinity constant.

If the sorption isotherm is linear (n = 1), sorption will be independent from concentration and can be determined by the sludge-water distribution coefficient $K_{d,sec}$ (L $kg_{dw\ sludge}^{-1}$):

$$K_{d,sec} = \frac{C_s}{C_w} \qquad (3\text{-}2)$$

For comparison with literature data, $K_{d,sec}$ values were normalized to the fraction of total organic carbon f_{OC} (kg_{OC} $kg_{dw\ sludge}^{-1}$) resulting in the K_{OC} (L kg_{OC}^{-1}):

$$K_{OC} = \frac{K_{d,sec}}{f_{OC}} \tag{3-3}$$

Assuming no significant biodegradation, the removal efficiency via withdrawal of excess sludge η_{sorb} (%) depends on the sludge production SP ($kg_{TSS}\ L^{-1}$) as follows:

$$\eta_{sorp} = \frac{K_f C_w{}^{n-1} SP}{1 + K_f C_w{}^{n-1} SP} \cdot 100 \tag{3-4}$$

The mass balance, i.e. the recovery Rec. (%) of the spiked compound concentration, was determined according to:

$$Rec. = \frac{C_s + C_w}{C_{0,spiked}} * 100 \tag{3-5}$$

The soluble concentration C_w was inserted in eq. 3-5 after subtraction of the natural soluble background concentration $C_{w,background}$:

$$C_w = C_{w,spiked} - C_{w,background} \tag{3-6}$$

For calculation of the sorbed concentration C_s inserted in eq. 3-5, the natural sorbed background concentration $C_{s,background}$ as well as the soluble concentration in the sludge sample after centrifugation derived from the remaining water content f_{water} ($L\ g_{dw\ sludge}^{-1}$) were considered:

$$C_s = \left(C_{s,spiked} - C_{w,spiked} * f_{water}\right) - \left(C_{s,background} - C_{w,background} * f_{water}\right) \tag{3-7}$$

Throughout the study, the $logD_{OW}$ was used instead of the $logK_{OW}$ considering that some target compounds are charged at the ambient pH of 6.8: For acidic compounds the $logD_{OW}$ is defined as

$$\log D_{OW} = \log K_{OW} + \log \frac{1}{1 + 10^{pH - pKa}} \tag{3-8}$$

and for basic compounds as:

$$\log D_{OW} = \log K_{OW} + \log \frac{1}{1 + 10^{pKa - pH}} \tag{3-9}$$

However, except for the basic compounds imazalil, fenpropimorph and tridemorph (~ 50% positively charged) and the acidic compounds mecoprop, BZP-4 and PBSA (~ 100% negatively charged), the non-charged species dominate at the ambient pH of 6.8 for all target compounds and thus the $logD_{OW}$ equals the $logK_{OW}$.

3.3 Results and Discussion

3.3.1 Sorption kinetics

Sorption equilibrium and mass balance

Figure 3-1 shows exemplarily the sorption kinetics of the biocides irgarol, thiabendazole and 1,2-benzisothiazolin-3-one (BIT) to secondary sludge with and without adding NaN_3 by comparing the ratio [%] of the measured sorbed, soluble and total concentration to the spike concentration of 10 µg L^{-1}. The total analyte concentration is calculated as the sum of the soluble and sorbed concentrations determined according to eq. 3-6 and 3-7, respectively. The sorption kinetics of all other examined analytes are shown in the Supplementary Information (Figure SI 3-1).

At least in the NaN_3 amended samples, the sorbed concentration was constant after 1.5 h, indicating that sorption equilibrium was reached except for BIT and 2-n-octyl-4-isothaizolin-3-one (OIT) being subject of significant biodegradation. This is consistent with the results from other studies determining also a fast sorption kinetic for sludge with sorption equilibrium times of less than 2 h (Andersen et al., 2005; Ternes et al., 2004b).

For 18 of 27 analytes the mass balance in sludge amended samples as well as in filtered control samples without sludge was in an acceptable range of 100 ± 30% indicating a good analytical method performance as well as no significant losses by biotransformation and volatilization. A significantly lower mass balance after 24 h was observed for the partly (~ 50%) positively charged analytes tridemorph, fenpropimorph and imazalil as well as the strong sorbing analytes triclosan and triclocarban in the control samples and indicated sorption to the glass vessels. However, due to a higher sorption affinity to secondary sludge, the mass balances of these analytes were also within the acceptable range of 100 ± 30% in the sludge samples. A significant degradation of more than 30% in the sludge samples without NaN_3 within 24 h was observed for terbuthylazine, fenpropimorph and tridemorph, of more than 60% for chlorophene, benzophenone-3 (BZP-3) and benzophenone-4 (BZP-4) and of more than 90% for BIT, OIT, benzophenone-1 (BZP-1) and benzophenone-2 (BZP-2) (Table 3-2).

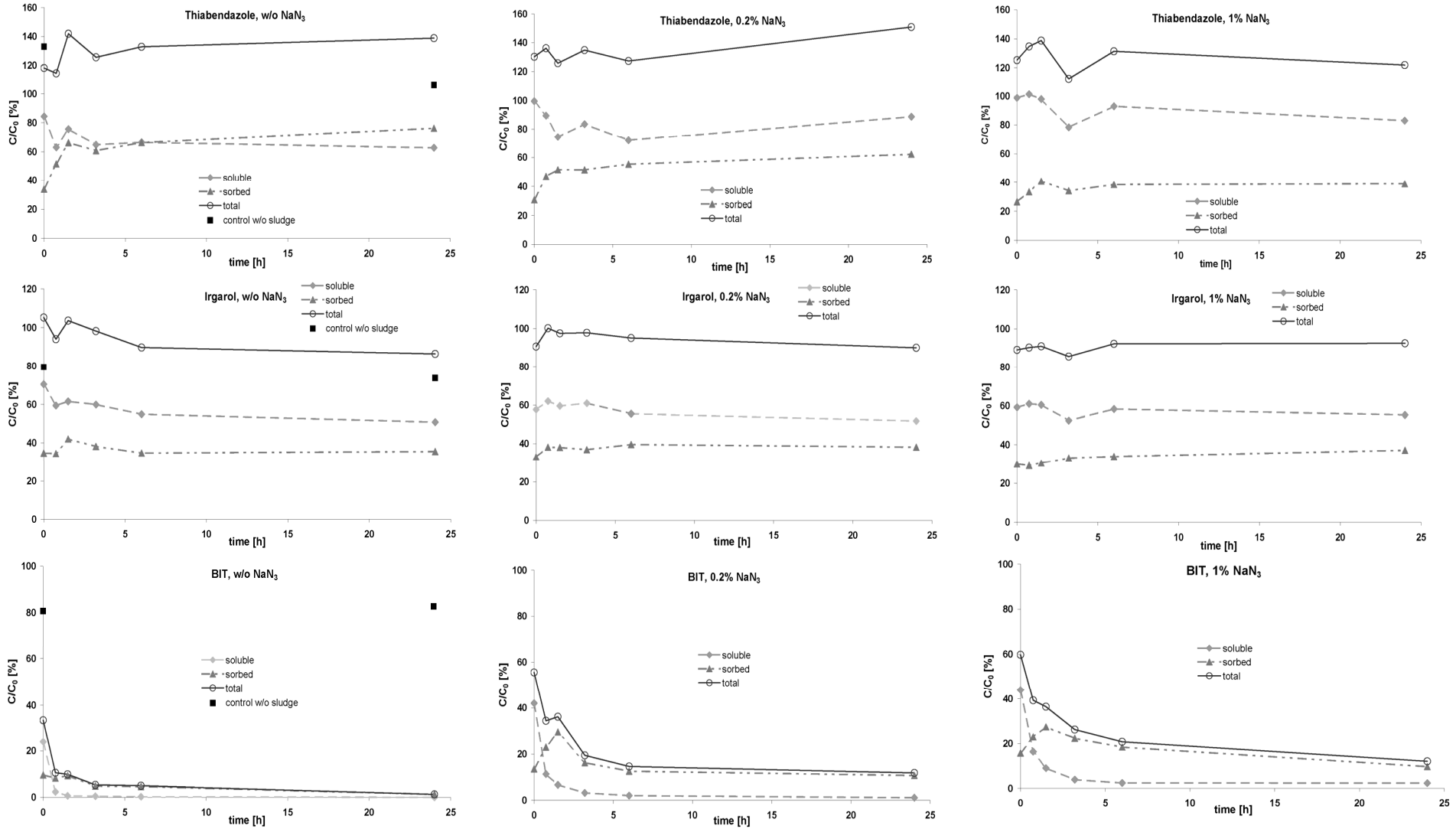


Figure 3-1. Ratio [%] of sorbed, soluble and the total concentration to the spike concentration of 10 µg L^{-1} over time for three selected biocides with and without addition of NaN_3 for microbial activity inhibition. The natural background concentrations as well as the water content of the sludge were assessed and considered for the calculation.

Except for terbuthylazine, dissipation was significantly reduced by addition of NaN_3 and can therefore mainly be attributed to biological transformation (Figure SI 3-1). The inhibition of the degradation of BIT and OIT was insufficient for reaching a sorption equilibrium even at a concentration of 1% NaN_3. An incomplete inhibition of the microbial activity by NaN_3 has also been observed by Ramil et al. (2010) who investigated the sorption of beta blockers in soils. However, the authors did not investigate whether the addition of NaN_3 also has an influence on the sludge-water distribution.

Influence of NaN_3 on the sludge-water distribution

For 18 out of 27 analytes, the addition of NaN_3 had a distinct influence on the sludge-water distribution (Table 3-2). While for the triazines such as irgarol (Figure 3-1), the ratio of sorbed and soluble concentration was comparable with different NaN_3 concentrations, the sorbed concentration was lower for most analytes including thiabendazole (Figure 3-1) and diuron (Figure SI 3-1). Similar results of the influence of NaN_3 on the sorption of diuron to soil were also reported by Gaillardon (1996). An inhibitory effect of NaN_3 on the sorption might be due to physico-chemical interactions, such as competition or change of the microbial surface caused by increased cell lysis. However, for analytes such as imazalil and triclosan the soluble concentration did not increase parallel to the decrease of the sorbed concentration, leading to a lower mass balance in the NaN_3 amended assays (Figure SI 3-1). Even though stable isotope-labeled surrogate standards have been used for analysis, this might be due the influence of NaN_3 on the extraction efficiency or matrix effects during ionization, which have shown to have a significant influence on the recoveries of the target analytes (Wick et al., 2010; chapter 2). Similarly, the severe overestimation of the soluble concentration of dimethomorph leading to a mass balance of up to 700% in the samples amended with 1% NaN_3, might be explained by matrix effects, i.e. strong ion enhancement in the ESI interface (Figure SI 3-1). For dimethomorph no stable isotope-labeled surrogate was available for analysis.

Further investigations are needed to fully understand the mode of action of NaN_3 on the sorption kinetics and chemical analysis, but the results confirmed that NaN_3 can have a negative impact on the reliability of sorption experiments. However, except for the fast degrading isothiazolones BIT and OIT, the distribution of the analytes between sludge and water phase, i.e. the $K_{d,sec}$ values, did not significantly change in the period from 1.5 to 6 h of incubation without using NaN_3 (Table 3-2). Thus, the dissipation by biological degradation was considerably slower than the sorption kinetics and reliable results of sludge-water distribution

were obtained within this time range. However, analyzing both the sorbed and soluble concentration was crucial to avoid an overestimation of the sludge-water distribution due to a faster dissipation of the soluble amount by biological degradation. If sorption equilibrium is not reached due to rapid biodegradation as observed for BIT and OIT, alternative methods for inactivation of sludge, such as autoclavation or freeze-drying, have to be applied to determine sludge-water distribution coefficients. However, for these rapidly biodegradable compounds the influence of sorption on their overall removal in WWTPs can be expected to be of minor importance.

Table 3-2. Results of the sorption equilibrium experiments for biocides and UV-filters. The recovery (Rec.) [%] of the spiked compound concentration after 1.5, 3.2 and 6 h of incubation without addition of NaN_3 is defined as the ratio of the measured total concentration, i.e. the sum of the sorbed and soluble concentration C_s and C_w, and the spiked concentration according to eq. 5. The average $K_{d,sec}$ [L $kg_{dw\ sludge}^{-1}$] was calculated from the sludge-water distribution coefficients determined after 1.5, 3.2 and 6 h of incubation. The range indicates the 95% confidence interval. w/o: without; ND: not determined.

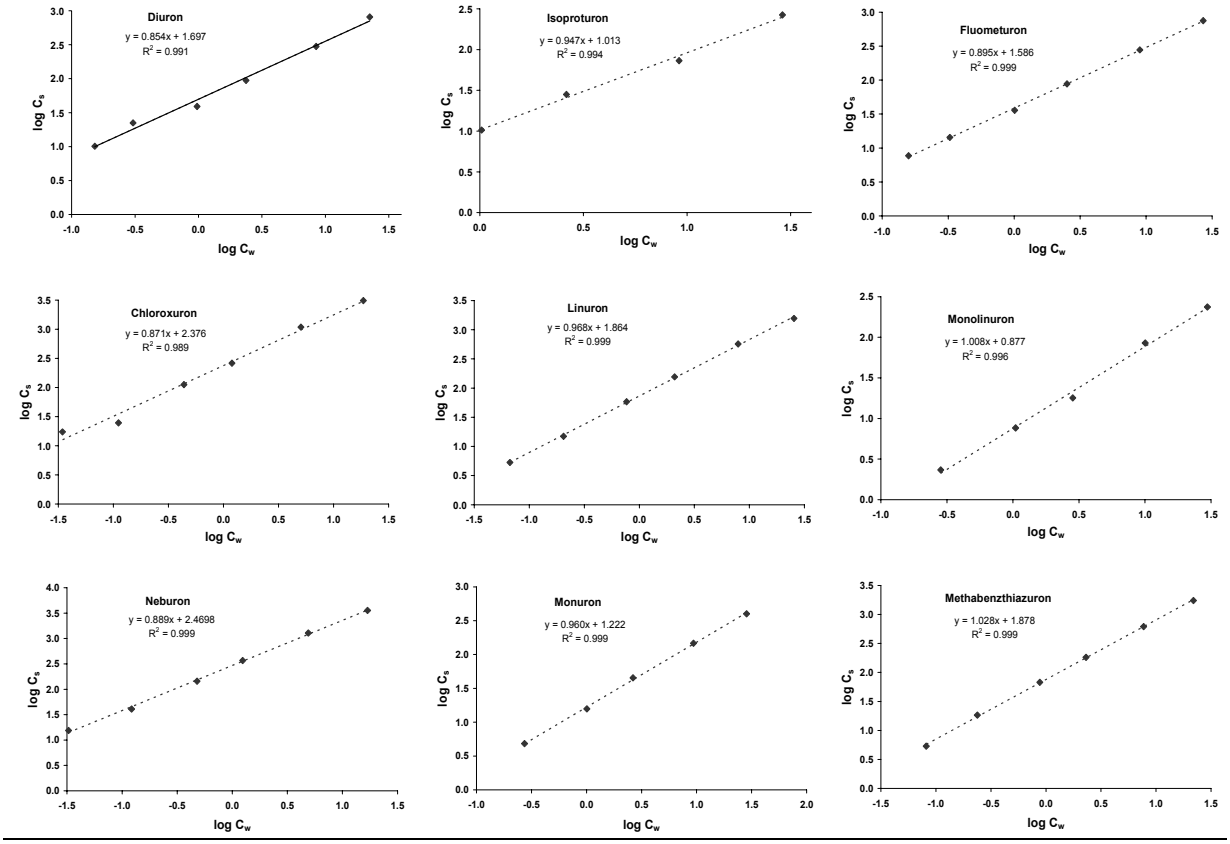
Analyte	1.5 h		3.2 h		6 h		average	Degree of dissipation w/o NaN_3 [a]	Influence of NaN_3 [b]
	Mass balance [%]	$K_{d,sec}$ [L kg_{TSS}^{-1}]	Mass balance [%]	$K_{d,sec}$ [L kg_{TSS}^{-1}]	Mass balance [%]	$K_{d,sec}$ [L kg_{TSS}^{-1}]	$K_{d,sec}$ [L kg_{TSS}^{-1}]		
Phenylurea herbicides									
Diuron	105	38	105	44	115	32	38 ± 7	o	lower C_s
Isoproturon	112	19	122	10	109	15	15 ± 5	o	lower C_s
Triazines									
Terbuthylazine	100	63	94	43	90	44	50 ± 13	+	no
Terbutryn	113	170	106	160	97	150	160 ± 10	o	no
Irgarol	104	150	98	140	90	140	140 ± 10	o	no
M1[c]	97	65	86	59	90	58	60 ± 4	o	no
Biocides									
Mecoprop	96	15	103	10	102	12	12 ± 3	o	lower C_s
Propiconazole	135	440	109	330	110	340	370 ± 70	o	lower C_s and Rec.
Tebuconazole	145	480	134	380	138	580	480 ± 110	o	lower C_s and Rec.
Imazalil	141	3000	128	2800	137	3200	3000 ± 200	o	lower C_s and Rec.
Carbendazim	136	25	117	16	129	11	20 ± 5	o	lower C_s
Thiabendazole	142	190	126	210	133	220	210 ± 20	o	lower C_s and higher C_w → Rec. const.
Dimethomorph	105	72	105	53	98	56	60 ± 12	o	higher C_w → Rec. >>100%
Fenpropimorph	136	1800	103	1400	91	1400	1500 ± 300	+	lower C_s and Rec.
Tridemorph	121	24000	83	14000	72	18000	19000 ± 6000	+	lower C_s and Rec.
BIT[d]	10	ND	5	ND	< 5	ND	ND	+++	ND
OIT[e]	< 5	ND	< 5	ND	< 5	ND	ND	+++	ND
DMST[f]	111	28	134	7	122	17	17 ± 12	o	no
DMSA[g]	119	22	106	14	116	17	18 ± 5	o	no
Triclosan	166	16000	119	16000	119	17000	16000 ± 1000	o	lower C_s and Rec.
Triclocarban	136	36300	126	39500	125	43500	40000 ± 4000	o	lower C_s and Rec.
Chlorophene	86	2700	61	1600	53	1700	2000 ± 700	++	lower C_s and Rec.
UV-filters									
BZP-1[h]	86	260	60	320	45	190	260 ± 70	+++	lower C_s and Rec.
BZP-2[h]	133	1100	85	1300	80	1400	1300 ± 200	+++	lower C_s and Rec.
BZP-3[h]	161	1500	137	1200	115	1200	1300 ± 200	++	lower C_s and Rec.
BZP-4[h]	95	12	83	6	83	7	8 ± 3	++	no
PBSA[i]	103	7	80	11	86	18	12 ± 6	o	variable C_w and Rec.

[a] o: <30%, >30%: + , >60%: ++ , >90%: +++
[b] Details about the influence of NaN_3 on the mass balance and sludge-water distribution are provided in Fig. 1 and Fig. A1 in the appendix.
[c] 2-Methylthio-4-tert-butylamino-6-amino-s-triazine, [d] 1,2-Benzisothiazolin-3-one, [e] 2-n-Octyl-4-isothaizolin-3-one,
[f] N,N-Dimethyl-N'-p-tolylsulfamide, [g] N,N-Dimethyl-N'-phenylsulfamide, [h] BZP: Benzophenone, [i] Phenylbenzimidazole sulfonic acid

3.3.2 Freundlich isotherms

Isotherm experiments were performed for all analytes for which the sorption kinetics were examined except for BIT and OIT, as for these two isothiazolinones sorption equilibria were not attained due to rapid biodegradation. Moreover, seven additional triazines and seven phenylurea herbicides were included in the isotherm studies. Based on the results from the sorption kinetics, sorption isotherm experiments were conducted without adding NaN_3 using an equilibrium time of 1.5 h. Freundlich isotherms were determined by linear regression of the logarithmized sorbed and soluble concentrations for all compounds for which the concentrations were above the limit of quantification for more than 4 spiking levels. This criteria was fulfilled for all analytes except BZP-4, mecoprop and DMSA (sorption too low) and tridemorph (sorption too high) and the Freundlich isotherms are shown in Figure 3-2.

Phenylurea herbicides



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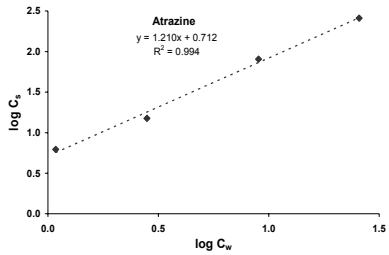
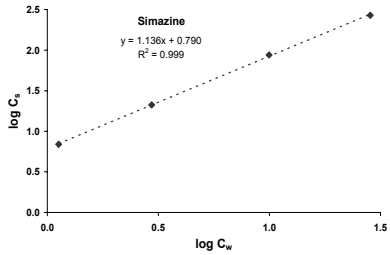
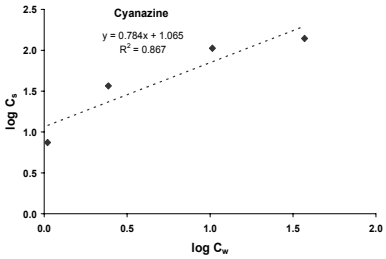
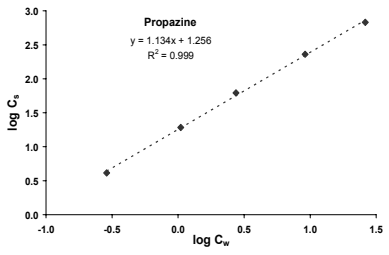
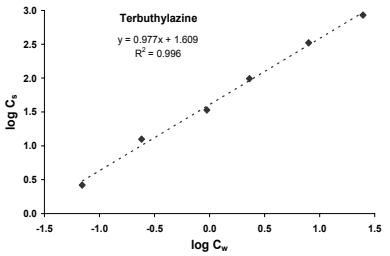
Triazines

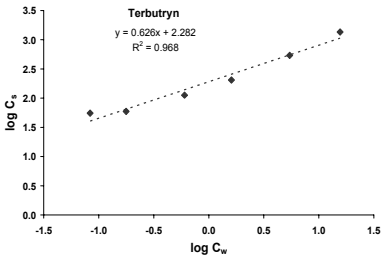


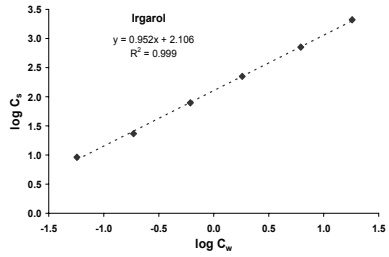


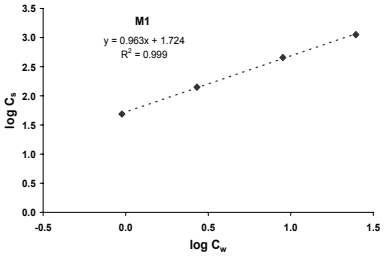


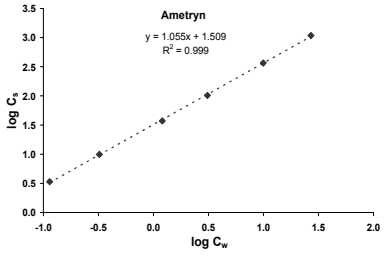


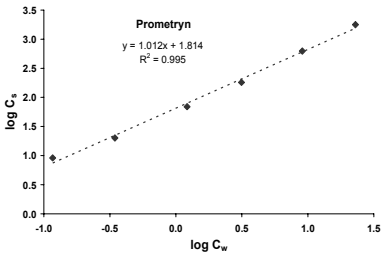


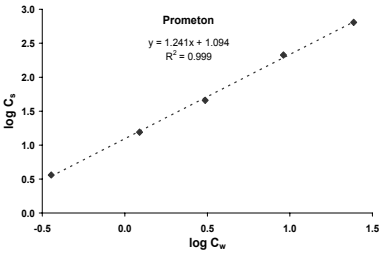




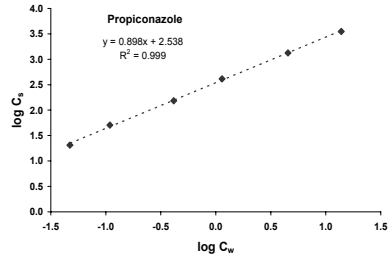


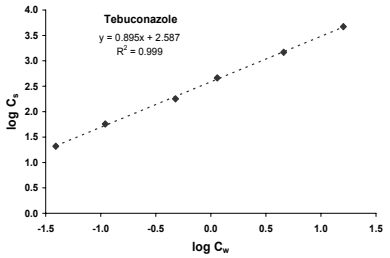


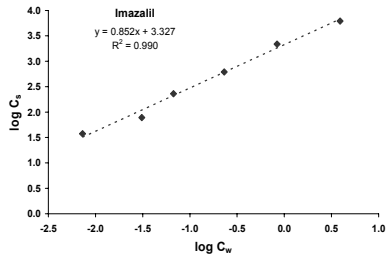


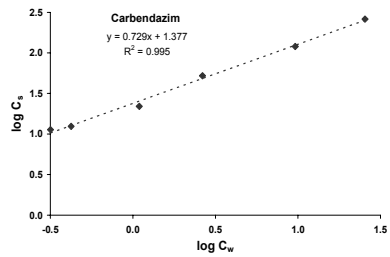


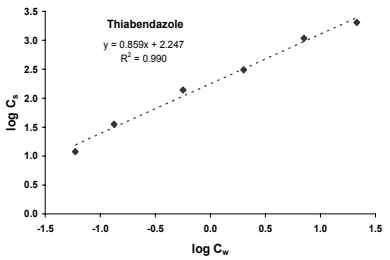
Biocides, UV-filters

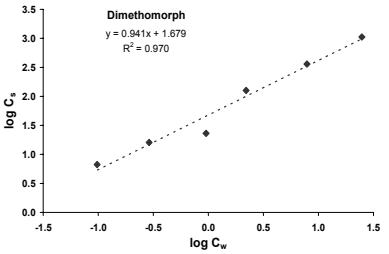












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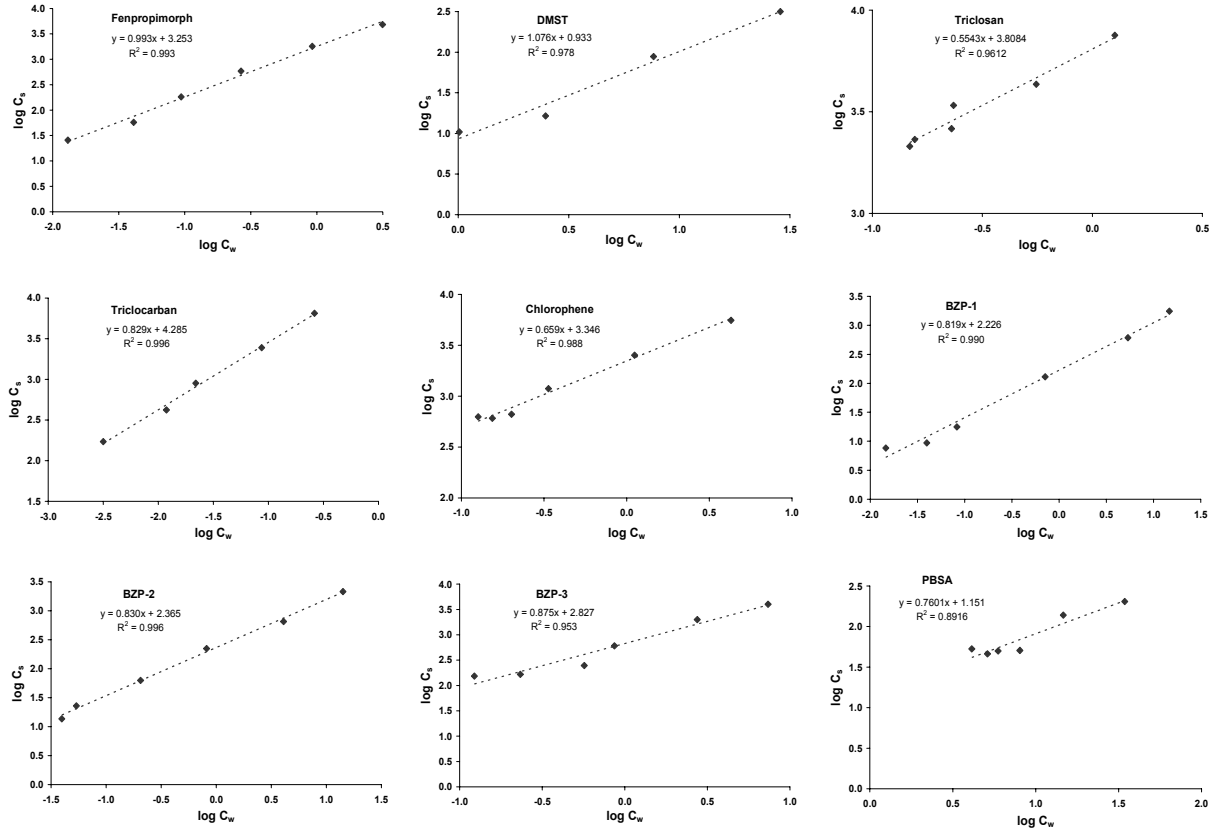

Figure 3-2. Sorption isotherms of triazines, phenylurea herbicides, biocides, and UV-filters on activated sludge. Lines represent Freundlich model fits to the sorption data (average of duplicate measurements). C_s: sorbed concentration (µg $kg_{dw\ sludge}^{-1}$), C_w: soluble concentration (µg L^{-1}).

For most compounds the spiked amount was recovered by more than 75% (Table 3-3), even though no NaN_3 was used for sludge deactivation. Lower recoveries were only determined for terbutryn (73 ± 8%), tridemorph (43 ± 21%), BZP-1 (40 ± 42%) and BZP-2 (55 ± 20%). However, the incomplete mass balance of these compounds is not supposed to significantly influence the sludge-water distribution, since analyte concentrations were determined both in the aqueous and solid phase. In addition, the results from the sorption kinetics showed no significantly influence of biological degradation on the sludge-water distribution coefficients of these compounds within 6 h of incubation (Table 3-2).

Table 3-3. Summary of the results of the Freundlich isotherm batch experiments conducted with secondary sludge from the nitrification zone of a biological treatment (TSS: 4 g_{TSS} L^{-1}, TOC: 29%, pH 6.8) using 6 different concentration levels (100 ng L^{-1} to 30 µg L^{-1}). The $K_{d,sec}$ values were calculated from the Freundlich parameters and the measured soluble concentration C_w for the 1 µg L^{-1} spiking level according to eq. 3-10 (actual total concentrations significantly higher than 1 µg L^{-1} due to the background concentrations are noted in parenthesis). The WWTP removal [%] by sorption was predicted for the same concentration level and a sludge production of 0.1 – 0.4 g_{TSS} L^{-1} according to eq. 3-4.

	log D_{OW}	pKa	Recovery [%]	R^2	n	K_f [$\mu g^{1-n} L^n kg^{-1}$]	$K_{d,sec}$ [$L\ kg_{dw\ sludge}^{-1}$] at 1 µg L^{-1}	log K_{OC}	log $K_{OC,LIT}$	Predicted WWTP removal [%]
Phenylurea herbicides										
Di uron	2.8[1]	-	91 ± 8	0.991	0.85 ± 0.11	48 ± 11	48	2.2	2.6[4]	<5
Isoproturon	2.5[1]	-	96 ± 9	0.994	0.95 ± 0.22	10 ± 5	9	1.5	2.1[5]	<5
Fluometuron	2.2[1]	-	90 ± 16	0.999	0.90 ± 0.03	39± 2	39	2.1	1.5-2.3, ∅2.7[6]	<5
Chloroxuron	3.4[2]	-	83 ± 19	0.989	0.87 ± 0.13	240 ± 70	270	3.0	3.2[4]	<5 – 10
Linuron	3.0[1]	-	95 ± 11	0.999	0.97 ± 0.03	73 ± 5	74	2.4	2.0-2.9, ∅2.7[6] 2.4[7]	<5
Monolinuron	2.2[1]	-	100 ± 7	0.996	1.01 ± 0.12	8 ± 2	8	1.4	1.4-3.3, ∅2.3[6]	<5
Neburon	3.8[2]	-	96 ± 10	0.999	0.89 ± 0.05	290 ± 30	320	3.0	3.3-3.6, ∅3.4[6]	<5 – 10
Monuron	1.8[2]	-	97 ± 7	0.999	0.96 ± 0.05	17 ± 1	17	1.8	1.4-3.3, ∅2.2[6], 1.5[7]	<5
Methabenz-thiazuron	2.6[2]	-	101 ± 8	0.999	1.03 ± 0.03	75 ± 5	76	2.4	2.7[2]	<5
Triazines										
Atrazine	2.5[1]	1.7[1]	95 ± 9	0.994	1.21 ± 0.28	5 ± 3	5	1.2	2.2[4], 1.7[7]	<5
Simazine	2.1[1]	1.6[1]	103 ± 7	0.999	1.14 ± 0.06	6 ± 1	6	1.3	2.1[4]	<5
Cyanazine	2.1[1]	12.9[1]	103 ± 20	0.867	≤1.7[d]	≤35[d]	ND	ND	1.6-2.4, ∅2.3[6]	<5
Propazine	2.9[3]	1.7[1]	101 ± 7	0.999	1.13 ± 0.07	18 ± 2	18	1.8	2.2[4]	<5
Terbuthylazine	3.0[1]	2.0[1]	95 ± 8	0.996	0.98 ± 0.09	42 ± 8	42	2.2	2.2-2.5[2]	< 5
Terbutryn	3.7[1]	4.3[1]	73 ± 8	0.968	0.63 ± 0.16	190 ± 60	230	2.9	2.9[4]	<5 – 10
Irgarol	3.7[3]	4.1[2]	88 ± 2	0.999	0.95 ± 0.04	130 ± 10	130	2.7	3.0[8]	<5 - 5
M1	-	-	106 ± 7	0.999	0.96 ± 0.08	53 ± 9	53	2.3	-	< 5
Ametryn	2.6[1]	10.1[1]	114 ± 13	0.999	1.06 ± 0.03	32 ± 2	33	2.1	2.6[4]	<5
Prometryn	3.1[1]	10.0[1]	117 ± 19	0.995	1.01 ± 0.10	65 ± 13	65 (1.5)	2.4	2.9[4]	<5
Prometon	2.9[2]	9.7[1]	107 ± 18	0.999	1.24 ± 0.08	12 ± 2	13	1.7	2.5[4]	<5
Biocides										
Mecoprop	-1.0[3]	3.1[1]	105 ± 10	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	1.1-1.4[2]	< 5
Propiconazole	3.7[1]	1.1[1]	92 ± 6	0.999	0.90 ± 0.04	350 ± 30	380	3.1	2.6-3.1, ∅2.8[6] log K_d = 2.9[9]	<5 - 15
Tebuconazole	3.7[1]	-	106 ± 10	0.999	0.89 ± 0.04	390 ± 30	420	3.2	2.8[5]	<5 - 15
Imazalil	3.7[1]	6.5[1]	89 ± 5	0.990	0.85 ± 0.12	2200 ± 600	3300	4.1	3.5-3.9, ∅3.6[6]	25 - 55
Carbendazim	1.5[1]	4.2[1]	89 ± 9	0.995	0.73 ± 0.07	24 ± 3	23	1.9	2.3-2.9, ∅2.5[6] log K_d < 2.7[10]	< 5
Thiabendazole	2.4[2]	4.7;12.0[1]	105 ± 10	0.990	0.86 ± 0.12	180 ± 50	190	2.8	3.0-3.5, ∅3.4[6]	< 5 - 10
Dimethomorph	2.7[1]	-	98 ± 10	0.970	0.94 ± 0.23	46 ± 22	44	2.2	2.5[2]	< 5
Fenpropimorph	4.1[1]	7.0[1]	78 ± 5	0.993	0.99 ± 0.12	1800 ± 500	1800	3.8	2.9-3.7[1]	15 - 40
Tridemorph	6.9[3]	6.5[1]	43 ± 21	ND[b]	ND[b]	ND[b]	ND[b]	ND[b]	3.4-4.0[1]	ND[d]
BIT	1.2[3]	-	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	-	ND[e]
OIT	2.5[1]	-	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	ND[c]	-	ND[e]
DMST	-	-	92 ± 13	0.978	1.08 ± 0.49	9 ± 8	10	1.5	-	< 5
DMSA	-	-	125 ± 18	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	-	< 5
Triclosan	4.8[3]	8.0[2]	92 ± 44	0.961	0.55 ± 0.16	6400 ± 1400	12000 (10.4)	4.6	4.3[11],4.7[12, 13]	55 - 85
Triclocarban	5.1[3]	-	95 ± 16	0.996	0.83 ± 0.10	19000 ± 7000	40000 (1.6)	5.1	3.8[11], 4.7[14]	80 - 95
Chlorophene	4.1[3]	9.6[2]	90 ± 28	0.988	0.66 ± 0.10	2200 ± 300	3800 (2.8)	4.1	-	30 - 60
UV-filters										
BZP-1	2.9[3]	8.0[2]	40 ± 42	0.990	0.82 ± 0.12	170 ± 50	260 (0.2)	3.0	-	<5 – 10
BZP-2	2.1[3]	8.0[2]	55 ± 20	0.996	0.83 ± 0.07	230 ± 40	300 (0.4)	3.0	-	<5 – 10
BZP-3	3.8[3]	8.0[2]	96 ± 19	0.953	0.88 ± 0.27	670 ± 260	720 (1.5)	3.4	-	5 - 20
BZP-4	-5.9[3]	0.7[2]	104 (n=2)	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]	-	< 5
PBSA	-1.1[3]	4.9; 0.7[2]	127 ± 39	0.892	0.76 ± 0.37	14 ± 12	9 (6.1)	1.5	-	< 5

[a] Sorption too low for determination of Freundlich isotherms. [b] Sorption too high for determination of Freundlich isotherms. [c] Biological transformation too fast for determination of Freundlich isotherms.[d] The sign "≤" indicates that the lower limit was below experimental resolution.

(1)Tomlin, 1994; (2) IUPAC, Pesticide Properties Database, 2010; (3) ALOGPS 2.1, Virtual Computational Chemistry Laboratory, 2005; (4) Liu and Qian, 1995 (soil); (5) Berenzen et al., 2005 (soil); (6) ARS Pesticide Properties Database, 2009 (soil); (7) Kördel et al., 1997 (sludge); (8) Tolosa et al., 1996 (sediment); (9) Kahle et al., 2008 (sludge); (10) Kupper et al., 2006 (sludge); (11) Agyin-Birikorang et al., 2010 (soil, biosolids); (12) Singer et al., 2002 (sludge); (13) Orvos et al. 2002 (sludge); (14) TCC Consortium, 2002 (sludge).

Concentration dependence

Except for the triazine cyanazine and the UV-filter PBSA, the correlation of determination (R^2) was > 0.95 and for most analytes even > 0.99, indicating a good fit of the Freundlich model. The Freundlich exponents n and the Freundlich affinity constants K_f together with the corresponding 95% confidence intervals are provided in Table 3-3. For 14 out of 34 analytes the Freundlich exponents n were significantly smaller than 1, indicating a non-linear sorption to secondary sludge. This implies a decreased sorption affinity to the sludge flocs with an increasing aqueous phase concentration of these compounds. Therefore, $K_{d,sec}$ values from the linear model determined at relatively high spiking concentrations may underestimate the sorption affinity at lower environmentally relevant concentrations. The effect of the concentration level on the $K_{d,sec}$ values of analytes with different sorption affinities is illustrated for triclosan, terbutryn and imazalil in Table 3-4.

Table 3-4. Influence of the compound concentration on the sludge-water distribution coefficients $K_{d,sec}$ [L $kg_{dw\ sludge}^{-1}$] and the predicted removal by activated sludge treatment for three compounds with Freundlich exponents < 1. The removal was predicted according to eq. 3-4 for a sludge production range of 0.1-0.4 g_{TSS} L^{-1} typical for municipal WWTPs.

	Triclosan (n = 0.55 ± 0.16)		**Terbutryn** (n = 0.63 ± 0.16)		**Imazalil** (n = 0.85 ± 0.12)	
Spike Conc.	$K_{d,sec}$ [L $kg_{dw\ sludge}^{-1}$]	Predicted removal range [%]	$K_{d,sec}$ [L $kg_{dw\ sludge}^{-1}$]	Predicted removal range [%]	$K_{d,sec}$ [L $kg_{dw\ sludge}^{-1}$]	Predicted removal range [%]
100	15000	60-86	480	5-16	5000	32-65
300	14700	60-86	370	4-13	3700	27-60
1000	12400	55-83	230	2-9	3300	25-57
3000	12300	55-83	160	2-6	2800	22-52
10000	8400	46-77	100	1-4	2300	19-48
30000	5800	37-70	70	1-3	1800	15-42

The $K_{d,sec}$ values shown in Table 3-4 were calculated from the measured soluble concentration C_w combining eq. 3-1 and 3-2 to:

$$K_{d,sec} = K_f * C_w^{n-1} \quad (3\text{-}10)$$

It follows from eq. 3-10 that the effect of a lower Freundlich exponent is more pronounced for analytes with a lower sorption affinity, i.e. a broader soluble concentration range. Consequently, the calculated $K_{d,sec}$ value of terbutryn is about seven times higher at a spiking level of 100 ng L^{-1} compared to 30000 ng L^{-1}, whereas it is less than three times higher for triclosan which has a similar Freundlich exponent. Therefore, possible concentration effects should be taken into account when comparing sludge-water distribution coefficients. However, considering that the WWTP influent concentrations are typically spanning less than two orders of

magnitude, the prediction of the removal efficiencies in WWTPs by sorption to excess sludge according to eq. 3-4 reveals that the bias in the predicted removal efficiency due to non-linear sorption effects is relatively low (Table 3-4).

Sludge-water distribution coefficients

For the comparison of the sludge-water distribution, the $K_{d,sec}$ values shown in Table 3-3 were calculated for the 1 µg L^{-1} spiking level based on eq. 3-10 to consider the non-linear sorption observed for some analytes. The calculated $K_{d,sec}$ values were in the same order of magnitude as those determined from the sorption kinetics, except for BZP-2 and BZP-3 (Table 3-2). In accordance with their high $logD_{OW}$ values of 5.1, 4.8 and 4.1, highest distribution coefficients of 40000, 12000 and 3800 L $kg_{dw\ sludge}^{-1}$ were calculated for the bactericides triclocarban, triclosan and chlorophene, respectively. In addition, values above 500 L $kg_{dw\ sludge}^{-1}$ were determined for the partly positively charged (~ 50%) biocides imazalil (3300 L $kg_{dw\ sludge}^{-1}$) and fenpropimorph (1800 L $kg_{dw\ sludge}^{-1}$) and the UV-filter BZP-3 (720 L $kg_{dw\ sludge}^{-1}$). According to eq. 3-4, sorption to sludge can be expected to significantly contribute to the removal of these analytes in WWTPs (>10%). Relatively high sorption removal efficiencies of 55-85% and 80-95% predicted for triclosan and triclocarban, respectively, are in accordance with their measured mass balance in full-scale plants (Heidler et al., 2006; Heidler and Halden, 2007). However, for most of the target analytes, the distribution coefficients were below 500 L $kg_{dw\ sludge}^{-1}$ and the removal by sorption to secondary sludge in WWTPs can be expected to be negligible (<10%).

The logarithmized organic carbon based distribution coefficients ($logK_{OC}$), which were determined within the current study for secondary sludge, were mainly within ± 0.5 log units of those available in literature for soil and sludge. Considering that systematic studies with different soils revealed already standard deviations of ± 0.3 log units (Schwarzenbach et al., 2003), the results are in good agreement with literature data. However, especially for analytes with a lower sorption affinity ($logD_{OW} < 3$), the $logK_{OC}$ values determined within the current study were generally in the lower range of those reported in literature for soils. In contrast, the $logK_{OC}$ values of the strongly sorbing compounds such as triclosan, triclocarban, imazalil and fenpropimorph were slightly higher.

3.3.3 Correlation of $logD_{OW}$ and $logK_{OC}$

Since the neutral species dominated under the conditions of the batch system, the hydrophobicity of the target analytes was expected to have the strongest influence on their sorption affinity to secondary sludge, except for the partly (~ 50%) positively charged compounds imazalil, fenpropimorph and tridemorph and the negatively charged acids mecoprop, PBSA and BZP-4. Therefore, the $logD_{OW}$ as a measure of the compound hydrophobicity was correlated with the logarithmized organic carbon based $K_{d,sec}$ ($logK_{OC}$) for all compounds for which $logK_{OC}$ values could be determined within the current study, except for the negatively charged UV-filter PBSA (Figure 3-3(a)).

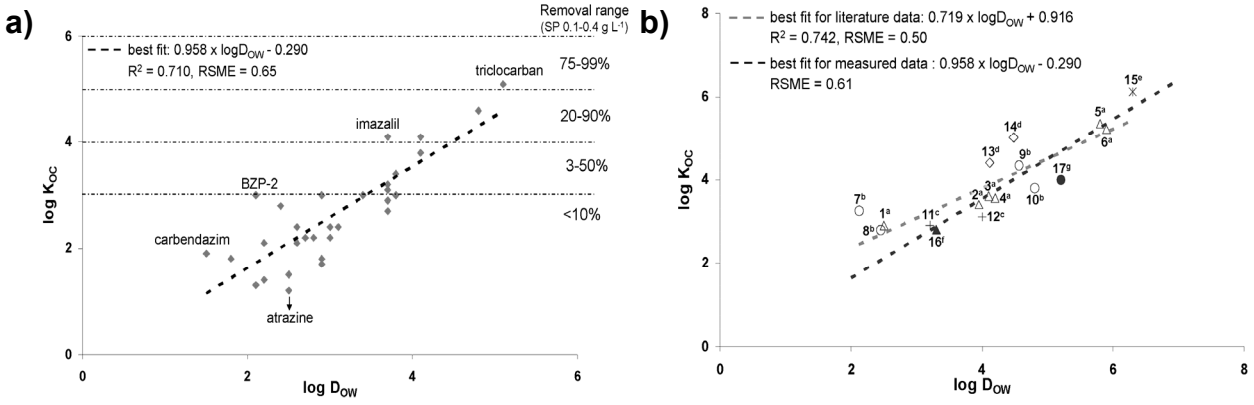

Figure 3-3. $LogK_{OC}$ values determined in secondary sludge versus $logD_{OW}$ values for a) 31 target compounds of the current study and b) 17 compounds different from the target compounds for which K_{OC} or K_d values for sludge were available from literature. The removal ranges were calculated according to eq. 3-4 assuming a sludge production (SP) in the range of 0.1-0.4 g_{TSS} L^{-1}.

(1) Carbamazepine, (2) Estradiol, (3) Estrone, (4) Ethinylestradiol, (5) Tonalide, (6) Galaxolide ([a]Carballa et al., 2008, digested sludge); (7) Methyl benzoate, (8) 2,5-Dichloroaniline, (9) Phenanthrene (10) Fenthion ([b]Kördel et al., 1997, freeze-dried sludge); (11) Clarithromycin, (12) Azithromycin ([c]Göbel et al., 2005, estimated from apparent K_d for full-scale samples); (13) Octylphenol, (14) Nonylphenol ([d]Isobe et al., 2001); (15) Clotrimazole ([e]Kahle et al., 2008, estimated from apparent K_d for full-scale samples); (16) Bisphenol A ([f]Zhao et al., 2008, autoclaved sludge); (17) Loratidine ([g]Radjenović et al., 2009b, estimated from apparent K_d for full-scale samples).

This single parameter linear free-energy relationship (sp-LFER) approach has been widely used to estimate the sorption affinity of certain groups of non-charged pollutants toward soils (Schwarzenbach et al., 2003). However, studies using this approach for sludge are rare (Carballa et al., 2008; Heidler and Halden, 2008; Kim et al., 2009; Urase and Kikuta, 2005). The linear regression in Figure 3-3(a) revealed a best fit linear model for the target analytes of $logK_{OC} = 0.958 \times logD_{OW} - 0.290$ featuring a R^2 of 0.71 and a root mean square error (RMSE) of 0.51. This confirms a general linear dependency of $logD_{OW}$ and $logK_{OC}$. However, the variability of the $logK_{OC}$ values of up to one log unit indicates the influence of further specific sorption mechanisms for certain analytes. For instance, the $logK_{OC}$ of imazalil was significantly higher than of other analytes with comparable $logD_{OW}$ values. This might be attributed to the fact that approximately 50% of the imazalil is positively charged at pH 7 and

thus electrostatic interaction with the mainly negatively charged sludge flocs significantly contributed to the overall sorption affinity. In contrast, even though fenpropimorph has a similar pKa as imazalil but a significantly higher $logD_{OW}$, its $logK_{OC}$ was in the same range as determined for imazalil. Thus, the different sorption behavior of these two analytes cannot be sufficiently described by the pKa and the $logD_{OW}$.

Further compound specific sorption mechanisms can also be assumed to determine the sorption behavior of atrazine and the UV-filters BZP-1 and BZP-2. While for atrazine with a $logD_{OW}$ of 2.5, the $logK_{OC}$ of 1.2 was unexpectedly low, the $logK_{OC}$ value of 3.0 measured for BZP-1 and BZP-2 was higher than estimated from their $logD_{OW}$ values of 2.9 and 2.1, respectively. In general, a higher variability of the $logK_{OC}$ was observed for compounds with lower $logD_{OW}$ values (< 3). This is in accordance with the theory that for polar compounds, capable of H-bonding, various types of interactions can occur which cannot be sufficiently described by only one parameter, i.e. the $logD_{OW}$ (Faria and Young, 2010). The application of multiparameter models with descriptors for both sludge and micropollutant characteristics might be a valuable approach for a more accurate prediction of sludge-water distribution of nonionic compounds. The use of such a multiparameter model to predict the sorption of hydrophobic micropollutants (PAHs and PCBs) to sludge particles by Barret et al. (2010) revealed the importance of several sludge predictors such as the protein content and the mineral density and micropollutant predictors such as the molar mass, the number of chlorines and the $logD_{OW}$. However, these approaches require an extensive sludge and molecule characterization which was not within the scope of the current study and will need further attention regarding the suitability of these approaches for emerging micropollutants of higher polarity.

To check whether the results for the dataset of the current study can be transferred to other compounds being non-charged at pH 7, a correlation of the $logD_{OW,}$ i.e. the $logK_{OW}$, with the $logK_{OC}$ was also performed with a dataset of 17 structurally diverse compounds with a $logD_{OW}$ between 2 and 6 for which K_d or K_{OC} data were available from literature (Figure 3-3(b)). The comparison of the best fit linear model for this dataset from literature (RSME = 0.50) with that from our dataset (RSME = 0.61) revealed that the latter also performed reasonably well for other compounds and sludge types which were not examined within this study. The results demonstrate that single parameter LFER approaches based on the $logD_{OW}$ can be applied for an estimation of the sorption affinity of non-charged micropollutants to sludge.

The D_{OW} prediction approach allows for a classification of non-charged compounds regarding their full-scale removal via withdrawal of excess sludge when other removal processes such as volatilization and biotransformation can be neglected (Figure 3-4). Removal is expected to be negligible for nonionic compounds with $logD_{OW} < 3.5$, whereas, depending on the sludge production, removal by <10 to 80%, 50 to 95% and >90% can be expected for $logD_{OW}$ values in the range of 3.5 to 5, 5 to 6 and >6, respectively.

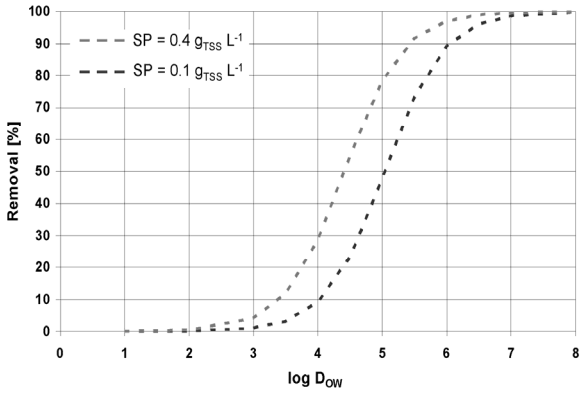

Figure 3-4. Predicted removal of nonionic compounds via withdrawal of excess sludge versus $logD_{OW}$ for a sludge production of 0.1 and 0.4 g_{TSS} L^{-1}. The removal was predicted using eq. 3-4 assuming a correlation of $logD_{OW}$ and $logK_{OC}$ according to the best fit linear model determined for the target analytes of the current study ($logK_{OC}$ = 0.958 x $logD_{OW}$ – 0.290; f_{OC} = 0.29 g_{OC} $g_{dw\ sludge}^{-1}$).

For compounds being charged at the ambient pH of the activated sludge treatment (pH 6-8), the sorption affinity and thus the removal by sorption in WWTPs strongly depends on their specific charge and is currently not reasonably predictable (Ternes et al, 2004; Urase and Kikuta, 2005). As observed within this study for imazalil, positively charged compounds tend to have stronger sorption affinities than expected based on their $logD_{OW}$ values due to electrostatic interactions with the mainly negatively charged surfaces of the sludge flocs. Consequently, negatively charged compounds are known to partition predominantly in the aqueous phase due to electrostatic repulsion (Ternes and Joss, 2007). For instance, it has to be considered that the WWTP removal by sorption might be overestimated for triclosan and the UV-filters BZP-1, BZP-2 and BZP-3 using distribution coefficients determined at neutral pH, since their pKa value of 8 is within the range typical for activated sludge (pH 6-8). At pH 7 the non-charged species dominate (90%), whereas the negatively charged species increase to 50% at a sludge pH of 8 and thus most likely reduce their sorption affinity.

3.4 Conclusions

Within the present study the sorption of 41 micropollutants to activated sludge was investigated. For 27 contaminants, sludge-water distribution coefficients and their concentration dependency was determined for the first time. Both are major prerequisites for assessing their fate in WWTPs and thus their potential release into the environment via treated wastewater or via sludge amendment to agricultural land.

Furthermore, the results of the present study imply important aspects for performing sorption experiments with fresh activated sludge. NaN_3 has been shown to be inappropriate for the deactivation of fresh sludge due to i) insufficient inhibition of microbial degradation and ii) its influence on the sludge-water distribution. To the best of our knowledge, this is the first study confirming that increasing NaN_3 concentrations can lead to decreasing compound amounts sorbed to activated sludge. In addition, the results show that the deactivation of sludge is not a prerequisite if biodegradation does not hinder the attainment of sorption equilibria. However, quantification of the analytes in both sludge and aqueous phase is crucial if biodegradation cannot be excluded, since analyzing only the dissolved concentration would lead to increased distribution coefficients.

The Freundlich model was shown to be appropriate to describe the sorption behavior of various micropollutants in contact to secondary sludge. Since for many compounds the Freundlich exponents were close to 1 and thus $K_f \approx K_d$, the linear model can be used in most cases for a rapid determination of sludge-water distribution coefficients. For the linear approach concentrations should be as close as possible to real concentrations in full-scale WWTPs.

For compounds being non-charged in the pH range typical for activated sludge treatment (pH 6-8), sludge-water distribution can be reasonable predicted using a simple LFER approach based on the $\log K_{OW}$ as a single descriptor. Even though this approach does not consider specific molecular interactions, which are especially important for polar compounds, it is a simple practical tool for a first assessment of the removal of nonionic compounds via withdrawal of excess sludge in WWTPs. Accordingly, nonionic compounds can be classified as not significantly removable (<10%) by sorption for $\log K_{OW}$ values <3.5, partly removable (10-95%) for $\log K_{OW}$ values of 3.5-6 and almost completely removable (>90%) for $\log K_{OW}$ values > 6.

3.5 Acknowledgements

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4. Fate of beta blockers and psycho-active drugs in conventional wastewater treatment

Arne Wick, Guido Fink, Adriano Joss, Hansruedi Siegrist, Thomas A. Ternes

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Abstract

The removal of beta blockers and psycho-active drugs was investigated in a representative conventional German WWTP by long-term measurement campaigns along different biological treatment processes. The activated sludge treatment with an elevated SRT of 18 d was the only process which led to a significant removal of certain beta blockers and psycho-active drugs. The removal efficiency was below 60% for all compounds except for the natural opium alkaloids codeine and morphine being removed by more than 80%. Primary biological transformation and sorption onto sludge as the main removal mechanisms were examined in lab-scale batch experiments. Sorption onto activated sludge was found to be negligible (< 3%). The biological transformation could be described by pseudo-first order kinetics and the transformation constants k_{biol} were used to predict the removal of beta blockers and psycho-active drugs in an activated sludge unit with a model. For most compounds the removal efficiencies measured on the full-scale WWTP were within the 95% confidence intervals predicted by the model. The results from full-scale measurements and modeling indicate that biological transformation in the nitrification tank together with parameters such as the sludge retention time and the temperature is crucial regarding the biological transformation of beta blockers and psycho-active drugs in conventional WWTPs.

4.1 Introduction

Beta blockers and psycho-active drugs such as opium alkaloids, tranquilizers and anti-depressants are highly consumed, either via prescription or via ingredients of illicit drugs. Several of these pharmaceuticals are metabolized in the body and hence, a mixture of the unaltered drug and its metabolites are excreted by urine and feces. Private households are known to be the main sources for pharmaceuticals in wastewater, but also hospitals, retirement homes and radiological practices contribute to a significant extent. Due to an incomplete removal in wastewater treatment plants (WWTP), pharmaceuticals are finally emitted into surface waters (Daughton and Ternes, 1999; Hummel et al., 2006; Ternes, 1998).

Beta blockers were reported to be found ubiquitously in influents and effluents of WWTPs in the ng L^{-1} to µg L^{-1} range and to be only partially eliminated by conventional biological treatment (Huggett et al., 2003; Lee et al., 2007; Ternes, 1998; Vieno et al., 2006). Examining 14 WWTPs in Canada, Lee et al. (2007) determined removal efficiencies of 40, 15, 9, 12 and 37% for atenolol, sotalol, metoprolol, propranolol and bisoprolol, respectively. Vieno et al. (2006) quantified the removal of beta blockers in three WWTPs in Finland and found removal efficiencies ranging from 45 to 92% for atenolol, 59 to 75% for sotalol and 0 to 26% for metoprolol. As a result of the incomplete removal during conventional wastewater treatment, beta blockers were also found in surface waters in the ng L^{-1} to low µg L^{-1} range (Bendz et al., 2005; Gros et al., 2006; Ternes, 1998; Vieno et al., 2006).

Except for the antiepileptic carbamazepine, information on the occurrence and fate of psycho-active drugs in WWTPs is very scarce. Carbamazepine is ubiquitously present in influents and effluents of WWTPs in the high ng L^{-1} to low µg L^{-1} range and is not significantly reduced during conventional biological treatment (Castiglioni et al., 2006; Joss et al., 2005; Miao and Metcalfe, 2003; Ternes, 1998; Ternes et al., 2007; Vieno et al., 2006). Hummel et al. (2006) found several psycho-active drugs being present in influents and effluents in up to 14 WWTPs. This study indicates that the concentrations of the natural opium alkaloids morphine and codeine are significantly reduced in WWTPs, while most other psycho-active drugs like the opioid methadone and the tranquilizer oxazepam are found in similar concentrations in influents and effluents. Psycho-active drugs could be detected in surface waters indicating also an incomplete removal in WWTPs. Among several psycho-active drugs found in rivers and streams in Southwest Germany, oxazepam, primidone and doxepin were the predominant compounds with maximum concentrations of 398, 594 and 219 ng L^{-1}, respectively

(Hummel et al., 2006). Kasprzyk-Hordern et al. (2007) detected codeine and tramadol in rivers in Wales and Poland with maximum concentrations of 34 and 2100 ng L^{-1}, respectively.

So far, studies regarding the ecotoxicity of beta blockers and psycho-active drugs indicate a potential hazard to aquatic ecosystems. Studies with fresh water invertebrates exposed to sediments spiked with the antiepileptic carbamazepine showed a significant and concentration-dependent decrease of abundance of the non-biting midge *Chironomus riparius.* The lowest observed effect concentration (LOEC) was as low as 140 µg kg^{-1} (Oetken et al., 2005). Adverse effects of morphine and carbamazepine on the immune system of the fresh water mussel *Elliptio complanata* were determined by Gagné et al. (2006). Huggett et al. (2002) observed a decrease in the number of eggs produced by the Japanese medaka *Oryzias latipes* after a 4-week exposure to 0.5 µg L^{-1} of the beta blocker propranolol.

To prevent the emission of these pharmaceuticals into the aquatic environment, which might have adverse effects on aquatic organisms, the focus of modern WWTPs has to be extended to their removal. But regarding the removal of beta blockers and psycho-active drugs in conventional wastewater treatment information about the main removal processes and its influencing parameters are still missing. The objective of this study was to obtain more comprehensive knowledge about the fate (sorption, biotransformation) of several beta blockers (Table 4-1) and psycho-active drugs (Table 4-2) in a conventional activated sludge treatment by comparing a modeling approach with long-term full-scale measurement campaigns.

Table 4-1. Beta blockers: chemical structures and CAS-No.

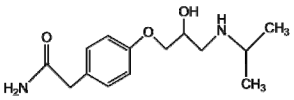
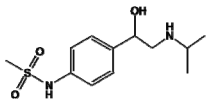
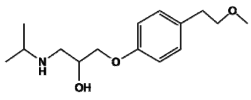
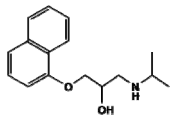
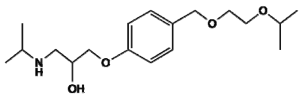
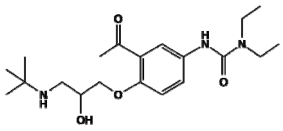
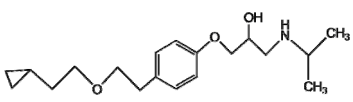
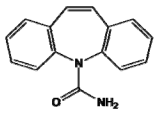
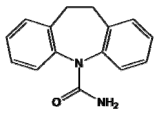
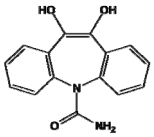
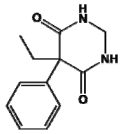
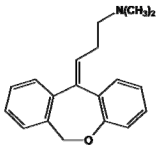
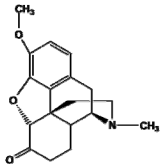
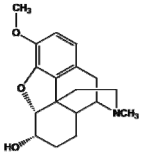
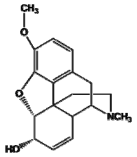
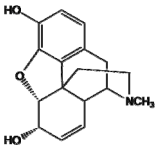
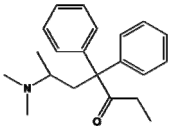
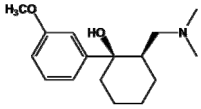
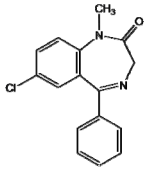
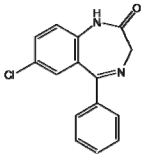
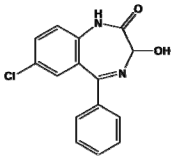
Compound CAS number	Chemical Structure	Compound CAS number	Chemical Structure
Atenolol (ATL) CAS: 29122-68-7		Sotalol (STL) CAS: 3930-20-9	
Metoprolol (MPL) CAS: 37350-58-6		Propranolol (PPL) CAS: 525-66-6	
Bisoprolol (BPL) CAS: 66722-44-9		Celiprolol (CPL) CAS: 56980-93-9	
Betaxolol (BXL) CAS: 63659-18-7			

Table 4-2. Psycho-active drugs: chemical structures and CAS-No.

Compound Application CAS number	Chemical Structure	Compound Application CAS number	Chemical Structure
Carbamazepine (CBZ) Antiepileptic CAS: 298-46-4		10,11-Dihydro-carbamazepine (DH-CBZ) Metabolite of Carbamazepine CAS: 3564-73-6	
10,11-Dihydroxy-10,11-dihydro-carbamazepine (DHH) Metabolite of Carbamazepine CAS: 125-28-0		Primidone (PMD) Antiepileptic CAS: 125-33-7	
Doxepin (DXP) Antidepressant CAS: 1668-19-5		Oxycodon (OCN) Opioid CAS: 76-42-6	
Dihydrocodeine (DHC) Opioid CAS: 125-28-0		Codeine (CDN) Opium alkaloid CAS: 79-57-3	
Morphine (MPN) Opium alkaloid CAS: 57-27-2		Methadone (MTD) Opioid CAS: 76-99-33	
Tramadol (TMD) Opioid CAS: 36282-47-0		Diazepam (DZP) Tranquilizer CAS: 439-14-5	
Nordiazepam (NZP) Tranquilizer CAS: 1088-11-5		Oxazepam (OZP) Tranquilizer CAS: 604-75-1	

4.2 Experimental Methods

4.2.1 Sampling campaigns

The monitoring was performed in a conventional German WWTP serving 1,350,000 population equivalents (PE) (Figure 4-1). On dry weather conditions this plant has a daily wastewater flow-through of approx. 200,000 m^3. Primary treatment consists of a screen, an aerated grit-removal tank and a primary clarifier. The primary effluent is directed to the activated sludge system consisting of two treatment units in series. The first treatment unit is completely aerated and operates with a hydraulic retention time (HRT) and solid retention time (SRT) of approx. 1 h and 0.5 d, respectively. After passing a secondary clarifier the wastewater is directed to a second activated sludge unit. Approx. one fourth of this unit consists of a stirred anaerobic compartment for denitrification, followed by an aerated compartment for nitrification. The second activated sludge unit is operated with a HRT and SRT of approx. 5 h and 18 d, respectively. After passing the tertiary clarifier about 70% of the tertiary effluent goes through a post-denitrification unit consisting of a fix-bed reactor before being discharged into the receiving river.

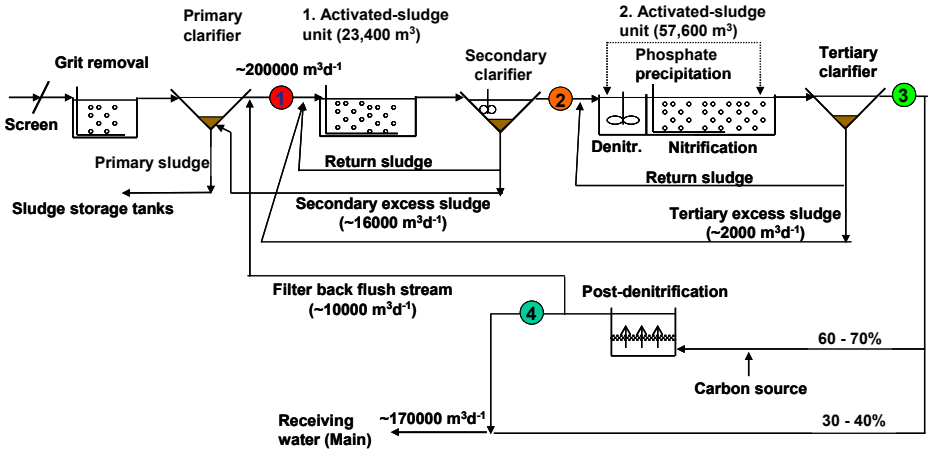


Figure 4-1. Flow scheme of the WWTP Frankfurt-Niederrad consisting of a mechanical treatment, two activated sludge units in series and a post-denitrification unit. The numbers indicate the sampling spots along the treatment lane. The flow rates represent typical values at dry weather conditions.

Weekly sampling campaigns were performed on the following dates: 19th – 25th March 2007, 7th – 13th May 2007 and 3rd – 9th July 2007. Samples were taken after primary clarification (spot 1), secondary clarification (spot 2), tertiary clarification (spot 3) and post-denitrification (spot 4). Each sampling campaign was conducted over an entire week taking two 48 h and one 72 h composite samples. Taking into account the total HRT of the WWTP

of approx 24 h, the primary effluent was sampled 24 h prior to the other locations. For the calculation of compound loads, the wastewater and sludge flows were determined throughout the sampling campaigns for the influent of the first activated sludge unit, the influent of the post-denitrification unit, the final effluent, the filter back flush stream and the secondary and tertiary excess sludge. Conventional WWTP parameters (pH, TOC, COD, BOD, NH_4-N, NO_3-N, PO_4-P) characterizing the wastewater along the different treatment processes are shown in Table SI 4-1. During an additional sampling campaign on 1st October 2007, 24 h composite samples were taken after secondary clarification (spot 2) and tertiary clarification (spot 3) without any time delay to assess the removal efficiency of the second activated sludge unit. All composite samples were stored at 4°C. The samples were immediately filtered (glass-fiber filter Whatman, GF6) and acidified to pH 3 with 3.5 M sulfuric acid to inhibit the microbial activity.

Secondary sludge for sorption and transformation batch experiments was taken as grab samples from the nitrification zone of the second activated sludge unit. The sludge samples were taken once for a sorption batch experiment on 10th September 2007 and twice for transformation batch experiments on 21st March 2007 and 1st October 2007. The sludge grab samples were directly brought into the lab and immediately used in the batch experiments.

4.2.2 Batch experiments

Sorption

A homogenous suspension (200 mL) of secondary sludge (9.01 g_{TSS} L^{-1}, pH 6.8, 0.25 g_{OC} g_{TSS}^{-1}) was filled into 250 mL amber glass bottles. In order to inhibit the microbial activity, 2 mL of an aqueous solution of sodium azide was added to reach a final concentration of 0.2% v/v. Psycho-active drugs were spiked to a concentration of 15 µg L^{-1} and the samples were slowly stirred for 2.5, 5 and 14 h. Three replicates were run in parallel. In each time series, two non-spiked samples were integrated as blank samples. After the defined exposure time, the slurries were centrifuged (15 min, 4000 rpm). While the supernatant was stored at 4°C for solid-phase extraction (SPE), the solid particles were freeze-dried for micropollutant analysis and the water content was determined.

Biological transformation

Two batch experiments examining the biological transformation kinetics were conducted with sludge from different seasons. The first experiment was started on March 21st 2007 and the second one on October 1st 2007. A homogenous suspension (40 mL) of activated

sludge (first experiment: 8.43 g_{TSS} L^{-1}, second experiment: 7.82 g_{TSS} L^{-1}) was filled into 500 mL amber gas wash bottles with a screw connector system and a top with a filter disc for purging exhaust gas. In order to minimize the possible impact of sorption, the sludge was diluted to a final volume of 400 mL with the final effluent. The bottles were continuously flushed with air to establish aerobic conditions (~ 8 mg O_2 L^{-1}). Two different methods were used to prevent a rapid increase of the pH due to the removal of CO_2: a) in the first batch experiment in March, the final effluent was buffered with 20 mM phosphate buffer, while b) in the second experiment in October, CO_2 was constantly mixed to the air flow using a rotameter (Figure 4-2). The latter method was established to ensure that the incubation system was not influenced by an additional nutrient source such as phosphate. The pH was controlled throughout the whole exposure time in a separate sample and rightly before sampling in the spiked samples used for chemical analysis. During both experiments pH was controlled at 7.2.

The sludge was equilibrated for 2 h before samples were taken for the determination of compound blank values. Beta blockers and psycho-active drugs were spiked to an initial concentration of 3 µg L^{-1}. After defined exposure times of 0, (10), 20 min, (30), 40 min, 1.5 h, 3 h, 6 h, 15 h, 24 h and 48 h (times in parenthesis: not sampled in the second experiment) the bottles were disconnected and the samples were acidified to pH 3 with 3.5 M sulfuric acid. For both batch experiments three replicates were run in parallel for each sampling time. Spiked duplicate samples without sludge served as negative controls. These samples were treated the same way as the spiked ones but with exposure times of 0, 24 and 48 h. Immediately after sampling, all samples were stored at 4°C in the dark and filtered (glass-fiber filter Whatman, GF6) within maximally 12 h after sampling.

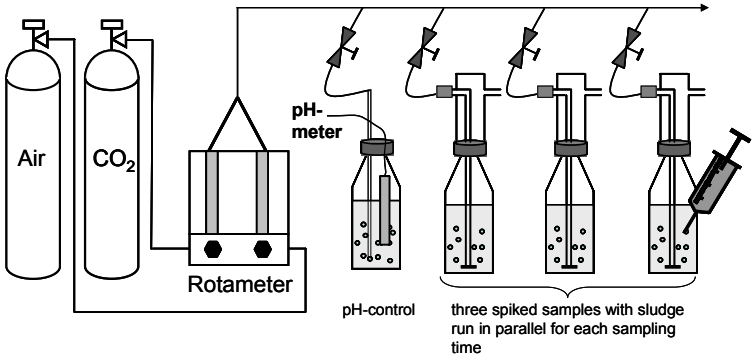


Figure 4-2. Schematic set-up of the batch reactors as used in the second batch experiment to determine the biological transformation kinetics. Exemplarily, triplicate samples for one sampling time and a separate sample used throughout the whole exposure time as pH-control are shown. An incubation volume of 400 mL was continuously purged with a mixture of compressed air and CO_2 to establish oxic conditions and a stable pH (7.2).

4.2.3 Chemical analysis

Psychoactive drugs

The analytical method used was already described in detail in Hummel et al. (2006). A brief description is listed below: The filtered aqueous samples were neutralized with 13.3 M ammonium hydroxide and 200 ng of an internal standard mix (1 µg mL^{-1} stock solution) was added prior to an enrichment by SPE with polymeric material (Oasis HLB, Waters). The detection was performed using reversed-phase liquid chromatography (Synergi Polar-RP, 150 x 3 mm, 4 µm, Phenomenex) coupled to electrospray mass spectrometry in the positive ionization mode (API 4000 tandem MS, Applied Biosystems).

Extraction of the freeze-dried sludge samples was conducted by pressurized liquid extraction (PLE) (ASE 200 Accelerated Solvent Extractor, 22 ml cells, cellulose filters (Dionex, Idstein, Germany) according to a method described in Stein et al. (2008). Briefly, 0.5 g of the freeze-dried sludge was filled into an extraction cell and 200 ng of the internal standard mix was added. PLE was accomplished with a mixture of water and methanol (50/50, v/v) at 100°C and 100 bar. The extract was diluted with groundwater to a final volume of 500 mL and further preparation was performed as described above for the aqueous samples.

Beta blockers

The analytical method was already described in detail in Ternes (2001) and was only slightly modified using another column for reversed-phase liquid chromatography according to Scheurer et al. (2010). The filtered aqueous samples were neutralized with 13.3 M ammonium hydroxide and 200 ng of an internal standard mix (1 µg mL^{-1} stock solution) was added prior to an enrichment by SPE on Bakerbond RP-C18 cartridges. The detection was performed using reversed-phase liquid chromatography (Synergi Polar-RP, 150 x 3 mm, 4 µm, Phenomenex) coupled to electrospray mass spectrometry in the positive ionization mode (API 4000 tandem MS, Applied Biosystems).

All psycho-active drugs and beta blockers were quantified using an internal calibration and the limit of quantification (LOQ) was defined as the second lowest calibration point in the regression as long as the calculated signal to noise ratio of the compounds in the native sample extracts was > 10.

4.2.4 Calculation of removal efficiencies and accuracy prediction

Due to the different HRTs of the different treatment processes and variations of the wastewater flow, the removal efficiencies of the treatment units were calculated using the

weekly compound loads as the sum of the 48 h and 72 h loads. Since loads withdrawn from the wastewater by the liquid fraction of the excess sludge and by the flush stream of the post-denitrification unit are not removed from the system by biological transformation or sorption (i.e. recycled to the influent), they were subtracted from influent loads of the different treatment units (Figure 4-1). The removal efficiencies were calculated normalized to the influent load without internal recycles. Since the flush stream of the post-denitrification unit and the secondary excess sludge are re-introduced into the system before the wastewater reaches the first activated sludge unit (prior to sampling spot 1), the real influent load is different from the measured load. It was also calculated by subtracting the compound load of the liquid fraction of the secondary excess sludge and the load of the flush stream. In contrast, assuming that the sorbed compound load of the secondary and the tertiary sludge are not resolved, the excess sludge can be regarded as removed from the system and has not to be considered in the following calculations:

$$R_{1.Bio} = \left(\frac{\sum F_{Bio,in} + \sum F_{tert.exsludge,(l)} - \sum F_{sec.\,clarifier,effl} - \sum F_{sec.exsludge,(l)} - \sum F_{deni,flush}}{\sum F_{Bio,in} - \sum F_{sec.exsludge,(l)} - \sum F_{deni,flush}} \right) \cdot 100 \qquad (4\text{-}1)$$

$$R_{2.Bio} = \left(\frac{\sum F_{sec.clarifier,effl} - \sum F_{tert.clarifier,effl} - \sum F_{tert.exsludge,(l)}}{\sum F_{Bio,in} - \sum F_{sec.sludge,(l)} - \sum F_{deni,flush}} \right) \cdot 100 \qquad (4\text{-}2)$$

$$R_{deni} = \left(\frac{f_{deni,Q} \cdot \sum F_{tert.clarifier,effl} - \sum F_{deni,effl} - \sum F_{deni,flush}}{\sum F_{Bio,in} - \sum F_{sec.sludge,(l)} - \sum F_{deni,flush}} \right) \cdot 100 \qquad (4\text{-}3)$$

$$R_{total} = \left(\frac{\sum F_{Bio,in} - \sum F_{sec.exsludge,(l)} - \left(1 - f_{deni,Q}\right) \cdot \sum F_{tert.clarifier,effl} - \sum F_{deni,effl}}{\sum F_{Bio,in} - \sum F_{sec.sludge,(l)} - \sum F_{deni,flush}} \right) \cdot 100 \qquad (4\text{-}4)$$

$R_{1.Bio}$	average weekly removal efficiency of the first activated sludge unit
$R_{2.Bio}$	average weekly removal efficiency of the second activated sludge unit
R_{deni}	average weekly removal efficiency of the post-denitrification unit
R_{total}	average weekly removal efficiency of the complete treatment
$F_{Bio,in}$	influent load of the first activated sludge unit as measured at sampling spot 1 (Figure 4-1)
$F_{sec.clarifier,effl}$	effluent load of the secondary clarifier, as measured at sampling spot 2 (Figure 4-1)
$F_{sec.exsludge,(l)}$	load contained in the liquid fraction of the secondary excess sludge; corresponds to withdrawn volume of secondary excess sludge multiplied by the soluble concentration measured at sampling spot 2 (Figure 4-1)
$F_{tert.clarifier,effl}$	effluent load of the tertiary clarifier, as measured at sampling spot 3 (Figure 4-1)

$F_{tert.exsludge,(l)}$	load contained in the liquid fraction of the tertiary excess sludge; corresponds to withdrawn volume of tertiary excess sludge multiplied by the soluble concentration measured at sampling spot 3 (Figure 4-1)
$f_{deni,Q}$	relative amount of the flow at sampling spot 3 (Figure 4-1) treated by the post-denitrification unit (60-70%)
$F_{deni,effl}$	effluent load of the post-denitrification unit as measured at sampling spot 4 (Figure 4-1)
$F_{deni,flush}$	load contained in the wastewater used for flushing the post-denitrification unit, corresponds to withdrawn volume for flushing multiplied by the soluble concentration measured at sampling spot 3 (Figure 4-1)

The accuracy of the removal efficiencies was predicted with Monte Carlo simulations varying all the input parameters simultaneously randomly according to their assumed distribution. Based on the distribution of the results from numerous randomly varied calculation runs (5000 runs), the removal was predicted as the mean and the 95%-confidence intervals as the 95%-percentiles. Consequently, the distribution and accuracy of each input parameter had to be characterized. Regarding the measurements of the compound concentrations a log-normal distribution over the measuring range was assumed. The corresponding total geometric standard deviation was calculated from the three duplicate measurements of the weekly sampling campaigns as the relative standard deviation of the analytical method at a specific sampling site according to Joss et al. (2006a). For the single 24 h composite samples from the October measurement the geometric standard deviation was calculated from the results of the triplicate analyses. The error of the daily wastewater flow measurement was assumed to be normally distributed with a relative standard deviation of 10%. Since an assumption was made for the error distribution and the error of the input variables have not been shown to be independent, the simulation approach allows at best to predict the 95% confidence intervals.

4.3 Results and Discussion

4.3.1 Removal in the full-scale WWTP

Except for the tranquilizers diazepam and nordiazepam, the opioid oxycodon and the beta blocker betaxolol, all other target compounds were always detected in the WWTP (Table 4-3). The predominant psycho-active drugs were the antiepileptic carbamazepine and its metabolite 10,11-dihydro-10,11-dihydroxycarbamazepine (DHH) with medians in the WWTP influent of 0.66 and 2.2 µg L^{-1}, respectively. Carbamazepine was not removed at all, while DHH showed a slightly lower median in the WWTP effluent. Other psycho-active drugs were found with median influent concentrations in a range of 0.061 (dihydrocodeine) to 0.30 µg L^{-1} (morphine). Significantly lower effluent concentrations were observed only for the natural and semi-synthetic opium alkaloids morphine, codeine and dihydrocodeine as well as for the antiepileptic primidone. Sotalol and metoprolol were found to be the predominant beta blockers with medians in the influent of 0.87 and 0.81 µg L^{-1} and only slightly lower effluent median concentrations of 0.71 and 0.64 µg L^{-1}, respectively. A more considerable reduction of the concentration from 0.54 µg L^{-1} in the influent to 0.30 µg L^{-1} in the effluent was observed for the beta blocker atenolol.

Table 4-3. Occurrence of psycho-active drugs and beta blockers in WWTP influent (influent first activated sludge unit) and effluent (effluent tertiary clarifier), limit of quantification (LOQ), number of samples with concentrations above LOQ, median, 90th percentile and maximum.

	influent					effluent				
Substances	**LOQ [µg L^{-1}]**	**no. of samples > LOQ**	**median [µg L^{-1}]**	**P90 [µg L^{-1}]**	**max. [µg L^{-1}]**	**LOQ [µg L^{-1}]**	**no. of samples > LOQ**	**median [µg L^{-1}]**	**P90 [µg L^{-1}]**	**max. [µg L^{-1}]**
Antiepileptics										
Carbamazepine	0.020	9	0.66	0.94	1.0	0.010	9	0.74	0.92	1.2
DH-CBZ	0.010	7	0.023	0.030	0.032	0.050	8	0.020	0.032	0.034
DHH	0.020	9	2.2	2.8	4.1	0.010	9	1.6	2.3	2.6
Primidone	0.020	9	0.23	0.40	0.42	0.010	9	0.14	0.22	0.25
Antidepressants										
Doxepin	0.020	9	0.10	0.13	0.13	0.010	9	0.14	0.19	0.19
Opioids										
Oxycodon	0.020	0	-	-	-	0.010	0	-	-	-
Dihydrocodeine	0.020	9	0.061	0.11	0.14	0.010	9	0.038	0.051	0.073
Codeine	0.020	9	0.12	0.14	0.16	0.010	9	0.022	0.025	0.025
Morphine	0.020	9	0.30	0.39	0.44	0.010	8	0.025	0.028	0.029
Methadone	0.010	9	0.088	0.11	0.13	0.005	9	0.087	0.11	0.12
Tramadol	0.020	6	0.24	0.44	0.47	0.010	6	0.23	0.34	0.37
Tranquilizers										
Diazepam	0.020	0	-	-	-	0.010	0	-	-	-
Nordiazepam	0.020	0	-	-	-	0.010	0	-	-	-
Oxazepam	0.020	6	0.14	0.18	0.19	0.010	6	0.13	0.17	0.18
Beta blockers										
Atenolol	0.010	9	0.54	0.72	0.91	0.005	9	0.30	0.34	0.37
Sotalol	0.010	9	0.87	1.1	1.3	0.005	9	0.71	1.0	1.2
Metoprolol	0.010	9	0.81	0.97	1.2	0.005	9	0.64	1.0	1.1
Propranolol	0.005	9	0.040	0.053	0.073	0.003	9	0.040	0.053	0.058
Bisoprolol	0.010	9	0.21	0.29	0.38	0.005	9	0.21	0.24	0.27
Celiprolol	0.010	9	0.10	0.13	0.16	0.005	9	0.12	0.14	0.16
Betaxolol	0.005	4	0.006	8	0.009	0.003	1	-	-	-

In Figure 4-3 the percental reduction of the compound load by the different treatment units as well as the compound fraction not removed, is shown relative to the influent load without internal recycles for the two sampling campaigns in May and July (see eq. 4-1 to 4-4). The removal was calculated for all target compounds except for DH-CBZ, oxycodon, hydrocodon, diazepam, nordiazepam and betaxolol, since the influent concentrations of these compounds were below or close to its corresponding LOQs. Consistent with the median concentrations only the influent load of the natural opium alkaloids morphine and codeine was reduced by more than 80% during both sampling campaigns. For carbamazepine, oxazepam, propranolol and celiprolol the overall load reduction was below 20% and can be assumed insignificant. Removal efficiencies beyond 60% during at least one sampling campaign were only determined for primidone, doxepin, dihydrocodeine and atenolol. The reduction of compound loads was mainly limited to the second activated sludge unit. Except for primidone, no significant removal of the influent load could be observed during the first activated sludge treatment for both campaigns. Significant removal in the post-denitrification unit was only determined in July for the beta blockers metoprolol and bisoprolol, but the removal efficiencies were below 20% for both compounds.

The difference in the removal efficiency between the first and the second activated sludge unit is presumably caused by the increased SRT from 0.5 to 18 d. Similar conclusions were drawn by Clara et al. (2005) who determined a critical SRT of at least 10 d for bisphenol-A, ibuprofen, bezafibrate and the natural estrogens. The diversity of metabolic activities might increase with the SRT due to a diversification of the microbial community or a diversification of the available enzymes within the microbial community caused by a lower sludge loading with bulk organics (Clara et al., 2005; Ternes et al., 2004a).

For the sake of clarity and since in most case not significant, negative values are omitted in Figure 4-3. However, significant negative removal can be recognized from Figure 4-3 by a mass balance significantly higher than 100% as observed in May for doxepin (approx. - 40 and -120% in the first and second activated sludge unit, respectively) and in July for dihydrocodeine (approx. -40% in the first activated sludge unit). Assuming no systematic analytical error for example due to the high matrix content of the influent of the first activated sludge unit, negative values can be explained by the cleavage of conjugates. For instance, Rana et al. (2008) reported that more than 90% of the tricyclic antidepressant doxepin is excreted in urine as glucuronide conjugate. The glucuronide conjugate is also known to be one of the major metabolites of dihydrocodeine (Hufschmid et al., 1995). The occurrence and mi-

crobial cleavage of conjugates is well known to influence the mass balance in WWTPs (Andersen et al., 2003; Sedlak and Pinkston, 2001; Ternes et al., 1999). Variations in the ratio of deconjugation and biological transformation of the deconjugated compound may explain the variability of the measured removal for some compounds such as doxepin, dihydrocodeine and metoprolol (see 6.5.2). Although it has been reported that carbamazepine is partially excreted as glucuronide conjugate and not significantly removed by biological treatment (Maggs et al., 1997; Miao and Metcalfe, 2003), no significant increase of the carbamazepine load was observed in this study. However, the occurrence of conjugates was not determined and therefore needs to be addressed in future studies.

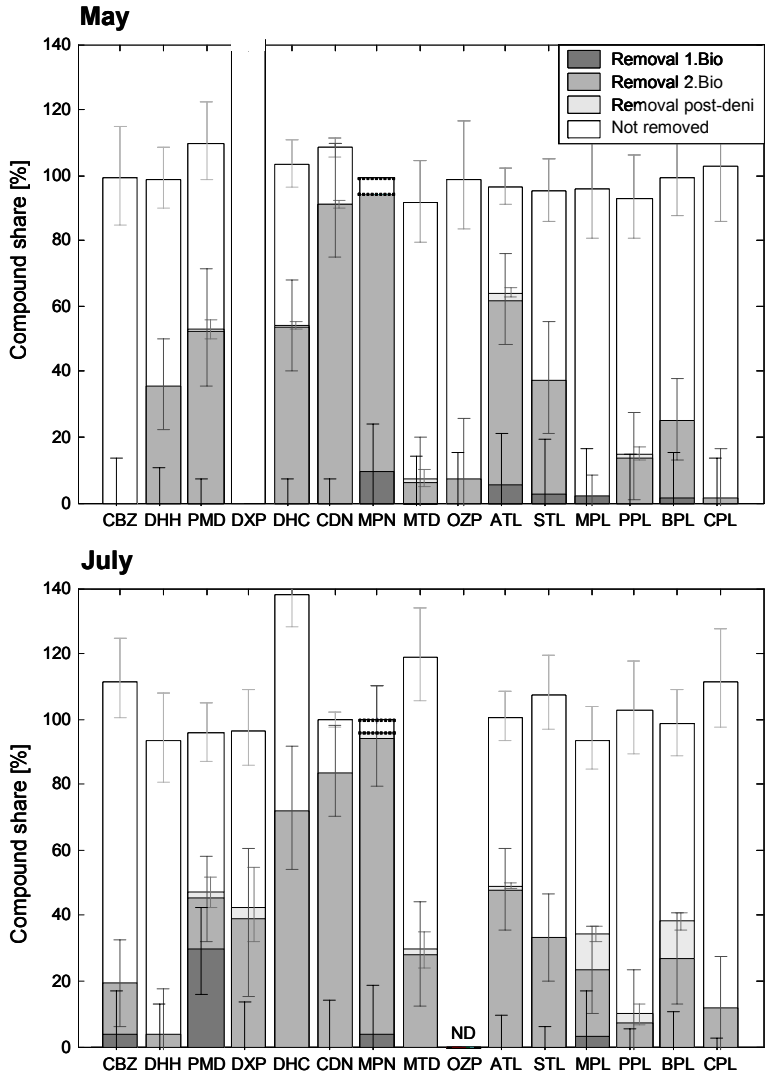


Figure 4-3. Percental reduction of the weekly loads of beta blockers and psycho-active drugs by the different WWTP treatment units relative to the influent load of the first activated sludge unit during two sampling campaigns in May and July. The white sections represent the compound load ratios not removed by the treatment processes. Mass balances higher than 100% result from negative removal values. The dashed lines for morphine (MPN) indicate minimum values for the measured removal due to concentrations below the LOQ of 20 ng L^{-1}. Error bars indicate the 95% confidence intervals derived from Monte Carlo simulations (5000 runs). ND: not determined.

4.3.2 Solid-water distribution coefficients for secondary sludge ($K_{d,sec}$)

Table SI 4-2 in the Supplementary Information shows that for all target compounds recoveries exceeded 80%, except for the natural opium alkaloids morphine and codeine. For these compounds the recovery decreased significantly during the sampling period. Since morphine and codeine featured also the highest k_{biol} values of all target compounds (see 4.3.3), it can be assumed that both compounds were removed by microbial transformation, even though all samples contained 0.2% NaN_3. However, since for all tested psychoactive drugs the ratio between the sorbed and the soluble compound concentration was not significantly different between the three sampling time points, sorption was assumed to be in equilibrium with the liquid phase throughout the sampling period. Therefore, mean sorption coefficients with 95%-confidence intervals were calculated from the data of the three replicates at three sampling times (n = 9; Table 4-4).

Table 4-4. Sorption coefficients for activated sludge $K_{d,sec}$ and biological transformation rate constants k_{biol} observed in two batch experiments with activated sludge (0.25 g_{OC} g_{TSS}^{-1}) taken from the aerated zone of the second activated sludge unit of the WWTP Frankfurt.

	Sorption		Transformation	
Compounds	**$K_{d,sec}$ [L kg_{TSS}^{-1}]**	**K_{OC} [L kg_{OC}^{-1}]**	**k_{biol} (March) [L g_{TSS}^{-1} d^{-1}]**	**k_{biol} (Oct.) [L g_{TSS}^{-1} d^{-1}]**
Antiepileptics				
Carbamazepine	17 ± 1	68 ± 4	n.a.	≤ 0.10
DH-CBZ	12 ± 1	48 ± 2	n.a.	≤ 0.10
DHH	7 ± 1	28 ± 5	≤ 0.10	0.16 ± 0.04
Primidone	7 ± 1	28 ± 4	n.a.	≤ 0.1
Antidepressants				
Doxepin	139 ± 23	566 ± 93	0.29 ± 0.06	0.68 ± 0.12
Opioids				
Oxycodon	14 ± 2	55 ± 7	n.a.	0.21 ± 0.15
Dihydrocodeine	12 ± 1	48 ± 3	n.a.	1.8 ± 1.0
Codeine	14 ± 1	55 ± 3	4.7 ± 0.5	4.8 ± 0.6
Morphine	12 ± 2	49 ± 7	n.a.	13.5 ± 3.4
Methadone	76 ± 4	308 ± 16	0.24 ± 0.04	0.18 ± 0.06
Tramadol	47 ± 1	53 ± 12	≤ 0.11	≤ 0.13
Tranquilizers				
Diazepam	53 ± 1	216 ± 5	n.a.	≤ 0.16
Nordiazepam	65 ± 2	263 ± 7	n.a.	0.13 ± 0.06
Oxazepam	13 ± 3	191 ± 6	≤ 0.12	≤ 0.10
Beta blockers				
Atenolol	38 ± 33 [a]	n.a.	1.9 ± 0.2	1.1 ± 0.1
Sotalol	18[b]	58[b]	0.40 ± 0.12	0.43 ± 0.04
Metoprolol	65[b]	215[b]	0.35 ± 0.11	0.40 ± 0.07
Propranolol	343[b]	1138[b]	0.36 ± 0.07	0.46 ± 0.03
Bisoprolol	40[b]	131[b]	0.64 ± 0.09	0.77 ± 0.04
Celiprolol	85[b]	283[b]	0.24 ± 0.15	0.18 ± 0.10
Betaxolol	n.a.	n.a.	6.0 ± 0.7	6.0 ± 0.8

The range indicates the 95% confidence interval (CI). The sign "≤" indicates that the lower limit was below experimental resolution, i.e. CI > k_{biol} or k_{biol} < 0.1. n.a.: data not available, K_{OC}: organic-carbon normalized K_d

[a] Maurer et al. (2007) ([L kg_{COD}^{-1}])

[b] Scheurer et al. (2010)

All tested psycho-active drugs exhibited a very low sorption tendency onto secondary sludge of the second activated sludge unit at neutral pH. While the highest $K_{d,sec}$ value of approx. 140 L kg_{TSS}^{-1} was determined for the antidepressant doxepin, the $K_{d,sec}$ values of most of the tested compounds were below 20 L kg_{TSS}^{-1}. Higher values were only observed for the tranquilizers diazepam (53 L kg_{TSS}^{-1}), nordiazepam (65 L kg_{TSS}^{-1}) and oxazepam (47 L kg_{TSS}^{-1}) as well as for the synthetic opioid methadone (76 L kg_{TSS}^{-1}).

Maurer et al. (2007) and Scheurer et al. (2010) determined sludge-water distribution coefficients of beta blockers. In both studies the highest $K_{d,sec}$ value was found for propranolol with 317 L kg_{COD}^{-1} and 343 L kg_{TSS}^{-1}, respectively (assumption: chemical oxygen demand (COD) ≈ total suspended solids concentration (TSS)). $K_{d,sec}$ values for atenolol, sotalol, celiprolol and bisoprolol were below 100 L kg_{TSS}^{-1}.

For $K_{d,sec}$ values less than 500 L kg_{TSS}^{-1} the removal by sorption in a WWTP with a typical sludge production of 0.2 g_{TSS} L^{-1} is less than 10% (Ternes et al., 2004b) and hence does not significantly contribute to the removal of psycho-active drugs and beta blockers in activated sludge units. Regarding the average sludge production of 0.09 g_{TSS} L^{-1} in the second activated sludge unit of the examined WWTP Frankfurt-Niederrad even less than 3% of the examined beta blockers and psycho-active drugs were removed by sorption.

4.3.3 Biological transformation

Two independent batch experiments with sludge from different seasons (March and October) and with a slightly different experimental set-up (pH stabilization by using a phosphate buffer and by purging with CO_2) were conducted. Typical biological transformation curves obtained for the removal of beta blockers and psychoactive drugs in the batch experiments are shown in Figure 4-4.

A significant dissipation over the sampling period of 48 h could be observed for 8 (morphine, codeine, dihydrocodeine, doxepin, oxycodon, methadone, DHH-carbamazepine, and nordiazepam) out of 15 selected psycho-active drugs and for all selected beta blockers. In contrast, no significant decrease in concentration was observed for the psycho-active drugs hydrocodone, tramadol, diazepam, oxazepam, carbamazepine, DH-carbamazepine and primidone. Control experiments run without biologically active sludge but with final effluent, confirmed that the removal was caused due to the interaction with sludge. As a consequence, the transformation rate can be linked to the total suspended solids concentration TSS present in solution. An exponential decrease of the concentration over time was observed for all the compounds for which a removal beyond the experimental resolution limit was measured.

Since the impact of sorption on the dissipation of psycho-active drugs and beta blockers can be ruled out (see 4.3.2), the compound removal can be described by pseudo first-order kinetics as suggested by Joss et al. (2006a) and Schwarzenbach et al. (2003):

$$\frac{dC_w}{dt} = -k_{biol} \cdot TSS \cdot C_w \qquad (4\text{-}5)$$

C_w soluble compound concentration [µg L^{-1}]
t incubation time [d]
k_{bio} biological transformation rate constant [L g_{TSS} d^{-1}]
TSS total suspended solids concentration [g_{TSS} L^{-1}]

The logarithmized form of the solution of this differential equation

$$-\ln\left(\frac{C_{w,0}}{C_{w,t}}\right) = k_{biol} \cdot TSS \cdot t \qquad (4\text{-}6)$$

$C_{w,0}$ initial soluble compound concentration [µg L^{-1}]

was used to calculate the biological transformation rate constants (k_{biol}) determining the slope of the linear regression line of a semi-logarithmic plot of C/C_0 via time t (Table 4-4). The 95%-confidence intervals of the transformation rate constants were calculated from the prediction bands of the linear regression. Except for atenolol, doxepin and DHH the k_{biol} values derived from the March experiment were within the 95% confidence interval of those derived from the batch experiment in October. Since fewer compounds were examined during the first batch experiment and mainly comparable results were achieved during both experiments, the k_{biol} values derived from the 2nd experiment in October were used for comparison and the prediction of full-scale removal efficiencies.

In October, transformation rate constants higher than 1.0 L g_{TSS}^{-1} d^{-1} were only found for the opium alkaloids morphine (13.5 ±3.4 L g_{TSS}^{-1} d^{-1}), codeine (4.8 ± 0.6 L g_{TSS}^{-1} d^{-1}) and dihydrocodeine (1.8 ± 1.0 L g_{TSS}^{-1} d^{-1}) as well as for the beta blockers betaxolol (6.0 ± 0.8 L g_{TSS}^{-1} d^{-1}) and atenolol (1.1 ± 0.1 L g_{TSS}^{-1} d^{-1}). Except for doxepin (0.68 ± 0.12 L g_{TSS}^{-1} d^{-1}), the k_{biol} values determined for all other tested psycho-active drugs were below 0.3 L g_{TSS}^{-1} d^{-1}. The transformation rate constants of the beta blockers celiprolol, metoprolol, sotalol, propranolol and bisoprolol were all in the range of 0.18 ± 0.10 to 0.77 ± 0.04 L g_{TSS}^{-1} d^{-1}. Considering a classification scheme of Joss et al. (2006b) for the full-scale removal, only the natural opium alkaloid morphine can be assumed to be reduced by > 90%, while the other tested psychoactive drugs and beta blockers are expected to be removed between 20 and 90%.

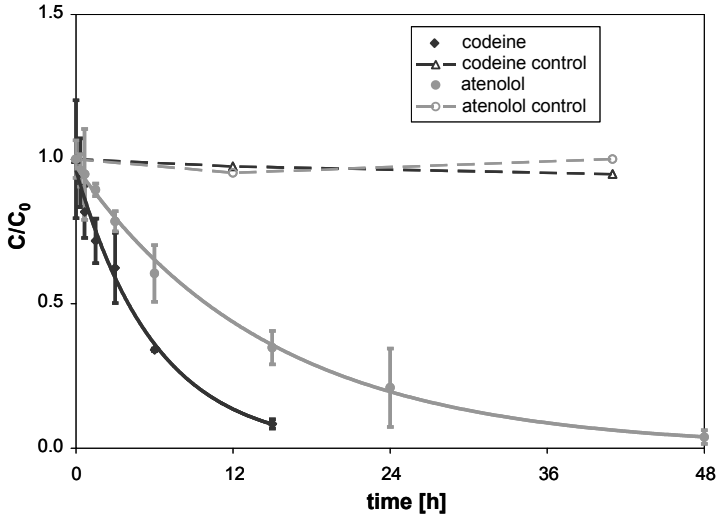


Figure 4-4. Biological transformation of the beta blocker atenolol and the psycho-active drug codeine in batch experiments. Batch experiments were performed in triplicate with activated sludge from the aerated zone of a conventional activated sludge unit diluted with the final effluent. Control experiments were performed in duplicate with final effluent without sludge. Error bars indicate the 95% confidence intervals.

4.3.4 Predicting the removal using a modeling approach

Model features

The removal of the psycho-active drugs and beta blockers was predicted for the second activated sludge unit, since the load reduction in the full-scale measurement campaigns occurred predominantly in this unit. Based on a prediction model of Joss et al. (2006b), the second activated sludge unit was assumed to represent a cascade of completely mixed reactors (CSTR) of comparable size and biological activity. Since sorption batch experiments in this study and literature data confirmed that sorption was not significant for all selected target compounds, the original model could be simplified using the discretized form of the differential eq. 4-6 as the basis formula for predicting the removal in a full-scale reactor. As a result, the following equation describing the removal based on biological transformation in the second activated sludge unit was obtained:

$$\check{R}_{2.Bio} = \left(1 - \frac{C_{w,out}}{C_{w,in}}\right) \cdot 100 = \left(1 - \frac{1}{(1+R)\cdot\left[1+\left(k_{biol}\cdot SS\cdot\frac{HRT}{n\cdot(1+R)}\right)\right]^{n} - R}\right) \cdot 100 \qquad (4\text{-}7)$$

$\check{R}_{2.Bio}$ predicted removal in the second activated sludge unit [%]
$C_{w,out}$ soluble compound concentration in the effluent [µg L^{-1}]
$C_{w,in}$ soluble compound concentration in the influent [µg L^{-1}]
HRT hydraulic retention time [d]
R sludge recycle [-]; $R = Q_{sludge\ recycle}/Q_{wastewater}$ with Q = flow rate [$m^3\ d^{-1}$]
n number of compartments [n]

To account for the temperature influence, the transformation rate constants k_{biol} were multiplied by a temperature correction factor T_{corr} based on the Arrhenius equation:

$$T_{corr} = e^{\kappa\cdot(T_{ref} - T)} \qquad (4\text{-}8)$$

κ temperature coefficient [K^{-1}]
T_{ref} temperature of the batch experiments (reference temperature) [K]
T temperature in the activated sludge unit of the full-scale WWTP [K]

The temperature coefficient κ is defined as:

$$\kappa = -\frac{E_a}{R\cdot T_m^2} \qquad (4\text{-}9)$$

E_a activation energy [J mol^{-1}]
R universal gas constant [J mol^{-1} K^{-1}]
T_m $(T_{ref}-T)/2$

This model is well-used for describing the influence of temperature on biological reaction rates in biological wastewater treatment (Clara et al. 2005; Li et al., 2005; Tchobanoglous et al., 2003). The temperature coefficient κ for organic micropollutants like pharmaceuticals is expected to be in the range of 0.03 - 0.09 K^{-1} (Joss et al., 2006a; Li et al., 2005). Therefore, in the current study a κ of 0.06 ± 0.02 K^{-1} was used for the prediction within the Monte Carlo Simulation.

Biological transformation constants were determined in this study under aerobic conditions with sludge taken from the nitrifying zone. To account for the denitrifying zone of the second activated sludge unit, two scenarios were used to predict its efficiencies. Scenario 1): It was assumed that the transformation rate constants of the denitrifying zone are not significantly different from those of the nitrifying zone ($k_{biol,Deni} \approx k_{biol,Nitri}$; HTR of both nitrification and denitrification considered). Scenario 2): No significant transformation in the denitrifying

zone occurred ($k_{biol,Deni} \approx 0$); this was implemented by taking into account only the HRT of the nitrifying zone.

Since the second activated sludge unit is about 5 time longer as wide, the number of compartments n was estimated to be between 2 and 4. In case of scenario 2) the number of compartments was estimated between 1 and 3. The uncertainty linked to the number of compartments was also considered in the Monte Carlo simulation by varying n continuously between the assumed minimum and maximum value. The hydraulic retention time (HRT), the total suspended solids concentration (TSS) and the sludge recycle (R) were provided by the WWTP personnel. For the Monte Carlo simulation these parameters were assumed to be normally distributed with a relative deviation of 10%.

Predicted versus measured removal in the second activated sludge unit

In Figure 4-5 the modeled removal efficiencies for beta blockers and psycho-active drugs are compared with those calculated from the compound loads measured in the effluent of the secondary clarifier (sampling spot 2) and in the effluent of the tertiary clarifier (sampling spot 3). The model prediction was accomplished using scenario 1) ($k_{biol,Deni} \approx k_{biol,Nitri}$) and scenario 2) ($k_{biol,Deni} \approx 0$). For the sampling campaigns in July, May and March the removal was calculated from weekly loads, while the sampling in October allowed only for the calculation from 24 h loads. The 95%-confidence intervals of the measured and modeled removal were derived from Monte Carlo simulations (5000 runs). They indicate that removal below 20% is insignificant as expected when considering the uncertainties of the analytical methods used (Hummel et al., 2006; Scheurer et al., 2010). For higher removal efficiencies uncertainties of ± 10-20% have to be taken into account.

Especially for the sampling campaigns in October and July the removal of most target compounds could be quite well predicted. The differences between the modeled values of scenario 1) ($k_{biol,Deni} \approx k_{biol,Nitri}$) and scenario 2) ($k_{biol,Deni} \approx 0$) were small in comparison to the uncertainties. Regarding the significant lower transformation rates previously observed for other pharmaceuticals in absence of molecular oxygen (Joss et al., 2004; Joss et al., 2006a), a better fit using scenario 2) had been expected. But even though the prediction using scenario 2) resulted in slightly better fits for the 24 h sampling campaign in October, the experimental setup does not allow discriminating between the two scenarios. However, considering the small differences between the two scenarios, the prediction model indicates that independent

of the actual values for $k_{biol,Deni}$ (provided that $k_{biol,Deni} \leq k_{biol,Nitri}$) the nitrification zone was most crucial for the observed removal of the selected beta blockers and psycho-active drugs.

Significantly reduced removal efficiencies (negative values are found) measured in October, May and March for doxepin and in May and March for metoprolol could not be predicted with the modeling approach used. Presumably, at least one crucial factor influencing the removal is not yet considered in the model. As discussed before (see 4.3.1), the occurrence and microbial cleavage of conjugates could be responsible for an underestimation of the removal efficiencies for some compounds. Consequently, the real removal efficiencies might be significantly higher as already supposed by Maurer et al. (2007) for the beta blocker metoprolol. Similarly, Fono and Sedlak (2005) and MacLeod et al. (2007) suggested that a significant decrease of the enantiomeric fraction (EF) of the beta blocker propranolol during biological treatment was caused by the cleavage of S-(-)-propranolol enriched conjugates excreted in urine. These findings support that the lower removal of propranolol in comparison to the predicted removal determined in the current study might also be the result of microbial deconjugation.

The removal of primidone was underestimated by the model. Since the transformation constant of primidone was below the experimental resolution, only an upper limit of removal of approx. 10% could be predicted using the upper 95% confidence value of k_{biol}. But except for the October campaign, the measured removal was between 30 and 50%. However, the transformation constant of primidone was only determined during the batch experiment in October. Thus, in contrast to the results for other target compounds it cannot be excluded that the sludge might have lost its capability of transforming primidone.

Influence of temperature and number of compartments

In the March campaign for most compounds the removal efficiency of the second activated sludge unit was significantly lower, although the transformation rate constants determined in parallel batch experiments were similar to those determined in October. The only difference was the lowered temperature of down to 10.4°C in the treatment tank in contrast to the temperature during the other sampling campaigns (16.9 – 19.4°C). Hence, the reduced removal efficiency might be due to kinetic reasons. Significant lower removal rates for several pharmaceuticals in winter were also observed by Castiglioni et al. (2006). Reduced rate constants due to lower temperatures were considered by the temperature correction term of the model and consequently the modeled removal efficiencies in the March campaign were

also significantly reduced. Nevertheless, a complete inhibition of the removal found for example for dihydrocodeine and sotalol could not be predicted. Presumably, there exists a temperature below which a biological removal of certain beta blockers and psycho-active drugs are completely inhibited. However, to finally confirm the compound-specific influence of the temperature on the biological removal, batch experiments under different temperatures are needed.

The number of compartments (n) estimated on experiences from other WWTPs might also have an influence on the removal. However, especially for the slowly transformed compounds (removal < 40%) the influence of the number of compartments is negligible. For the other compounds the good quality of the predicted values indicates that the estimated range of 1-3 (scenario 1) and 2-4 (scenario 2) was a quite realistic assumption.

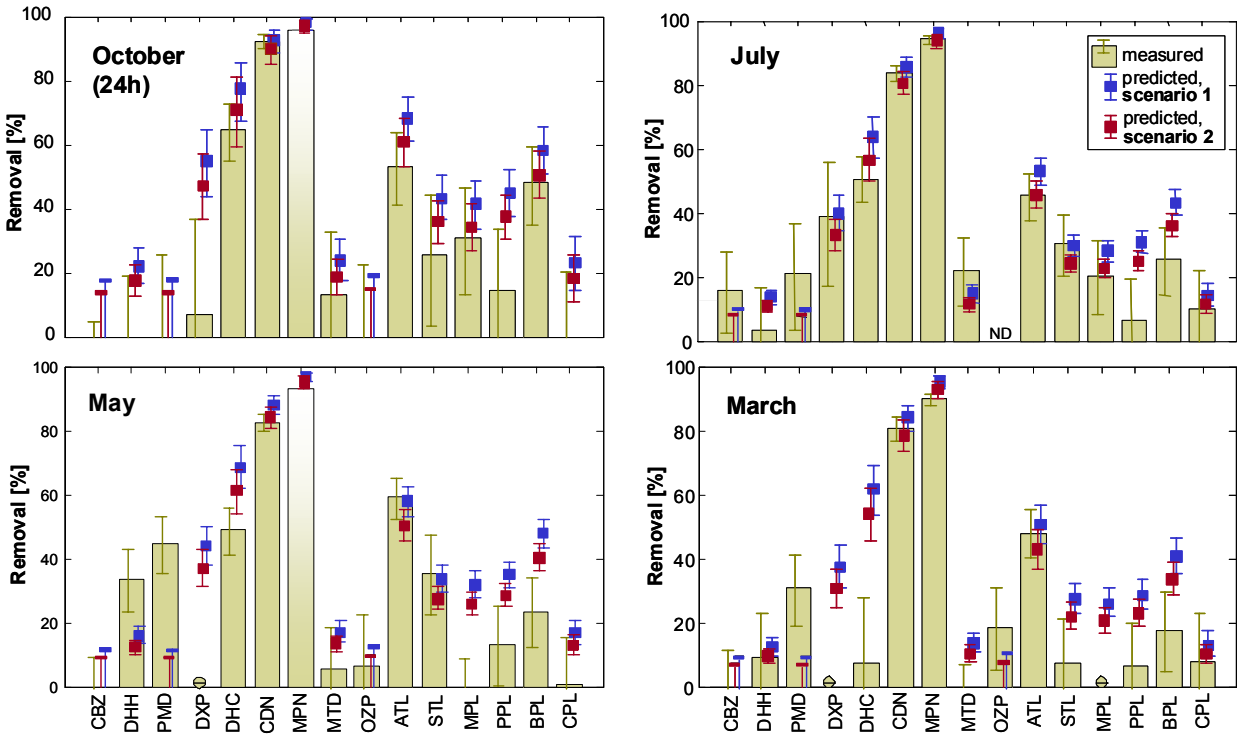


Fig. 4-5. Measured versus predicted removal of beta blockers and psycho-active drugs in an activated sludge unit of a municipal WWTP calculated for three weekly sampling campaigns in July, May and March and a 24 h sampling campaign in October. Columns show the removal calculated from the measured compound loads while the squares show the removal predicted considering scenario 1) with $k_{biol,Deni} \approx k_{biol,Nitri}$ (grey dots) and scenario 2) with $k_{biol,Deni} \approx 0$ (black dots). Faded columns for morphine (MPN) indicate minimum values for the measured removal due to effluent concentrations laying below the LOQ of 20 ng L^{-1}. Measured removal efficiencies significantly lower than zero are indicated by the minus sign. Dashes instead of squares represent upper limits of the predicted removal for those compounds for which only upper limits of k_{biol} could be identified. Error bars indicate the 95% confidence intervals derived form Monte Carlo simulations (5000 runs).

4.4 Conclusions

The results of this study revealed that even state-of-the-art WWTPs with multiple biological treatment units and high SRTs are insufficient barriers to prevent the emission of beta blockers and psycho-active drugs into the receiving waters with up to now unforeseen effects on the aquatic ecosystem. While the opium alkaloids morphine and codeine are removed by more than 80%, most compounds such as the beta blocker sotalol or the antiepileptic primidon are only barely removable. For some compounds such as the beta blocker celiprolol or the antidepressant oxazepam no significant removal can be detected. Sorption batch experiments within this study and literature data confirmed that sorption onto activated sludge is negligible and thus, any removal of the examined beta blockers and psycho-active drugs can be exclusively referred to as biological transformation.

A comprehensive mass balance of the biological treatment units calculated from average weekly compound loads of all important streams indicates that activated sludge units designed for COD removal with a high substrate loading and low SRTs do not significantly contribute to the removal of any of the examined beta blockers and psycho-active drugs. In contrast, the results demonstrate the importance of the second activated sludge unit with high SRT achieving at least partial removal of the target compounds. Further biological treatment by a fixed filter-bed operated under denitrifying conditions is also incapable of reducing the load of beta blockers and psycho-active drugs to a significant extent.

Using a model based on pseudo-first-order transformation kinetics and the corresponding transformation constants determined in batch experiments, the removal of beta blockers and psycho-active drugs in an activated-sludge unit could be predicted quite well. In most cases the predicted removal efficiencies were consistent with those derived from full-scale measurement campaigns. Hence, information from lab-scale transformation batch experiments are a useful tool to at least semi-quantitatively predict the fate of pharmaceuticals like beta blockers and psycho-active drugs in WWTPs.

However, for some beta blockers and psycho-active drugs the cleavage of conjugates might lead to a significant bias between the observed and predicted removal. A significantly lower and even negative removal measured occasionally for compounds such as doxepin and metoprolol indicates that deconjugation interferes to a variable extent with biological transformation of the deconjugated compound.

Additionally, for a quantitative prediction of the removal in the biological treatment units, a detailed correlation with parameters such as the temperature and the transformation constants for different redox conditions are crucial and needs to be addressed in coming up studies. The comparison of the results from the measurement campaigns and the modeling did not allow for an assessment of any removal under anoxic conditions, e.g. in the denitrification tank of the second activated sludge unit. However, independent of the removal efficiency of the denitrification tank, the two different modeling scenarios identified the nitrification tank as the unit being most responsible for the observed removal of beta blockers and psychoactive drugs along the treatment processes.

4.5 Acknowledgements

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5. Elucidation of the transformation pathway of the opium alkaloid codeine in biological wastewater treatment

Arne Wick, Manfred Wagner and Thomas A. Ternes

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Abstract

Codeine, an opium alkaloid, was transformed in aerobic batch experiments with activated sludge into several transformation products (TPs). For eight TPs, the chemical structures were unambiguously identified by a multi-step approach using results from high-resolution mass spectrometry (HR-MS) and 1D and 2D nuclear magnetic resonance (NMR) experiments. For an additional 10 TPs, tentative structures were proposed. Most of the TPs identified, exhibited only slightly modified molecular structures featuring double bond shifts, introduction of hydroxy groups or amine demethylation. The transformation pathway of codeine in contact with activated sludge is characterized by a combination of biologically and chemically mediated reactions. Biological oxidation of codeine leads to the formation of the α,β-unsaturated ketone codeinone, which is the precursor for further abiotic and biotic transformation due to its high chemical reactivity. An analytical method based on solid-phase extraction and LC tandem MS detection was developed to confirm the formation of several TPs in wastewater treatment plants (WWTPs). The mass balances were comparable to those obtained from batch experiments. A HR-MS screening approach of TPs from dihydrocodeine and morphine revealed that the knowledge from the codeine transformation pathway can be extrapolated to the distinct transformation pathways of these structurally related opium alkaloids. In total, 17 TPs were proposed for morphine and two TPs for dihydrocodeine.

5.1 Introduction

The natural opium alkaloid codeine is highly consumed, either by prescription or as an illicit drug. In Germany, approximately 2.4 t of codeine are annually used as an analgesic, mostly in combination with non-opioid substances or as an antitussive. Codeine has been detected in wastewater influents at a maximum concentration of 540 ng L^{-1} in Germany and up to 10 µg L^{-1} in Wales (Hummel et al., 2006; Kasprzyk-Hordern et al., 2008). Ecotoxicity data of opium alkaloids is rare, but indicates a potential impact on the immune system of vertebrates (Gagné et al., 2006). While sorption was shown to be of minor importance, biological transformation was identified to remove about 80% of the codeine load during biological treatment of a WWTP with a sludge age of 18 d (Wick et al., 2009). For the majority of the organic trace contaminants, microbial transformation does not lead to mineralization, but rather to the formation of a multitude of transformation products (TPs) (Kosjek et al., 2009). These TPs might have a similar or in some cases an enhanced ecotoxicological potential relative to that of the parent compound (Sinclair and Boxall, 2003; Van Zelm et al., 2010).

In the past, the elucidation of biotransformation and chemical transformation of micropollutants at environmental relevant concentrations was extremely time consuming or even impossible due to the limited analytical capabilities. In recent years, sophisticated hybrid mass spectrometry systems (e.g. triple quadrupole linear ion trap mass spectrometers: Qq-LIT-MS, quadrupole time-of-flight mass spectrometers: Qq-TOF-MS, linear ion trap quadrupole Fourier transformation mass spectrometers: LTQ-FT-MS) have been shown to be powerful tools for the structural identification of TPs (Helbling et al., 2010a; Kosjek and Heath, 2008; Radjenović et al., 2009a). For example, Medana et al. (2008) identified 23 photochemical TPs of atenolol using the capability of a LTQ-Orbitrap-MS for analyzing the fragmentation pathways of the TPs by high-resolution MS^n experiments. Kosjek et al. (2009) identified a TP of diclofenac and a TP of clofibric acid formed in activated sludge bioreactors using Qq-TOF-MS.

Multi-step analytical approaches are frequently crucial for the successful identification of TPs. Nuclear magnetic resonance (NMR) is one option for a complementary technique for identification and confirmation of TP structures as long as isolation of sufficient quantities can be accomplished. The identification of a total of 46 microbial TPs of iodinated X-ray contrast media (Kormos et al., 2009; Schulz et al., 2008) and six ozonation products of β-lactam antibiotics (Dodd et al., 2010) by multinuclear NMR experiments in addition to

Qq LIT-MS and LTQ-Orbitrap-MS, respectively, showed the successful application of NMR techniques for TP identification.

The objective of this study was to identify TPs of codeine formed in laboratory batch experiments with activated sludge under aerobic conditions. Identification was accomplished using a multi-step analytical approach including HR-MS and 1D and 2D-NMR techniques. Furthermore, we investigated the transformation pathway of codeine as well as the transferability of the results to full-scale wastewater treatment plants (WWTPs) and to other structurally related opium alkaloids such as morphine and dihydrocodeine.

5.2 Experimental Methods

5.2.1 Chemicals

Codeine and dihydrocodeine were purchased from Th.Geyer (Renningen, Germany) and hydrocodone was purchased from Sigma-Aldrich (Schnelldorf, Germany). Codeine-d_6, used as the surrogate standard, and the analytical reference standards codeinone, 14 hydroxycodeinone and pseudomorphine were purchased from LGC Promochem (Wesel, Germany). 14-Hydroxycodeine was purchased from Campro Scientific (Berlin, Germany). Reference standards of TP 300(1), TP 302, TP 332(1), TP 264, TP 346 and TP 348 were not commercially available, and therefore were isolated and purified as described below. The purity of these TPs was assessed by HPLC-UV and exceeded 90%.

DMSO-d_6 (isotopic enrichment 99.96%) was used as solvent for NMR and was purchased from Deutero GmbH (Kastellaun, Germany). All other solvents (n-heptane, acetone, methanol and acetonitrile) were picograde and purchased from LGC Promochem (Wesel, Germany).

5.2.2 Batch systems with activated sludge

For the batch experiments, sludge was taken from the nitrification zone of the activated sludge tank of a conventional German WWTP. The WWTP has a designed capacity of 320 000 population equivalents (PE) and a daily flow rate of 60000 m^3. The activated sludge unit is characterized by a hydraulic retention time (HRT) and a sludge retention time (SRT) of 6 h and 12 d, respectively. Details about the treatment processes at this WWTP can be found in Wick et al. (2010). Fresh sludge (total suspended solids, TSS: ~ 4 g_{SS} L^{-1}; total organic carbon, TOC: ~ 0.3 g g_{SS}^{-1}) was transported to the laboratory and experiments were initiated on the same day. In general, the sludge was diluted with groundwater, free of all target compounds, to minimize the impact of sorption and analytical interference with matrix components. The activated sludge was continuously stirred in 500 mL amber gas wash bottles and flushed with a mixture of air and carbon-dioxide (~98/2; v/v) using a rotameter. This ensured aerobic conditions (6-8 mg O_2 L^{-1}) and a stable pH (7.0 ± 0.2). The pH and oxygen concentration was continuously monitored in a control sample and after each sampling in the spiked samples. Details about the aeration with carbon-dioxide-supplemented air are provided in Elphick et al. (2005). Depending on the specific application, different concentrations (between 2 µg L^{-1} and 200 mg L^{-1}) of the respective target compounds were separately spiked

into the sludge slurry after an equilibration time of 1 h (Table 5-1). The duration of the experiments was chosen in a way that at least 80% of the initially spiked concentration was transformed. Samples were taken just before and at specified time intervals after spiking. For direct injection, samples were filtered through 0.45 µm syringe filters made of regenerated cellulose (Spartan, Whatman, USA), and formic acid was added to a final concentration of 0.2% (see SI). The samples were kept frozen (-25°C) until analysis. In those cases where enrichment was necessary (i.e. batch systems with a spiking concentration of 2 µg L^{-1}), 50 mL samples were taken and acidified to pH 3 with 3.5 M sulfuric acid, filtered through glass fiber filters (GF/6, Whatman) and stored at 4°C. The samples were subjected to solid-phase extraction (SPE) as described below within 24 h after sampling.

Non-spiked sludge blanks were always run in parallel. For certain experiments spiked autoclaved diluted sludge as well as autoclaved groundwater and ultrapure water were used to confirm the occurrence of abiotic transformation processes. Samples from the autoclaved controls were taken with sterile pipette tips and the gas stream used for aeration was directed through 0.2 µm sterile syringe filters to avoid any microbial contamination during incubation. An overview of the details about the different batch experiments is given in Table 5-1.

Table 5-1. Overview of the different batch experiments.

Compounds	Spike conc.	Matrix	Duration	Analysis	Application
codeine	200 mg L^{-1}	diluted sludge (1:20, 0.2 g_{SS} L^{-1})	35 days	HPLC-UV, Qq-LIT-MS, LTQ-Orbitrap-MS, NMR	isolation and identification
codeine, codeine TPs[a]	200 µg L^{-1}	diluted sludge (1:10, 0.38 g_{SS} L^{-1})	7 days	LC-Qq-LIT-MS (SIM mode)	elucidation of transformation pathway
TP 298 (codeinone)	5 mg L^{-1}	diluted sludge (1:20, 0.2 g_{SS} L^{-1}), autoclaved diluted sludge, groundwater and ultrapure water	10 days	LC-Qq-LIT-MS (full scan and SIM mode), DOC measurement	quantification, mass balance, differentiation between chemical and microbial transformation
codeine and TP 298 (codeinone)	2 µg L^{-1}	diluted sludge (1:20, 0.18 g_{SS} L^{-1}), undiluted sludge (3.6 g_{SS} L^{-1})	70 h	LC-Qq-LIT-MS (SIM mode, enrichment via SPE)	quantification, occurrence and mass balance at environmental-relevant concentrations
morphine, dihydrocodeine, hydrocodone	0.5 mg L^{-1}	diluted sludge (1:20, 0.2 g_{SS} L^{-1}), autoclaved diluted sludge	6 d	LC-LTQ-Orbitrap-MS (full scan and MS^2)	screening for codeine-like TPs, identification of further TPs

[a] codeine TPs used: TP 300(1), TP 314, TP 316 and TP 332

5.2.3 Environmental samples from WWTPs

Influent (after primary clarification) and effluent samples were collected from four conventional WWTPs (WWTP 1-4) to determine the formation of codeine TPs during waste-

water treatment. In addition, grab samples of activated sludge were taken from the nitrification zone of the activated sludge tank of WWTP 1 to determine the sorbed concentrations. All four sampled WWTPs are conventional plants equipped with a mechanical treatment (screen, grit removal, primary clarifier) followed by an activated sludge treatment with nitrification and denitrification, phosphate removal and a final clarifier. Before discharge into the receiving water, the effluent of the final clarifier of WWTP 3 is directed through a filtration system. The designed capacity, daily flow rate, HRT and SRT of the activated sludge treatment of the WWTPs were as follows: WWTP 1: 30000 PE, 9000 m^3 d^{-1}, 32 h (including settling tanks), 16 d; WWTP 2: 1350000 PE, 200000 m^3 d^{-1}, 5 h, 18 d; WWTP 3: 285000 PE, 35500 m^3 d^{-1}, 36 h, 16 d; WWTP 4: 230000 PE, 67400 m^3 d^{-1}, 12 h, 29 d.

Samples were taken as 24 h (WWTP 1), 48 h (WWTP 2) and 1 week (WWTP 3) composite samples as well as grab samples (WWTP 4) from the influent (after primary clarification) and from the effluent before discharge into the receiving water. All samples were taken during dry weather periods in amber, solvent-rinsed glass bottles, acidified to pH 3 with 3.5 M sulfuric acid, filtered through glass-fiber filters (GF/6, Whatman) and stored at 4°C until SPE. The activated sludge samples were centrifuged and the aqueous phase was treated in the same manner as the wastewater samples. The solid phase was freeze-dried, spiked with 50 ng of the surrogate codeine-d_6 and extracted with a Dionex ASE 200 instrument (Sunnyvale, CA, USA) according to a procedure described in Wick et al. (2010).

5.2.4 TP isolation via semi-preparative HPLC-UV

After the concentrations of codeine were reduced by more than 90%, the batch samples were centrifuged and filtered through 1 µm glass fiber filters (GF/6, Whatman) and 0.45 µm cellulose sodium nitrate filters (Sartorius, Göttingen, Germany). The filtrate was frozen and concentrated to a final volume of 20 to 50 mL by freeze-drying. To fractionate and collect the TPs, a Waters HPLC-UV system consisting of a Waters 717 plus autosampler, column oven, Waters 600 controller with quaternary pump, in-line degasser, and Waters 2487 dual-wavelength absorbance detector (operated at 213 and 280 nm) was used. Isolation of individual TPs was achieved by chromatographic separation on a semi-preparative Synergi Polar-RP column (250 x 10 mm, 4 µm) from Phenomenex (Aschaffenburg, Germany). Mobile phase A consisted of 0.2% formic acid and a mixture of acetonitrile, methanol and formic acid (50/50/0.2, v/v) was used as mobile phase B. The applied gradient elution was as follows: start of the run with 10% B, kept isocratic for 22 min, increase to 25% B within 18 min, kept isocratic for 5 min, increase to 50% B within 20 min, kept isocratic for 20 min,

increase to 80% B within 0.1 min, kept isocratic for 5 min, return to the initial conditions within 0.1 min which were hold for the last 20 min. The flow rate was kept constant at 2 mL min^{-1} and the column oven temperature was set to 50°C. The sample volume injected was individually optimized based on the peak performance (150 to 500 µL).

Individual fractions were collected with an automated sample collector (Advantec SF-2120 super fraction collector, Techlab GmbH, Erkerode, Germany) based on the retention time of the peaks in the chromatogram. The composition and purity of the fractions were determined by HPLC-UV and LC tandem MS. If the purity of a TP was found to exceed 90% in the collected fraction, it was freeze-dried to complete dryness. Otherwise the volume of the solution was reduced by freeze-drying and purified again using HPLC-UV. The isolated TPs were used as analytical reference standards and for identification by mass spectrometry and NMR analysis.

5.2.5 Identification of TPs

Qq-LIT-MS

Determination of the nominal masses as well as MS^2 and MS^3 spectra of codeine and its isolated TPs were performed on a Qq-LIT-MS (MDX Sciex 4000 Q Trap, Applied Biosystems, Langen, Germany) by direct injection using a syringe pump (10 µL min^{-1}). Electrospray ionization (ESI) was used in the positive ionization mode. For MS^2 fragmentation, the collision energy was set to 40 eV, while for MS^3 fragmentation different excitation energies in the range of 20-200 mV were applied. For the mass spectrometric analysis of non-isolated codeine TPs, the Qq-LIT-MS was interfaced with an Agilent HPLC system (Agilent 1200 Series, Agilent Technologies, Waldbronn, Germany). Chromatographic conditions and ESI source conditions were similar to those used for the analysis of samples from the batch experiments and WWTPs (see 5.2.6).

LTQ-Orbitrap-MS

The mass spectrometric information obtained from Qq-LIT-MS experiments were supplemented with high-resolution MS analyses using a LTQ-Orbitrap-MS (LTQ Orbitrap Velos, Thermo Scientific, Bremen, Germany) with ESI operated in positive and negative ionization mode. Measurement of exact masses (mass accuracy < 5 ppm) allowed for the determination of the elemental composition of molecular ions and its product ions.

Product ions were obtained using collision-induced dissociation (CID) at a normalized collision energy of 25-35%. Isolated TPs were subjected to the LTQ-Orbitrap-MS by direct

injection with a syringe pump at a flow rate of 10 µl min^{-1}. For product ion analysis of the non-isolated codeine TPs as well as for the screening of batch samples for the occurrence of TPs from morphine and dihydrocodeine, the LTQ Orbitrap was interfaced with a Thermo Scientific Accela U-HPLC system (Accela pump and autosampler). Chromatographic conditions were the same as for LC tandem MS analysis (see 5.2.6).

The MS source parameters for the LC-LTQ-Orbitrap-MS measurements were set as follows: capillary temperature, 275 °C; capillary voltage, 3.0 kV; heater temperature, 380 °C; sheath gas flow rate, 40 AU; aux gas flow rate, 15 AU; S-lens RF level, 69%.

Data dependent acquisition was used to give rise to spectra as follows: A full scan (100 – 700 m/z; positive mode) was performed followed by MS^2 and MS^3 scans for the two most intense ions with intensities of >10000 and >1000, respectively. CID (collision induced dissociation, MS^2 and MS^3) and HCD (high-energy collision dissociation, only MS^2) with normalized collision energies of 35 % and 60%, respectively, were used for fragmentation. In addition, dynamic exclusion was applied (exclusion of masses for which three MS^n experiments have been performed; exclusion duration, 30 s) enabling also MS^n experiments for less abundant ions, e.g. during co-elution of different substances. External calibration was performed before the analysis of each batch to assure accurate mass determinations with a resolution of 60,000.

Identification of TPs via NMR

The isolated TPs (i.e. 0.7 mg to 6 mg of TPs) and 15 mg of codeine were dissolved in 0.8 mL DMSO-d_6. One exception was TP 364, which was dissolved in 0.8 mL D_2O. NMR experiments were recorded at 17.3 T (Bruker Avance 700, Rheinstetten, Germany) operating at a controlled temperature of 298 ± 0.1 K with a 5 mm z-gradient BBI ^1H/X probe. ^1H-NMR spectra were measured at 700 MHz, and ^{13}C-NMR spectra were measured at 176 MHz. Homo- and heteronuclear chemical shift correlations were determined by 2D-NMR techniques such as $^1H,^1H$-COSY and $^1H,^{13}C$-HSQC.

The spectra were referenced as follows: for the residual DMSO-(H)-d_5 $\delta(^1H)$ = 2.49 ppm and DMSO-d_6 $\delta(^{13}C)$ = 39.5 ppm and for HDO $\delta(^1H)$ = 4.8 ppm and $\delta(^{13}C)$ = DSS (sodium-3-trimethyl-1-propanesulphonat as an internal standard) in D_2O at 0 ppm.

A typical ^1H-NMR spectrum required between 8 and 128 transients acquired with a 9 µs 90° pulse, 14000 Hz spectral width and 5 s recycling delay, used to obtain qualitative information. ^{13}C-NMR experiments were obtained with both spin-echo (attached proton test)

and other ^{1}H decoupled ^{13}C-NMR acquisition methods using pulse sequences provided by the Bruker Topspin 2.1 software.

The homonuclear 2D experiments (COSY) were run by using 8192 points in f2 and 512 points in f1. Before Fourier transformation, the data was zero filled to 1024 points in f1 and multiplied by a window function (sine bell) in both dimension. The spectroscopic width was typically 14000 Hz in both dimensions (f1 and f2).

The 2D ^{1}H ^{13}C heteronuclear single quantum correlations (HSQC) were recorded with the following parameters: $^{1}J_{CH}$=145 Hz for optimizing observable intensities of cross peaks from one bond ^{1}H-^{13}C correlation, a relaxation delay of 2 s, while typical π/2 pulse width for ^{1}H and ^{13}C were 9 μs and 14.5 μs, respectively. The HSQC experiment used double inept transfer with decoupling (trim pulse in the inept transfer) during acquisition. The spectroscopic width for the proton window was 14000 Hz (20 ppm) and for the carbon 35000 Hz (200 ppm). All the 2D HSQC experiments were run by using between 4096 and 8192 points in f2 and 512 points in f1. Before Fourier transformation, the data were zero filled to 1024 points in f1 and multiplied by a window function (q-sine bell) in both dimensions.

5.2.6 Analysis of samples from batch experiments and WWTPs

Solid-phase extraction (SPE)

Different types of adsorbents (Oasis HLB, 200 mg, 33 μm and Oasis MCX, 60 mg, 30 μm, both Waters, Milfort, USA), pH values of the samples and different elution solvents were tested using 500 mL of groundwater spiked with 200 ng L^{-1} of codeine and each available TP. Additional groundwater samples were spiked with 100 μL of a sample from a batch experiment (initial codeine concentration: 200 mg L^{-1}, incubation day 27) containing most TPs to assess also the absolute recoveries of TPs for which no reference standards were available. Based on these results (Table SI 5-1), two different SPE procedures have been established. Method 1 was the optimal SPE procedure for codeine and all main TPs and was used for qualitative and quantitative analysis. An alternative method 2 was used for a qualitative monitoring of TPs with an acidic moiety, as these were not amenable to method 1. For both SPE methods, Oasis MCX cartridges (60 mg, 30 μm, Waters, Milfort, USA) were washed and conditioned with 1 x 2 mL heptane, 1 x 2 mL acetone, 3 x 2 mL methanol and 4 x 2 mL groundwater (adjusted to pH 3.0 with 3.5 M sulfuric acid). The samples (45 mL of the sample from the batch reactors, 100 mL of raw wastewater and 200 mL of treated wastewater) were spiked with 50 ng codeine-d_6 used as surrogate and then passed through the cartridges at a

flow rate of < 5 mL min^{-1}. The solid-phase material was dried by a continuous stream of nitrogen. For method 1, elution was accomplished with 4 x 2 mL of a mixture of acetonitrile and 25% ammonium hydroxide (95/5, v/v), while for method 2 acetonitrile was substituted by methanol. The extracts were evaporated to 100 µL using a gentle stream of nitrogen and filled up to a final volume of 1 mL with 0.2% formic acid (see also S24 in the Supplementary Information).

Liquid Chromatography-Tandem Mass Spectrometry

Analysis of the samples were performed by LC tandem MS using an Agilent HPLC system (Agilent 1200 Series, Agilent Technologies, Waldbronn, Germany) coupled to Qq-LIT-MS (MDX Sciex 4000 Q Trap, Applied Biosystems, Langen, Germany) with ESI operated in positive ionization mode. Chromatographic separation was accomplished using two coupled Synergi Polar-RP columns (150 mm x 3 mm, 4 µm) from Phenomenex (Aschaffenburg, Germany). Mobile phase A consisted of a mixture of 5 mM ammonium formate, methanol, acetonitrile and formic acid (94/3/3/0.2, v/v) and 0.1% formic acid in methanol was used as mobile phase B. The applied gradient elution was as follows: start of the run with 0% B, kept isocratic for 10 min, increase to 30% B within 10 min, kept isocratic for 6 min, increase to 80% B within 1 min, kept isocratic for 5 min, return to the initial conditions within 0.1 min which were hold for the last 9.9 min. The column oven was set to 50°C, the flow rate was kept constant at 0.5 mL min^{-1} and the sample volume injected was 25 µL.

Detection was accomplished by selected ion monitoring (SIM) monitoring two transitions for quantification (q_1) and confirmation (q_2). The compound specific parameters such as declustering potential, collision energy, and the cell exit potential were optimized for all TPs for which reference standards were available (Table SI 5-2). Optimization was performed in continuous flow mode via direct injection of standard solutions (500 ng mL^{-1}) with a syringe pump (10 µL min^{-1}). The MS/MS transition and corresponding MS parameters of the other TPs were taken from the results of the MS/MS experiments conducted for identification. Selected LC-MS/MS chromatograms of opium alkaloids and TPs of codeine and dihydrocodeine are shown in Figure SI 5-1.

Quantification and method validation

An external calibration with 10 calibration points ranging from 0.2 to 400 ng mL^{-1} was used for quantification. Quadratic fitting ($y = ax^2 + bx + c$) with a weighing factor of 1/x was

applied. The limit of quantification (LOQ) was defined as the second lowest calibration point as long as the calculated signal to noise ratio (S/N) of the compounds was >10 for the first transition (q_1) used for quantification and >3 for the second transition (q_2) used for confirmation. To consider the different sample volumes used for the influent, effluent and activated sludge samples, the corresponding LOQs were calculated by multiplying the LOQs derived from the external standard calibration by a factor of 10, 5 and 3.5, respectively. Still the criteria of a S/N ratio >10 for quantification and >3 for conformation had to be fulfilled.

Absolute recoveries were determined by comparing the peak area of spiked samples with the peak area in an external standard containing the same amount of analyte. For this, 500 mL groundwater, 200 mL WWTP effluent, 100 mL WWTP influent and 0.3 g activated sludge were spiked with codeine and its TPs to a concentration level of 0.4 µg L^{-1}, 1.0 µg L^{-1}, 2.0 µg L^{-1} and 1.3 µg g_{SS}^{-1}, respectively. The natural background concentration was considered by subtraction of the peak area determined in non-spiked samples. The relative recoveries describing the accuracy of the entire analytical procedure were calculated as the ratio of the spiked concentrations and the quantified concentrations

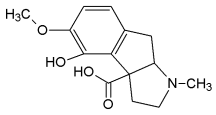
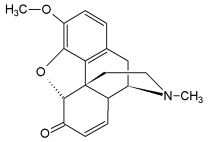
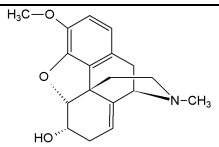
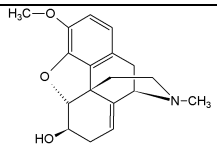
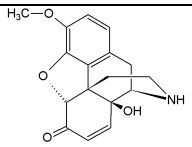
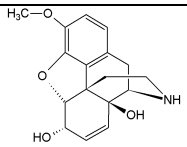
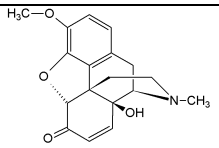
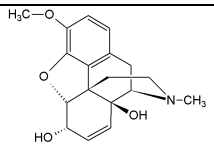
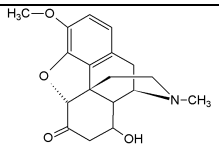
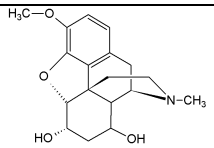
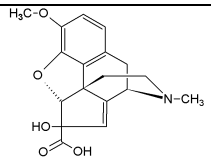
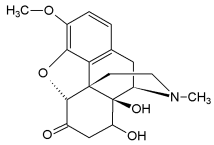
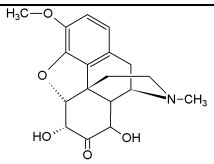
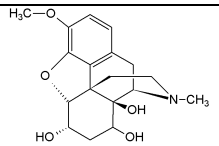
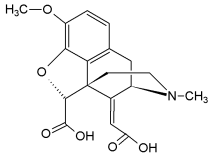
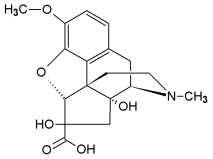
5.3 Results and Discussion

5.3.1 Isolation and identification of codeine TPs

Concentrations of codeine up to 200 mg L^{-1} and longer incubation periods of up to 35 d were used in order to isolate high quantities of TPs for identification and preparation of analytical reference standards. However, the results obtained from the batch experiments performed at elevated codeine concentrations were qualitatively similar to those performed at environmental relevant concentrations (see S27 in the Supplementary Information and section 5.3.2).

In the batch experiments spiked with 200 mg L^{-1}, codeine was found to be continuously transformed following zero-order kinetics. After 25 to 35 d codeine was completely dissipated. The dominant TPs were isolated and further purified for their identification and for the preparation of reference standards (Figure SI 5-2). Elucidation of the chemical structures of codeine TPs was mainly performed by Qq-LIT-MS and LTQ-Orbitrap-MS. For those TPs which could be isolated in sufficient quantities, 1D-and 2D-NMR experiments were performed. The identified codeine TPs are listed in Table 5-2 together with the techniques used for identification and confirmation.

Table 5-2. Overview of the elemental composition determined by HR-MS and the identified or proposed chemical structures of codeine TPs as well as the techniques used for identification and confirmation. For those TPs isolated by HPLC-UV coupled to a fraction collector, the isolated quantities and estimated purities are noted in brackets.

MW $[M+H]^+$ (error in ppm)	Proposed structure	Identification methods	MW $[M+H]^+$ (error in ppm)	Proposed structure	Identification methods
TP 264* 264.12312 (0.32) $C_{14}H_{18}NO_4$ [1.4 mg, 93%]		Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY, HSQC	**TP 298** 298.14376 (-0.1) $C_{18}H_{20}NO_3$	 Codeinone	Qq-LIT-MS, LTQ-Orbitrap-MS, authentic standard
TP 300(1) 300.15924 (-0.6) $C_{18}H_{22}NO_3$ [5.8 mg, 94%]	 Neopine	Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY, ^{13}C-NMR, HSQC,	**TP 300(2)** 300.15952 (0.3) $C_{18}H_{22}NO_3$ [0.7 mg, 90%]	 Isoneopine	Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY, HSQC,
TP 300(3)*[a] 300.12262 (-1.4) $C_{17}H_{18}NO_4$ [<0.4 mg]	 14-Hydroxy-N-desmethyl-codeinone [a]	Qq-LIT-MS, LTQ-Orbitrap-MS	**TP 302** 302.13883 (-0.5) $C_{17}H_{20}NO_4$ [1.1 mg, 96%]	 14-Hydroxy-N-desmethylcodeine	Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY, HSQC
TP 314 314.13841 (-0.9) $C_{18}H_{20}NO_4$	 14-Hydroxycodeinone	Qq-LIT-MS, LTQ-Orbitrap-MS, authentic standard ^{1}H-NMR, ^{1}H,^{1}H-COSY, HSQC	**TP 316(1)** 316.15399 (-1.1) $C_{18}H_{22}NO_4$	 14-Hydroxycodeine	Qq-LIT-MS, LTQ-Orbitrap-MS, authentic standard ^{1}H-NMR, ^{1}H,^{1}H-COSY, ^{13}C-NMR, HSQC
TP 316(2) 316.15420 (-0.4) $C_{18}H_{22}NO_4$	 8-Hydroxy-7,8-dihydrocodeinone	Qq-LIT-MS, LTQ-Orbitrap-MS	**TP 318** 318.16954 (-1.2) $C_{18}H_{24}NO_4$ [0.9 mg, 98%]	 8-Hydroxy-7,8-dihydrocodeine	Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY
TP 320 320.14849 (-2.4) $C_{17}H_{22}NO_5$ [2.0 mg, 90%]	no final structure carboxylic acid N-CH_3 and aromatic ring A preserved	Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY,	**TP 330*** 330.13351 (-0.1) $C_{18}H_{20}NO_5$		Qq-LIT-MS, LTQ-Orbitrap-MS
TP 332(1) 332.14929 (0.1) $C_{18}H_{22}NO_5$ [3.0 mg, 88%]	 8,14-Dihydroxy-7,8-dihydrocodeinone	Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY, ^{13}C-NMR, HSQC	**TP 332(2)*** 332.14924 (-0.1) $C_{18}H_{22}NO_5$	 8-Hydroxy-7-oxo-7,8-dihydrocodeine	Qq-LIT-MS, LTQ-Orbitrap-MS
TP 334* 334.16502 (0.4) $C_{18}H_{24}NO_5$ [<0.4 mg]	 8,14-Dihydroxy-7,8-dihydrocodeine	Qq-LIT-MS, LTQ-Orbitrap-MS	**TP 346*** 346.12810 (-1.2) $C_{18}H_{20}NO_6$ [1.7 mg, 90%]		Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY, HSQC
TP 348* 348.14407 (-0.3) $C_{18}H_{22}NO_6$ [1.3 mg, 90%]		Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY, HSQC	**TP 364** 364.13879 (-0.8) $C_{18}H_{22}NO_7$ [1.4 mg, 95%]	no final structure dicarboxylic acid N-CH_3 and aromatic ring A preserved probably α-oxo carboxylic acid moiety	Qq-LIT-MS, LTQ-Orbitrap-MS, ^{1}H-NMR, ^{1}H,^{1}H-COSY, HSQC

* Tentative structures that need further confirmation by ^{13}C-NMR.

[a] Formation of a N-hydroxylamine is also in accordance with the accurate mass and the fragmentation pattern.

Identification by Qq-LIT-MS and LTQ-Orbitrap-MS

Structural elucidation of the TPs was performed by examining the fragmentation pathways obtained from Qq-LIT-MS (MS^2, MS^3, precursor ion scan) as well as LTQ-Orbitrap-MS (up to MS^5). The determination of the accurate mass of the parent ions and product ions with the LTQ-Orbitrap-MS allowed to unambiguously identify the elemental composition of the TPs and their product ions. The interpretation of the mass spectral data and the prediction of fragmentation pathways were supported by literature data on the MS fragmentation of opium alkaloids (Penannen et al., 2001; Raith et al., 2003). The most important fragment losses and the assignment to specific structural features of codeine and its TPs, used for their identification, are listed in Table 5-3.

Table 5-3. Overview about characteristic losses of neutral fragments and their use for structural elucidation of codeine TPs.

Fragments	MW	Interpretation	TPs
C_3H_7N	57.0578	Cleavage of the unaltered ring E containing nitrogen	Codeine, TP 264, TP 298, TP300(1), TP 300(2), TP 316(1), TP 316(2), TP 318, TP 330, TP 332(2), TP 334, TP 348
C_2H_5N	43.0422	Cleavage of the N-demethylated ring E containing nitrogen	TP 302
C_4H_7N	69.0578	Cleavage of the unaltered ring E after rearrangement → Hydroxyl group at the C-14 position, double bond between C-7 and C-8	TP 314, TP 332(1)
C_3H_5N	55.0422	Cleavage of the N-demethylated ring E after rearrangement → Hydroxyl group at the C-14 position, double bond between C-7 and C-8	TP 300(3)
C_2H_4O	44.0262	Cleavage of acetaldehyde from ring C → Hydroxyl group in ring C, no substituent in α-position	Codeine, TP300(1), TP 300(2), TP 302, TP 316(1), TP 318, TP 332(1), TP 334, TP 348, TP 364
C_2H_2O	42.0106	Cleavage of ketene from ring C → Keto group in Ring C, no substituent in α-position	TP 298, TP 314, TP 316(2), TP 330, TP 332(1), TP 348
CH_3OH	32.0262	Cleavage of the methoxy group	Codeine, TP 264, TP 298, TP 300(1), TP 300(2), TP 300(3), TP 302, TP 314, TP 316(1), TP 316(2), TP 318, TP 320, TP 332(1)
H_2O	18.0106	Cleavage of a hydroxyl group with a removable hydrogen in α-position	Codeine, TP 300(1), TP 300(2), TP 300(3), TP 302, TP 314, TP 316(1), TP 316(2), TP 318, TP 320, TP 330, TP 332(1), TP 332(2), TP 334, TP 348, TP 364
H_2CO_2	46.0055	Cleavage of a carboxyl group with a removable hydrogen in α-position	TP 264, TP 330, TP 346, TP 348, TP 364
CO_2	43.9898	Cleavage of a carboxyl group without a removable hydrogen in α-position	TP 330, TP 346, TP 348,

To illustrate the general procedure applied for the elucidation of chemical structures of evolving TPs via Qq-LIT-MS and HR-MS, the identification of TP 302, TP 314 and

TP 316(1) is described. In contrast to codeine, a dehydration was observed as the initial reaction in the fragmentation pathways of these TPs (Figure 5-1). Based on the fragmentation pathway of the opioid oxycodon (Penannen et al., 2001), the abundant product ion formation by dehydration was attributed to the presence of a hydroxyl group at the C-14 position of TP 302, TP 314 and TP 316(1). The stability of these product ions can be explained by an extensive delocalization of the positive charge. For TP 302 and TP 316(1), the loss of water was succeeded by the cleavage of C_2H_4O (acetaldehyde) from ring C. This reaction was also observed for codeine, indicating that the positions C-6, C-7 and C-8 of TP 302 and TP 316(1) remained unaltered. In contrast, the loss of C_2H_2O (ketene) from TP 314 after its dehydration was attributed to the oxidation of the hydroxyl group at the C-6 position to a keto group. The molecular formula of TP 302 indicated that either a demethylation of the tertiary amine or the methoxy group of the benzene ring A occurred. However, the cleavage of C_2H_5N from TP 302 instead of C_3H_7N, as observed for codeine and 14-hydroxycodeine, confirmed the N-demethylation. Based on these findings, TP 302, TP 316(1) and TP 314 were identified as 14-hydroxy-N-desmethylcodeine, 14-hydroxycodeine, and 14-hydroxycodeinone, respectively.

Similar considerations lead to the tentative identification of the other TPs. The proposed fragmentation pathways and accurate masses of the product ions are shown in Figure SI 5-3 and Table SI 5-3, respectively.

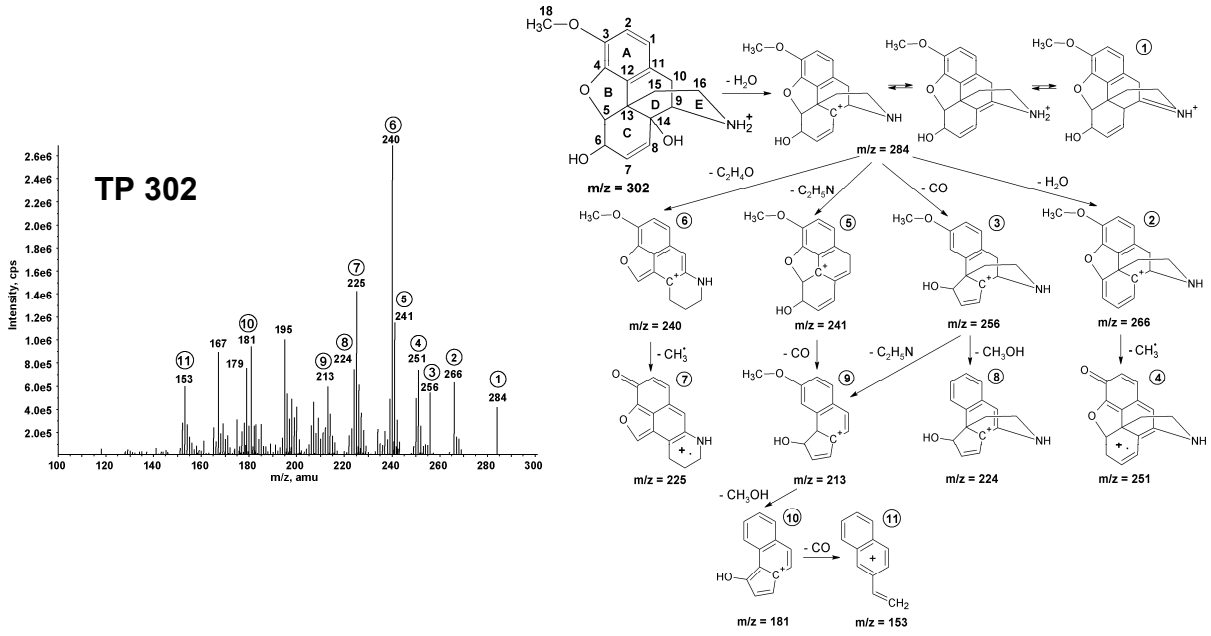


Figure 5-1. MS^2 spectra of TP 302 determined by Qq-LIT-MS (CE 40 eV). The corresponding fragmentation pathway was proposed based on MS^3 and precursor ion scans using Qq-LIT-MS as well as MS^n experiments using LTQ-Orbitrap MS. The accurate masses and elemental compositions of the precursor and product ions determined by LTQ-Orbitrap-MS are listed in Table SI 5-3.

Identification by NMR

NMR spectra (^{1}H-NMR, ^{13}C-NMR, ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-HSQC) were used for confirmation of the TP chemical structures proposed by mass spectrometry techniques (Figure SI 5-4). Identification was accomplished by the elucidation of characteristic chemical shifts (e.g. vinylic protons of the double bond in *ring C*) and cross peaks between adjacent protons (^{1}H,^{1}H-COSY) as well as between directly bonded protons and carbons (^{1}H,^{13}C-HSQC). Structural confirmation by NMR and authentic standards resulted in the unambiguous identification of 8 TPs (TP 298, TP 300(1), TP 300(2), TP 316(1), TP 314, TP 302, TP 318, TP 332(1)) (Table 5-2). TP 300(1) and TP 300(2) were identified as the stereoisomers neopine ((S)-OH-isomer) and isoneopine ((R)-OH-isomer), featuring a double bond shift from the 7,8 position to the 8,14 position. This was further confirmed by comparing the chromatographic retention times and fragmentation patterns of both substances with those of neopine and isoneopine synthesized via chemical reduction of neopinone (Gollwitzer et al., 1993). Details are provided in the Supplementary Information (S46).

However, NMR analysis could not be accomplished for TP 316(2), 300(3), TP 330, TP 332(2) and TP 334 because insufficient quantities were isolated (Table 5-2). For TP 264, TP 348 and TP 346, the information obtained from NMR experiments was not sufficient to finally elucidate their chemical structures. ^{13}C-NMR would be required for their unambiguous identification, therefore only tentative structures are given (Table 5-2).

5.3.2 Transformation under environmental conditions

Activated sludge batch systems

Codeine was incubated for 70 h in diluted and undiluted aerated activated sludge at a concentration of 2 µg L^{-1} to examine the TP formation under environmental relevant conditions. Application of the validated analytical method 1, based on SPE with Oasis MCX cartridges and LC tandem MS analysis, allowed for the quantification of codeine and seven TPs (codeinone, neopine, isoneopine, TP 316(1), TP 314, TP 332(1), TP 302). In addition, samples were also monitored for TPs for which analytical reference standards were not available. To determine the occurrence of TPs not applicable to the validated SPE method 1 (i.e. TP 264, TP 320, TP 346, TP 348 and TP 364), samples were extracted in parallel with an alternative SPE method 2.

More than 90% of the initial concentration of codeine was removed in the diluted sludge after 48 h (Figure 5-2a). Parallel to the transformation of codeine, codeinone, neopine, isoneopine, TP 300(3), TP 302, TP 314, TP 316(1) and TP 332(1) were formed. Except for

TP 300(3), these TPs could be quantified with the analytical method developed. Based on the mass balance determined, these TPs contributed to about 25% of the initial codeine concentration. Codeinone was the main TP at the beginning of the incubation period accounting for 24% of the transformed codeine after 11 h. The subsequent decrease of codeinone was accompanied by an increase of the other TPs accounting for 18 % of the initial codeine concentration after 48 h.

The same TPs, similar relative TP concentrations and a comparable mass balance were also observed during the incubation of codeinone (Figure 5-2b). This indicated that codeinone is a precursor of the other TPs identified. Similar results were also obtained with undiluted activated sludge (Figure SI 5-5).

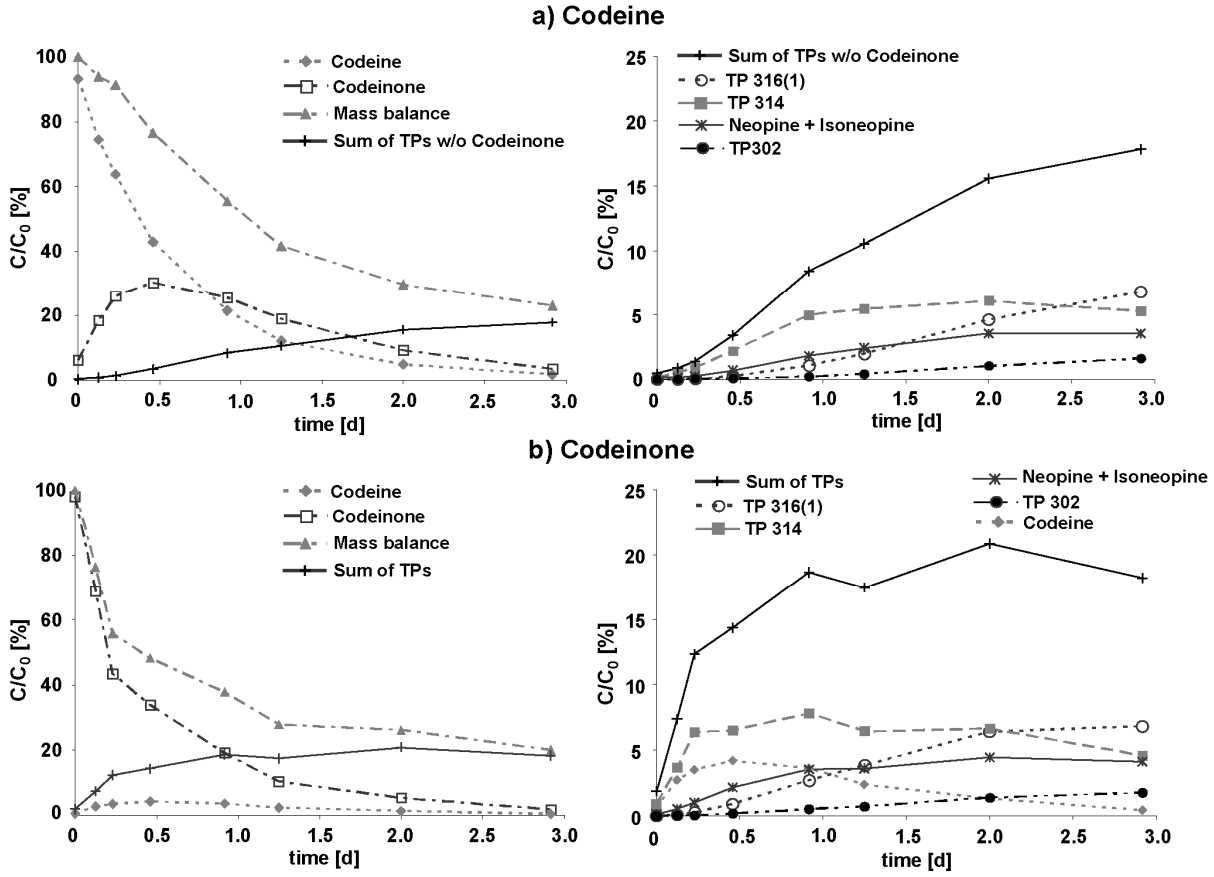


Figure 5-2. Mass balance [%] in incubation experiments of a) codeine and b) codeinone using diluted activated sludge (1:20 with groundwater, 0.18 g_{SS} L^{-1}). Codeine and codeinone were spiked separately at a concentration of 2 µg L^{-1}. The mass balance includes all TPs for which analytical standards were available and which were applicable to SPE method 1 (codeine, codeinone, neopine, isoneopine, TP 316(1), TP 314, TP 302 and TP 332(1)). Calculations were done on a molar basis.

To further examine the role of codeinone with regard to the incomplete mass balance and to distinguish between biotic and abiotic transformation processes, additional experiments with codeinone (5 mg L^{-1}) were performed, using i) *diluted sludge*, ii) *autoclaved diluted*

sludge, iii) *autoclaved groundwater* and iv) *autoclaved ultrapure wate*r. The relatively high initial concentration allowed for the determination of dissolved organic carbon (DOC) and for the screening of yet unknown TPs via full scan MS measurements.

For the *diluted sludge*, the same TPs as those observed for the low codeinone concentration (2 µg L^{-1}) were detected and their quantification led to a similar mass balance of approximately 25% after 7 d (Figure 5-3a). Since the DOC remained constant throughout the experiments, a significant mineralization, formation of bound residues or incorporation in the biomass can be ruled out as reasons for the incomplete mass balance (Figure SI 5-6b). Therefore, the gap in the mass balance is most likely caused by the transformation of codeinone into unknown TPs, which were not applicable to the analytical procedure applied

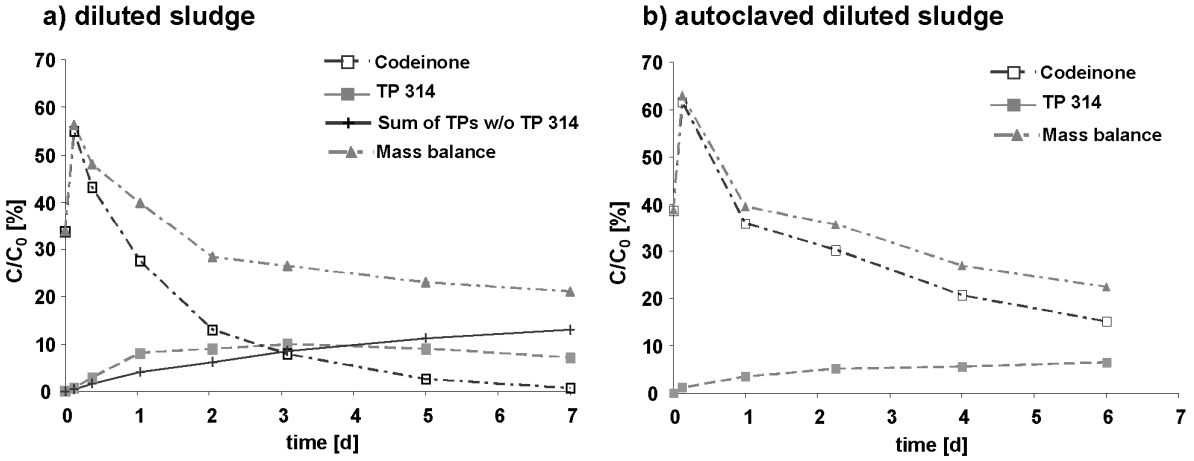


Figure 5-3. Mass balance [%] in incubation experiments of codeinone using a) sterile (autoclaved) and b) non-sterile diluted activated sludge (1:20 with groundwater, 0.20 g_{SS} L^{-1}). Codeinone was spiked at a concentration of 5 mg L^{-1}. The mass balance includes all TPs for which analytical standards were available and which were applicable to the developed SPE method 1 (codeine, codeinone, neopine, isoneopine, TP 316(1), TP 314, TP 302 and TP 332(1)). The low mass balance at the beginning is attributed to the initially incomplete dissolution of spiked codeinone. Calculations were done on a molar basis.

With the exception of TP 314, none of the TPs observed in non-sterile sludge was present in the *autoclaved diluted sludge* (Figure 5-3b). Since the quantities of TP 314 and fraction of unknown TPs, probably responsible for the incomplete mass balance, were comparable to results from non-sterile sludge, their formation was attributed to abiotic reactions. This was supported by the fact that in the autoclaved ultrapure water and groundwater about 50% of the initial spiked quantity of codeinone was transformed within 6 d (Figure SI 5-6a). Full scan MS measurements indicated that the incomplete mass balance in autoclaved groundwater and ultrapure water is mainly attributable to the formation of TP 316(2) (results not shown). TP 316(2) was identified as 8-hydroxy-7,8-dihydrocodeinone. Its formation can be explained

by a nucleophilic attack of water at the β-position of the α,β-unsaturated keto moiety of codeinone.

Even though TP 316(2) was also present in the diluted and autoclaved diluted sludge, it could not account for the fraction of unknown TPs in these experiments. However, the reaction mechanism described above indicates that codeinone is also highly reactive towards other nucleophilic functional groups present in activated sludge such as thiols and amines. Hence, it was hypothesized that the attack of nucleophiles on codeinone led to the formation of non-detected TPs in contact with sterile and non-sterile activated sludge. This hypothesis is also supported by results from Nagamatsu et al. (1986) and Walker et al. (2000), who reported the covalent binding of codeinone to cysteine in glutathione and proteins, respectively.

In summary, the incomplete mass balance of codeine is most likely caused by the abiotic transformation of its biological TP codeinone into unknown TPs, which were not amenable to the analytical procedure applied. Additional experiments, in particular with ^{14}C-labeled codeine, are necessary to confirm this hypothesis, and to elucidate the identity of the so far unknown TPs.

Transformation pathway of codeine

To elucidate the individual steps in the transformation pathway of codeine, incubation batch experiments were performed with neopine, TP 314, TP 316(1) and TP 332(1). Individual TPs were independently spiked at 200 µg L^{-1} in diluted activated sludge as well as autoclaved groundwater. The results are summarized in Figure 5-4.

As mentioned before, the incubation of codeinone revealed the formation of neopine and isoneopine. Codeinone has been shown to be in equilibrium with neopinone and involves the rearrangement of the double bond from the 7,8 to the 8,14 position (Gollwitzer et al., 1993). It can be assumed that the transformation of codeinone to the isomers neopine and isoneopine, which was only observed for non-sterile activated sludge, proceeds via a biologically mediated non-stereoselective reduction of the abiotic intermediate neopinone. In contrast, the biological reduction of codeinone was stereoselective leading only to the (S)-6-OH-isomer codeine.

Batch experiments spiked with neopine revealed that this TP is rather persistent. Although neopine differs from codeine only by the position of the double bond in ring C, the oxidation of the 6-OH group back to neopinone/codeinone was not observed.

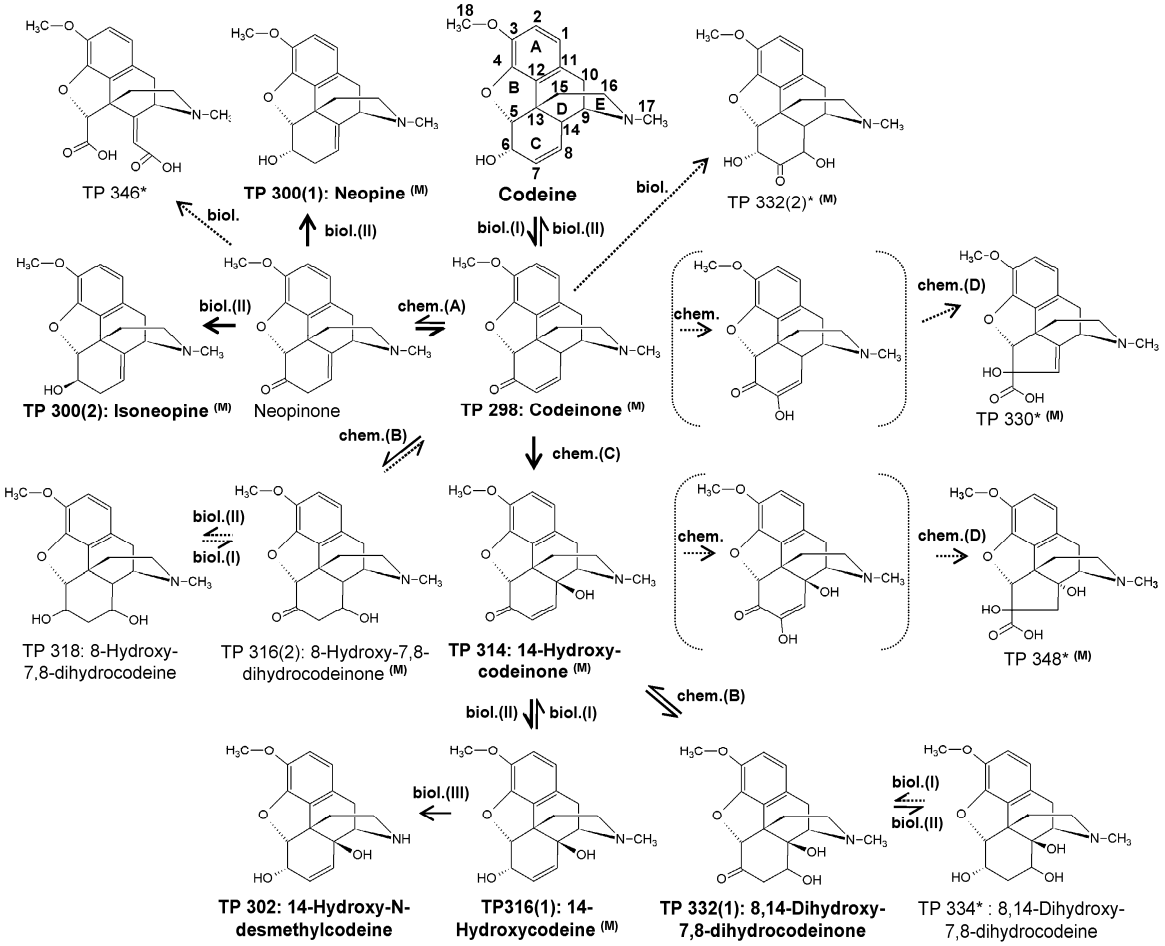


Figure 5-4. Proposed transformation pathway of codeine determined from laboratory batch experiments with codeine and codeine TPs in diluted activated sludge as well as autoclaved controls under aerobic conditions. Major TPs are printed in bold. The dashed arrows indicate predicted reactions which could not be directly confirmed in batch experiments. Tentative chemical structures of TPs are marked with an asterix. The index (M) indicates that corresponding TPs were also detected for Morphine (O-CH_3 at C-3 replaced by OH). Proposed intermediates in the formation of TPs are shown in parenthesis. The roman numerals I-III and the letters A-D indicate biotic and abiotic reactions, respectively: I) oxidation, II) reduction, III) N-demethylation; A) double bond shift, B) nucleophilic addition, C) hydroxylation, D) benzylic acid rearrangement.

TP 314 (14-hydroxycodeinone) was detected with increasing quantities in sterile ultrapure water, sterile groundwater and sterile diluted sludge spiked with codeinone. This indicates that TP 314 is formed by the abiotic oxidation of codeinone, probably catalyzed by matrix components. However, the contribution of enzymatic reactions to the formation of TP 314 in non-sterile sludge cannot be fully excluded. Due to its α,β-unsaturated keto group, TP 314 is susceptible to the attack of nucleophiles such as water at the C-8 position leading to the formation of TP 332(1) (8,14-dihydroxy-7,8-dihydrocodeinone). Furthermore, TP 314 is biologically reduced to TP 316(1). While in autoclaved groundwater TP 316(1) was not detected, almost 90% of the initial concentration of TP 314 was transformed into TP 316(1) within 7 d

in the diluted sludge. Interestingly, only the (S)-6-OH-isomer of TP 316(1) (available as reference standard) was detected, indicating that this reaction is stereoselective.

Finally, the removal of TP 316(1) (14-hydroxycodeine) could be attributed to the microbial N-demethylation, yielding TP 302 (14-hydroxy-N-desmethylcodeine), and oxidation of the 6-OH group yielding TP 314. In accordance to the formation of TP 316(1) from TP 314, TP 332(1) was found to be biologically reduced to TP 334.

In summary, the transformation of codeine in activated sludge under aerobic conditions and at a neutral pH can be mainly attributed to various interactions of three biologically mediated reactions, i.e. I) oxidation, II) reduction at the C-6 position, and III) N-demethylation, and three abiotic reactions, i.e. A) double bond shift from the 7,8 to the 8,14-position, B) addition of nucleophiles at the C-8 position, and C) hydroxylation at the C-14 position.

Further reactions, leading to several minor TPs, were identified in incubation experiments spiked with codeine and codeinone. In particular, the formation of TP 264, TP 300(3), TP 330 and TP 348 was observed with autoclaved sludge (Figure SI 5-7) and autoclaved groundwater and can therefore be attributed to abiotic reactions. In contrast, TP 332(2) and TP 346 were only detected in non-sterile sludge indicating their formation by biotic reactions.

Assuming a α-diketone precursor formed by water addition at the α-position followed by autoxidation, the abiotic formation of TP 330 and TP 348 might be explained by a benzylic acid rearrangement (Nace and Inaba, 1962). A plausible explanation for the formation of TP 332(2) is the biologically mediated epoxidation followed by hydrolysis or dioxygenation of the 7,8 double bond of codeinone (Neilson and Allard, 2008; Schwarzenbach et al., 2003). Transformation of codeinone to TP 346 might be the result of an oxygen-insertion between the C-6 and C-7 position of neopinone catalyzed by a monooxygenase followed by hydrolysis of the lactone intermediate (Neilson and Allard, 2008; Schwarzenbach et al., 2003).

Comparison with mammalian metabolism of codeine

In mammals, codeine is mainly metabolized to the 6-O-glucuronide conjugate and to a minor extent to morphine and norcodeine (up to 10% each) by O-demethylation of the benzylic methoxy group and N-demethylation of the amine moiety, respectively (Armstrong and Cozza, 2003; Gutstein and Akil, 2006; Vree and Verwey-Van Wissen, 1992). Morphine and norcodeine are partly transformed to normorphine by N-demethylation and O-demethylation, respectively. All these metabolites can be conjugated to their corresponding 6-O-glucuronides and in case of morphine and normorphine also to 3-O-glucuronides (Armstrong and Cozza,

2003; Gutstein and Akil, 2006; Vree and Verwey-Van Wissen, 1992). Moreover, there is evidence that codeinone, neopine, hydrocodone, dihydrocodeine and isodihydrocodeine can be formed to a limited extent in mammalian metabolism (Al-Amri et al., 2005; Cone et al., 1983; Nagamatsu et al., 1985).

Except for codeinone and neopine, which were major TPs in the batch experiments with activated sludge, none of the mammalian metabolites have been detected as TPs in the present study. Consequently, these results indicate that certain reactions such as O-demethylation, glucuronidation, and reduction of the 7,8 double bond, are mainly restricted to the mammalian metabolism of codeine, whereas the oxidation of the 6-OH group and the consecutive chemically and biologically mediated reactions shown in Figure 5-4 are crucial for the microbial transformation pathway observed in contact with activated sludge.
Furthermore, the comparison reveals that N-demethylation of the tertiary amine of codeine is part of both the mammalian metabolism and microbial transformation pathway in activated sludge. However, the formation of norcodeine by N-demethylation was not observed in the batch reactors.

Comparison with transformation pathways observed in cultures of single bacteria strains

Codeinone, 14-hydroxycodeinone and 14-hydroxycodeine have also been detected during the incubation of codeine with enriched cultures of single bacterial strains (Lister et al., 1999). In accordance to the results of the current study, the formation of 14-hydroxycodeine was assumed to proceed via the formation of 14-hydroxycodeinone rather than by direct hydroxylation of codeine. Although the hydroxylation at the C-14 position was attributed to an enzymatic reaction, Zhang et al. (2005) found a chemical mechanism of a catalytic autoxidation of codeinone to 14-hydroxycodeinone, which is consistent with the results of the present study.

In contrast, the microbial formation of neopine and isoneopine, which is an important pathway in the transformation of codeine in contact with activated sludge, has not been reported in studies with single bacterial strains. The same holds true for 14-hydroxy-N-desmethylcodeine. Furthermore, the mixed microbial community in the batch experiments with activated sludge was incapable of reducing codeinone to hydrocodone, a reaction which was observed for the bacterial strain *Pseudomonas putida* (Lister et al., 1999).

5.3.3 Occurrence of codeine TPs in municipal wastewater treatment plants

The analysis of raw and treated wastewater from four municipal WWTPs revealed that codeine is removed by 35 to 75%. The predominant TPs in batch experiments (codeinone, 14-hydroxycodeinone, 14-hydroxycodeine, neopine and isoneopine) were detected in almost all WWTP effluents (Table 5-4).

Table 5-4. Occurrence of codeine and its TPs in treated and raw wastewater and activated sludge, method validation results (LOQ and relative recoveries; n = 3). Parent compound removal [%] and TP formation [%] were calculated on a molar basis. The statistical errors are given as 95% confidence intervals (n = 3).

			Codeine	Codeinone	14-Hydroxy-codeinone	14-Hydroxy-codeine	Neopine	Isoneopine	∑ TPs
WWTP 1 [a]	influent	[ng L^{-1}]	634 ± 52	17 ± 2	<LOQ	<LOQ	<LOQ	<LOQ	17
	effluent	[ng L^{-1}]	413 ± 71	22 ± 3	2.5 ± 0.9	4.3 ± 1.0	18 ± 6	19 ± 5	66
	removal[e]/ formation[f]	[%]	35	2.2	1.1	1.9	7.9	8.6	22
	sludge	[ng g_{SS}^{-1}]	57	37	4.5	<LOQ	<LOQ	4.0	46
	filtrate	[ng L^{-1}]	608	19	<LOQ	4.9	22	22	68
	$K_{d,sludge}$	[L kg^{-1}]	90	1950	>1800	<400	<90	180	
WWTP 2 [b]	influent	[ng L^{-1}]	273 ± 30	11 ± 2	<LOQ	<LOQ	<LOQ	<LOQ	11
	effluent	[ng L^{-1}]	115 ± 9	27 ± 2	3.5 ± 0.2	<LOQ	4.7 ± 0.5	5.0 ± 0.1	40
	removal[e]/ formation[f]	[%]	58	10	2.2	<LOQ	3.0	3.2	18
WWTP 3 [c]	influent	[ng L^{-1}]	469	10	<LOQ	<LOQ	7.9	<LOQ	18
	effluent	[ng L^{-1}]	119	32	8.4	7.8	10	21	79
	removal[e]/ formation[f]	[%]	75	6.3	2.4	2.2	0.6	5.9	17
WWTP 4 [d]	influent	[ng L^{-1}]	440	7.3	<LOQ	<LOQ	<LOQ	<LOQ	7
	effluent	[ng L^{-1}]	232	49	3.2	3.4	9.2	24	89
	removal[e]/ formation[f]	[%]	47	20	1.5	1.6	4.4	11	39
LOQ	influent	[ng L^{-1}]	5	5	5	5	5	5	
	effluent	[ng L^{-1}]	2.5	2.5	2.5	2.5	2.5	2.5	
	sludge	[ng g_{SS}^{-1}]	2.0	2.0	2.0	2.0	2.0	2.0	
	filtrate	[ng L^{-1}]	2.5	2.5	2.5	2.5	2.5	2.5	
Relative recovery	influent	[%]	99 ± 11	95 ± 13	109 ± 23	107 ± 16	92 ± 15	ND	
	effluent	[%]	91 ± 9	98 ± 11	100 ± 2	94 ± 11	89 ± 12	ND	
	sludge[g]	[%]	96 ± 14	55 ± 16	89 ± 21	67 ± 17	73 ± 13	ND	

[a] influent and effluent: 24 h composite sample (n = 3), sludge samples: grab samples (n =1); [b] 48 h composite sample (n = 4); [c] 5 d composite sample from daily grab samples (n = 1); [d] grab samples (n = 2).
[e] Codeine removal = (codeine influent conc. – codeine effluent conc.) / codeine influent conc.
[f] TP formation = (TP effluent conc. – TP influent conc.) / (codeine influent conc. – codeine effluent conc.)

Effluent concentrations of the sum of all TPs were observed to be up to 90 ng L^{-1}. Codeinone and the sum of both neopine isomers were the dominant TPs with maximum effluent concentrations of approximately 50 and 40 ng L^{-1} accounting for 20 and 17% of the transformed codeine influent concentration, respectively. Codeinone was already present in the influent samples indicating either transformation of codeine to codeinone in the sewers and during primary clarification or in human metabolism.

The mass balances in the four investigated WWTPs (17-39%) were in good agreement with the results from batch experiments (20-40%). Since the same main TPs were observed, the results from the batch experiments can be at least qualitatively transferred to full-scale WWTPs. Further research is needed to assess the occurrence and cleavage of the codeine-6-glucuronide in WWTPs, which might lead to a bias in the codeine mass balance. However, the successful prediction of the full-scale removal of codeine by a modeling approach using pseudo-first-order transformation coefficients derived from similar batch experiments indicated no significant influence of the cleavage of codeine-6-glucuronide on the mass balance of codeine in WWTPs (Wick et al., 2009).

Based on the measured soluble and sorbed concentrations of codeinone in activated sludge of WWTP 1, an apparent sludge-water partition coefficient $K_{d,sludge}$ of approximately 2000 L kg^{-1} was estimated. Although this K_d value has to be considered as a rough estimate due to the non-guaranteed sorption equilibrium, it reveals appreciable partitioning of codeinone to the solid phase of activated sludge in full-scale WWTPs.

Although the opium alkaloid morphine is known to have the potential to adversely affect the immune system of invertebrates (Gagné et al., 2006), the ecotoxicological potential of codeine and its TPs remains unknown. However, compounds with α,β-unsaturated ketone moieties such as codeinone were found to be highly reactive towards cellular nucleophiles resulting in DNA damage, formation of DNA and protein adducts, mutagenicity and enzyme inhibition (Janzowski et al., 2003; Nagamatsu et al., 1985; Yarbrough and Schultz, 2007).

5.3.4 Transformation of structurally related opium alkaloids

The transferability of the results for codeine to the structurally related opium alkaloids, morphine and dihydrocodeine, was elucidated in batch experiments with sterile (autoclaved) and non-sterile diluted activated sludge. The screening for evolving TPs of morphine and dihydrocodeine was performed by LC-LTQ-Orbitrap-MS using the accurate masses and comparison of MS^2 spectra. The results together with the corresponding chemical structures are summarized in Table SI 5-5 and SI 5-6.

For morphine, 10 TPs similar to those observed for codeine (only differing in the OH group at the C-3 position) were detected, including the main TPs morphinone and 14-hydroxymorphinone. Thus, transformation reactions comparable to those observed for codeine can also be assumed for morphine (Figure 5-5).

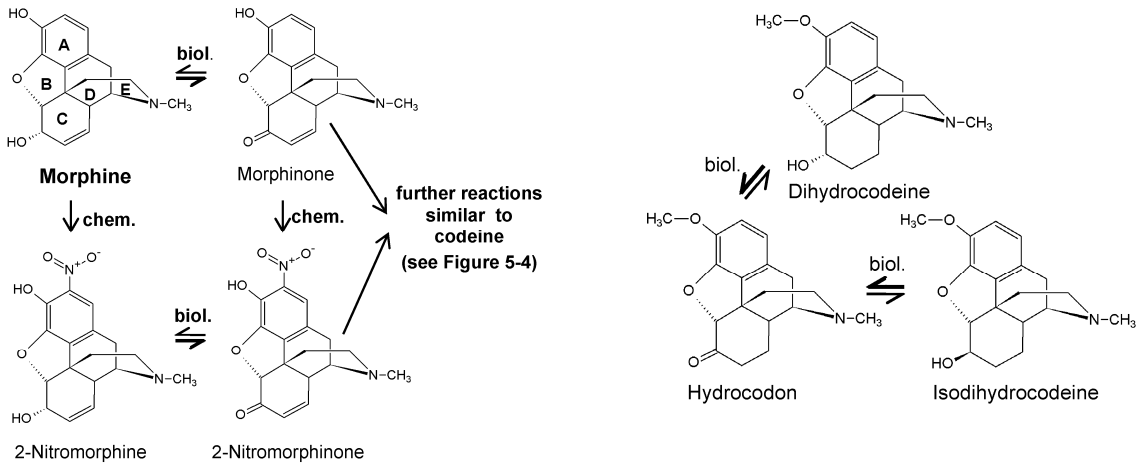


Figure 5-5. Transformation pathway of morphine and dihydrocodeine as determined in batch experiments incubating 0.5 mg L^{-1} of the target compounds in diluted activated sludge (0.04 g_{TSS} L^{-1}) under aerobic conditions.

In addition to the codeine-like TPs, an additional seven TPs were tentatively identified for morphine including the morphine dimer pseudomorphine and 2-nitro-derivatives. Pseudomorphine and 2-nitromorphine were also detected in the autoclaved controls. Similar to the formation mechanism of pseudomorphine proposed in literature (Yeh and Lach, 1961), nitration might proceed via the formation of a radical quinone in the presence of oxygen. The enhanced chemical reactivity of morphine owing to the phenolic moiety is a plausible explanation for the significantly higher removal efficiencies usually observed in WWTPs (Hummel et al., 2006; Wick et al., 2009). The nitration of phenolic micropollutants in contact with activated sludge has been also shown in other studies (Chiron et al., 2010; Gaulke et al., 2008; Patnaik and Khoury, 2004) and the formation mechanisms and occurrence in real wastewater needs further attention due to the potential toxicity of nitro-substituted phenols (Chiron et al., 2010).

In contrast to morphine, the transformation of dihydrocodeine was primarily restricted to the biological oxidation of the 6-OH group to hydrocodone (Figure 5-5), which is also used as a strong analgesic drug. To a minor extent, hydrocodone was transformed back to dihydrocodeine and isodihydrocodeine by non-stereoselective enzymatic reduction. Further abiotic and biotic transformations of hydrocodone, as observed for codeine and morphine, were not found due to the missing double bond in the α,β-position. Consequently, a complete mass balance (90-115%) was obtained in batch experiments with dihydrocodeine (Figure 5-6).

These results highlight that even small differences in the chemical structure might have significant impacts on the transformation of micropollutants. For a comprehensive eluci-

dation of the transformation pathways of micropollutants in the environment, it is crucial to consider both abiotic and biotic transformations of the parent compound and its TPs.

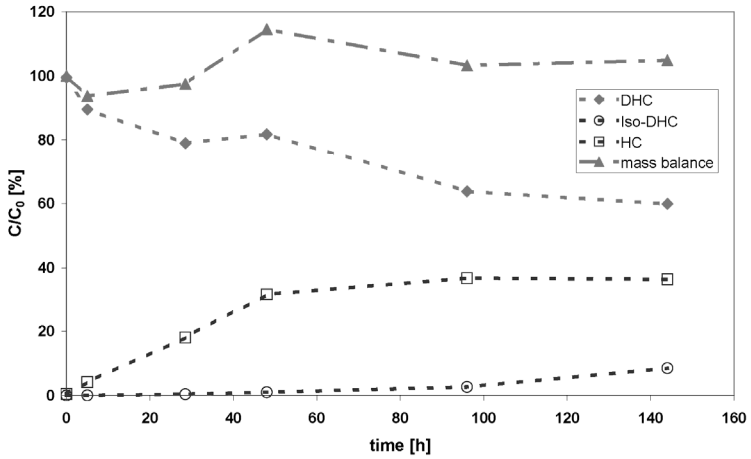


Figure 5-6. Mass balance [%] of dihydrocodeine spiked at a concentration of 500 µg L^{-1} to activated sludge diluted 1:20 with groundwater (0.2 g_{TSS} L^{-1}). After sample dilution (1:2.5) dihydrocodeine (DHC), isodihydrocodeine (Iso-DHC) and hydrocodon (HC) were measured with LC tandem MS in the SIM mode and quantified based on an external calibration with DHC and HC. Codeine-d_6 was used as surrogate.

5.4 Acknowledgements

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6. Final Conclusions

Due to the high number of chemicals, which are estimated to be continuously released into the aquatic environment, multi-residue analytical methods are required for determining the occurrence and fate of emerging organic micropollutants. The **multi-residue analytical method** developed and validated within this thesis allows for the rapid, reliable and sensitive detection and quantification of a broad spectrum of emerging micropollutants including various biocides, polar UV-filters and benzothiazoles in complex matrices (e.g. wastewater and activated sludge). The method validation revealed that matrix effects are a key factor effecting the sensitivity and reproducibility of multi-residue methods based on LC tandem MS analysis. This holds especially true for those emerging organic micropollutants for which no stable isotope-labeled surrogate standards were available. Using electrospray-ionization (ESI), ion suppression reduced the absolute recoveries by up to 90%. Even though atmospheric pressure chemical ionization (APCI) has recently been discussed as an alternative interface less susceptible to ion suppression, the comparison of both interfaces revealed a better performance of ESI with regard to sensitivity, precision and the susceptibility of APCI to ion enhancement.

The application of the analytical method for monitoring the **occurrence** of target compounds in different environmental matrices revealed high wastewater and surface water concentrations (up to the low $\mu g\,L^{-1}$ range) of several ingredients of personal care products (PCPs), such as the UV-filters BZP-4 and PBSA or the anti-dandruff climbazole. Since BZP-4 and PBSA are persistent and very polar negatively charged compounds, they are prone to contaminate drinking water. Even though the ecotoxicological potential of climbazole is currently unknown, its occurrence in environmental samples, reported for the first time within this study, raises concerns due to its biocidal activity. These results might result in the increased awareness of PCPs as environmental contaminants emitted by wastewater treatment plants (WWTPs) beyond the few well known contaminants such as the bactericides triclosan and triclocarban.

Moreover, the study exhibited that WWTP effluents are important point sources for the emission of the biocide irgarol, which is known to be toxic for macrophytes down to the low ng L^{-1} range. In contrast to previous monitoring studies of waterways and harbors, which assumed that the main emission of irgarol is the leaching from ship paints, the results of this work confirmed that the proposed environmental quality standard (EQS) of 2 ng L^{-1} will be exceeded in small streams with a high proportion of wastewater (see draft of the proposed

contaminants for the revision of the list of priority substances in the field of water policy - Annex X of Water Framework Directive, Directive 2000/60/EU).

The developed analytical method allowed for studying the **partition** of biocides and UV-filters as well as triazine and phenylurea herbicides to activated sludge, which is known to be one of the main removal processes determining the fate of organic micropollutants in WWTPs. Sodium azide (NaN_3), commonly used as a biocide in sorption experiments to inhibit the microbial transformation and thereby ensuring sorption equilibrium, was found to incompletely inhibit the microbial transformation of the target compounds and to significantly influences their sludge-water distribution. Hence, the use of NaN_3 in sorption experiments should be omitted if either an insufficient inhibition of the microbial activity or an impact of NaN_3 on the sorption behavior cannot be excluded. Moreover, in contrast to the OECD guideline 106, it is recommended to determine both the sorbed and the soluble concentration to exclude the influence of (bio)transformation on the sorption equilibrium.

For almost 30 contaminants studied, sludge-water distribution coefficients and their concentration dependency was determined for the first time. Both are major prerequisites for modeling their fate in WWTPs. In general, sorption onto activated sludge has been shown to be well described by the Freundlich model. However, since for most compounds sorption was independent from the actual concentration, the linear model is proposed to be an appropriate alternative for a faster screening of distribution coefficients. However, it has to be considered that the concentrations should be as close to environmental relevant concentrations as possible. Based on the obtained results, sorption onto activated sludge in full-scale WWTPs is expected to be only of minor importance (removal efficiency <10%) for the acidic (negatively charged under environmental pH-conditions) and nonionic target compounds with $\log D_{OW} < 3$. Moreover, the study indicated that nonionic compounds, for which currently no measured sludge-water distribution coefficients are available, can be classified regarding their WWTP removal by sorption based on a simple linear free energy relationship (LFER) approach using the $\log D_{OW}$ as a single descriptor. However, further research is needed to understand the sorption behavior of positively charged compounds, since the results confirmed that these compounds tend to have considerable higher sorption affinities due to ionic (Coulomb) interactions.

The **full-scale removal** of organic emerging micropollutants in a state-of-the-art WWTP was investigated by long-term measurement campaigns along different biological treatment units. Beta blockers and psycho-active drugs were selected as target compounds due to their ubiquitous presence in WWTPs and their expected ecotoxicological relevance. The measurements confirmed that a first highly loaded biological treatment unit, designed for chemical oxygen demand (COD) removal and characterized by a low sludge retention time (SRT) of 0.5 d, was incapable to significantly remove the selected target compounds. In contrast, a partial removal of several compounds, such as the natural opium alkaloids codeine and morphine, was observed in the second biological treatment unit with an elevated SRT of 18 d designed for nutrient removal. Accompanying lab-scale sorption and biotransformation experiments performed with activated sludge taken from this treatment unit revealed that the full-scale removal of beta blockers and psycho-active drugs was mainly due to primary biotransformation rather than sorption. Thereby, the study further confirmed that biological removal of organic micropollutants such as beta blockers and psycho-active drugs requires a SRT sufficient for nitrification (approximately >10 d).

The contaminant removal in the biotransformation batch experiments could be well described by pseudo-first order kinetics. By using the corresponding biotransformation kinetic constants (k_{biol}) for modeling the full-scale removal efficiencies in the second activated sludge unit, a good agreement with the measured removal efficiencies was obtained. Hence, this is one of the first studies which confirms that the specific biotransformation constants from batch experiments allows for a classification of the full-scale removal by biological transformation as proposed by Joss et al. (2006b).

However, for certain contaminants significant deviations between predicted and measured values were observed. Moreover, variations in the measured removal efficiencies between different measuring campaigns could not be completely explained by the modeling approach even though similar k_{biol} values were determined in batch experiments with sludge taken at different seasons. Hence, the dependency of the biotransformation constants on certain process parameters such as the SRT, redox conditions and temperature has to be further addressed in coming-up studies. It is deemed that a better understanding of the biological transformation processes behind the biological removal in WWTPs may contribute to a more reliable prediction and potentially leads to options for improving removal efficiencies.

These **transformation** processes were studied in detail for the structurally closely related opium alkaloids codeine, morphine, dihydrocodeine and hydrocodon using aerobic batch experiments with activated sludge. The unambiguous identification of the transformation products (TPs) revealed to be a particular challenge for compounds with multi-ring structures requiring at least two independent identification techniques such as high-resolution mass spectrometry (HR-MS) and nuclear magnetic resonance (NMR). It was elucidated for the first time that the opium alkaloids are neither mineralized nor incorporated into the biomass, but rather transformed into a multitude of TPs (in total 36 TPs were detected). The detection of some of the only slightly structurally modified TPs of codeine in WWTP effluents confirmed that the results are transferable to full-scale plants and highlights the conclusion that WWTPs are point sources of a multitude of TPs with unknown toxicity. It was demonstrated that even slight differences in the chemical structure of a contaminant might have significant impacts on the biotransformation rates and transformation pathways. For example, the position of a double bond (β- or γ-position), as seen for codeine and morphine, can determine whether a hydroxyl group is biologically oxidized or not. This highlights the specificity of certain enzyme catalyzed reactions during activated sludge treatment and the challenge of the prediction of transformation kinetics and pathways.

Moreover, another crucial outcome of this thesis is that the complexity of transformation pathways is further increased by chemical transformation reactions as shown for codeine and morphine. For these compounds, the high number of TPs and different degradability can only be explained by a combination of biotic enzyme catalyzed and abiotic reactions. Compounds with α,β-unsaturated keto moieties such as the primary biological TPs of morphine (morphinone) and codeine (codeinone) are chemically highly reactive leading to a further abiotic transformation. Since these compounds have potential to react with DNA and proteins, they are also of toxicological concern. Additionally, morphine was shown to be susceptible to abiotic nitration, which was previously also observed for the pharmaceutical acetaminophene (Chiron et al., 2010). Abiotic nitration might be a quite common transformation process of organic micropollutants during nitrification and deserves to be further examined due to the assumed toxicity of nitrated phenols (Patnaik and Khoury, 2004).

Hence, this study is one of the first approaches to improve the understanding of transformation processes in conventional biological wastewater treatment, which still has to be considered as a black box for the removal/transformation of most organic micropollutants. A stronger focus of coming-up studies on the dependency of biological and chemical transfor-

mation processes on specific structural features of the parent compounds may contribute to a more reliable prediction of transformation pathways facilitating faster screening approaches for TPs and their prioritization for risk assessment.

As an **overall conclusion**, the current WWTPs, even with nutrient removal units, are inappropriate to remove emerging organic contaminants. They are rather leading to the release of a multitude of TPs with unknown toxicity. Therefore, new mitigation strategies are crucial to:

1) meet the upcoming Environmental Quality Standards (EQS) values of the WFD.

2) minimize the potential impact of unregulated emerging organic micropollutants and their mostly unknown TPs on the aquatic ecosystem and the drinking water quality.

Options hereto may be the upgrade of conventional WWTPs with advanced physico-chemical treatment as outlined in Chapter 1 (1.5.4). However, the implementation of these techniques are connected with additional costs and energy consumption and in case of chemical oxidation bear the risk of the formation of toxic oxidation by-products. On a longer term, the elucidation of biological transformation pathways under different redox conditions as well as examining the enzymes involved, might also lead to technical options for an enhanced biological removal of organic micropollutants in terms of a more complete transformation connected with detoxification. Moreover, an improved source control by legal measures for limiting the use of environmental harmful consumer chemicals, labeling of compounds according to environmental friendliness or separate treatment of significant point sources (e.g. hospital wastewater, nursery homes, industrial wastewater) is an additional more sustainable option for impeding the emission of organic micropollutants.

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7. Supplementary Information (SI)

7.1 SI of chapter 2

Outline

Table SI 2-1. Absolute recoveries (in %) of biocides, UV-filters and benzothiazoles enriched from spiked groundwater (100 ng L^{-1}) using different SPE cartridges and extraction conditions. Deviations from the mean are given as 95% confidence intervals (HLB results: n = 4, all others n = 3). (*) Analytes determined in negative ionization mode. ND: not determined.

Cartridge	C18	MCX	ENV	Strata-XC	Stata X	HLB Effect of elution solvent				HLB Effect of matrix pH			
Matrix pH Elution solvent[a] Ratio [%]	pH 7 MeOH 100	pH 3 MeOH/NH_4OH[b] 95/5	pH 7 MeOH 100	pH 3 MeOH/NH_4OH[b] 95/5	pH 7 MeOH 100	pH 7 MeOH 100	pH 7 MeOH/AC[c] 80/20	pH 7 MeOH/AC[c] 60/40	pH 7 AC[c] 100	pH 8 MeOH/AC[c] 60/40	pH 7 MeOH/AC[c] 60/40	pH 6 MeOH/AC[c] 60/40	pH 5 MeOH/AC[c] 60/40
Biocides													
Propiconazole	77 ± 4	93 ± 51	172 ± 63	91 ± 42	88 ± 14	103 ± 4	105 ± 4	87 ± 5	93 ± 4	90 ± 5	89 ± 5	85 ± 5	84 ± 11
Tebuconazole	78 ± 7	100 ± 60	195 ± 83	103 ± 40	106 ± 28	97 ± 5	97 ± 8	88 ± 9	95 ± 7	92 ± 7	89 ± 9	88 ± 6	85 ± 8
Imazalil	76 ± 5	106 ± 75	149 ± 39	41 ± 9	44 ± 2	ND	ND	ND	ND	86 ± 6	90 ± 15	87 ± 10	84 ± 17
Carbendazim	80 ± 7	72 ± 3	72 ± 17	31 ± 5	44 ± 13	48 ± 8	53 ± 6	54 ± 2	15 ± 16	45 ± 10	42 ± 2	40 ± 1	33 ± 4
Thiabendazole	85 ± 3	99 ± 44	62 ± 7	22 ± 4	24 ± 1	33 ± 19	37 ± 2	41 ± 2	45 ± 10	30 ± 4	23 ± 3	25 ± 4	24 ± 2
Terbutryn	ND	ND	ND	ND	53 ± 9	68 ± 6	75 ± 5	81 ± 8	97 ± 6	60 ± 1	62 ± 6	58 ± 1	63 ± 3
Irgarol	ND	ND	ND	ND	57 ± 9	73 ± 9	74 ± 3	80 ±2	92 ± 5	75 ± 3	75 ± 2	73 ± 1	73 ± 5
M1	ND	ND	ND	ND	57 ± 6	48 ± 3	49 ± 2	53 ± 3	77 ± 9	50 ± 5	49 ± 3	46 ± 1	44 ± 3
Dimethomorph	94 ± 6	115 ± 68	31 ± 14	106 ± 35	102 ± 15	81 ± 7	83 ± 4	84 ± 3	92 ± 10	76 ± 1	78 ± 5	77 ± 1	76 ± 8
Fenpropimorph	< 5	< 5	42 ± 23	< 5	< 5	47 ± 4	47 ± 5	47 ± 2	73 ± 5	45 ± 9	52 ± 13	58 ± 5	62 ± 4
Tridemorph	ND	ND	ND	< 5	< 5	29 ± 6	27 ± 10	31 ± 6	51 ± 10	31 ± 9	39 ± 22	44 ± 6	60 ± 29
BIT	18 ± 9	87 ± 6	61 ± 20	34 ± 8	27 ± 13	51 ± 12	50 ± 19	44 ± 21	< 5	56 ± 17	61 ± 13	53 ± 15	48 ± 4
OIT	ND	ND	ND	ND	ND	90 ± 9	86 ± 5	84 ± 7	95 ± 11	82 ± 5	91 ± 11	92 ± 2	93 ± 4
DCOIT	ND	ND	ND	ND	< 5	70 ± 8	70 ± 11	75 ± 13	96 ± 8	37 ± 13	34 ± 27	71 ± 8	71 ± 11
DMST	ND	ND	ND	ND	74 ± 10	77 ± 12	82 ± 14	81 ± 10	93 ± 13	89 ± 3	85 ± 13	86 ± 11	85 ± 20
DMSA(*)	ND	ND	ND	ND	70 ± 5	68 ± 3	73 ± 2	74 ± 4	102 ± 9	77 ± 7	77 ± 5	71 ± 1	61 ± 3
IPBC	62 ± 10	123 ± 71	368 ± 136	77 ± 32	70 ± 82	80 ± 5	80 ± 2	84 ± 5	92 ± 7	80 ± 5	79 ± 2	80 ± 8	78 ± 12
Triclosan(*)	< 5	20 ± 20	85 ± 68	17 ± 18	23 ± 22	84 ± 7	83 ± 14	86 ± 10	61 ± 16	90 ± 24	83 ± 34	107 ± 9	86 ± 13
Triclocarban(*)	18 ± 11	39 ± 64	16 ± 18	31 ± 24	64 ± 64	77 ± 12	60 ± 15	58 ± 14	< 5	53 ± 28	55 ± 26	61 ± 11	45 ± 25
Chlorophene(*)	ND	ND	ND	58 ± 25	53 ± 13	86 ± 2	89 ± 9	93 ± 6	99 ± 10	80 ± 9	81 ± 8	75 ± 18	64 ± 13
UV-filter													
BZP-1(*)	74 ± 11	90 ± 9	74 ± 29	69 ± 18	57 ± 7	76 ± 5	79 ± 3	83 ± 3	9 ± 10	78 ± 7	77 ± 4	72 ± 4	65 ± 2
BZP-2(*)	80 ± 9	79 ± 5	58 ± 4	23 ± 20	40 ± 5	47 ± 2	51 ± 2	52 ± 3	< 5	48 ± 4	46 ± 6	42 ± 1	34 ± 2
BZP-3	10 ± 4	62 ± 111	13 ± 6	< 5	31 ± 13	89 ± 13	102 ± 8	96 ± 7	26 ± 11	86 ± 6	90 ± 15	87 ± 10	84 ± 17
BZP-4(*)	102 ± 8	49 ± 9	78 ± 12	72 ± 14	87 ± 4	85 ± 32	87 ± 5	93 ± 3	< 5	85 ± 8	92 ±20	91 ± 10	77 ± 20
PBSA	13 ± 1	78 ± 11	80 ± 25	91 ± 24	85 ± 11	86 ± 14	91 ± 5	96 ± 9	< 5	90 ± 6	89 ± 3	79 ± 5	74 ± 9
Benzothiazoles													
Benzothiazole	35 ± 32	80 ± 13	35 ± 21	26 ± 11	47 ± 29	101 ± 9	94 ± 28	97 ± 9	72 ± 14	98 ± 6	100 ± 37	130 ± 19	148 ± 2
MTBT	12 ± 4	31 ± 45	175 ± 162	< 5	19 ± 24	94 ± 4	84 ± 17	89 ± 12	84 ± 11	84 ± 11	81 ± 34	88 ± 1	84 ± 3
BTSA	5 ± 2	16 ± 3	89 ± 9	7 ± 1	86 ± 47	91 ± 7	103 ± 12	103 ± 17	126 ± 22	89 ± 5	95 ± 4	89 ± 14	67 ± 20
OHBT(*)	42 ± 2	96 ± 11	90 ± 6	46 ± 5	57 ± 8	56 ± 1	58 ± 3	60 ± 1	81 ± 18	64 ± 4	62 ± 5	55 ± 4	46 ± 6
Morpholinyl-BT	82 ± 13	58 ± 6	49 ± 27	55 ± 19	86 ± 21	58 ± 5	59 ± 3	61 ± 4	77 ± 4	61 ± 3	60 ± 4	57 ± 3	56 ± 6

[a] Elution solvent volume used: 4 x 2 mL; [b] 25% ammonium hydroxide solution; [c] Ac: Acetone

Table SI 2-2. Absolute recoveries (in %) of biocides, UV-filters and benzothiazoles extracted from spiked freeze-dried activated sludge (500 ng g_{TSS}^{-1}) using different PLE conditions. Deviations from the mean are given as 95% confidence intervals (n = 4). (*) Analytes determined in negative ionization mode. ND: not determined.

	Effect of extraction solvent			Effect of extraction temperature			
Solvent	**Acetone**	**MeOH**	**MeOH/H_2O[a]**	**MeOH/H_2O[a]**			
Temperature [°C]	**80**	**80**	**80**	**80**	**80**	**100**	**120**
No. of cycles	**3**	**3**	**3**	**3**	**4**	**3**	**3**
Biocides							
Diuron	49 ± 5	61 ± 3	59 ± 5	36 ± 7	35 ± 3	31 ± 5	25 ± 4
Isoproturon	72 ± 3	55 ± 12	56 ± 12	37 ± 4	36 ± 4	31 ± 3	25 ± 2
Mecoprop(*)	< 5	91 ± 5	114 ± 14	58 ± 3	59 ± 11	56 ± 10	45 ± 12
Propiconazole	13 ± 3	77 ± 19	80 ± 22	46 ± 9	38 ± 6	34 ± 4	27 ± 7
Tebuconazole	15 ± 3	81 ± 14	80 ± 17	55 ± 8	43 ± 6	42 ± 5	33 ± 12
Imazalil	48 ± 22	38 ± 6	45 ± 5	26 ± 6	20 ± 3	20 ± 1	15 ± 3
Climbazole	ND	26 ± 25	37 ± 25	ND	ND	ND	ND
Ketoconazole	ND	35 ± 22	21 ± 12	ND	ND	ND	ND
Carbendazim	25 ± 4	58 ± 12	47 ± 22	12 ± 5	17 ± 4	20 ± 3	18 ± 2
Thiabendazole	38 ± 17	26 ± 27	20 ± 7	7 ± 3	8 ± 2	10 ± 2	9 ± 1
Terbuthylazine	15 ± 3	50 ± 8	62 ± 25	51 ± 11	40 ± 3	42 ± 1	33 ± 11
Terbutryn	28 ± 6	41 ± 7	52 ± 19	30 ± 6	25 ± 1	25 ± 2	21 ± 2
Irgarol	25 ± 6	47 ± 10	46 ± 12	36 ± 3	29 ± 3	27 ± 2	23 ± 5
M1	50 ± 5	48 ± 6	54 ± 5	23 ± 3	22 ± 2	20 ± 1	16 ± 1
Dimethomorph	56 ± 12	100 ± 13	92 ± 24	53 ± 6	48 ± 8	43 ± 1	37 ± 3
Fenpropimorph	59 ± 22	44 ± 8	43 ± 10	14 ± 9	18 ± 4	21 ± 1	17 ± 4
Tridemorph	11 ± 11	13 ± 5	13 ± 4	< 5	6 ± 5	9 ± 1	8 ± 2
BIT	< 5	19 ± 10	36 ± 11	7 ± 1	12 ± 9	16 ± 6	14 ± 6
OIT	12 ± 2	30 ± 11	28 ± 13	20 ±6	21 ±14	18 ± 6	17 ± 3
DCOIT	<5	<5	<5	<5	<5	<5	<5
DMST	74 ± 4	79 ± 5	89 ± 31	35 ± 1	36 ± 9	34 ± 11	31 ± 3
DMSA(*)	ND	97 ± 4	83 ± 9	17 ± 1	19 ± 4	19 ± 2	17 ± 4
IPBC	<5	<5	<5	<5	<5	<5	<5
Triclosan(*)	<5	31 ± 10	52 ± 23	ND	ND	ND	ND
Triclocarban(*)	<5	34 ± 4	66 ± 5	55 ± 14	50 ± 9	55 ± 8	35 ± 18
Chlorophene(*)	<5	61 ± 4	85 ± 11	20 ± 2	17 ± 3	16 ± 3	12 ± 5
UV-filter							
BZP-1(*)	20 ± 3	38 ± 1	36 ± 3	29 ± 1	27 ± 6	24 ± 3	18 ± 2
BZP-2(*)	10 ± 2	16 ± 1	18 ± 1	13 ± 2	15 ± 3	14 ± 3	10 ± 1
BZP-3	< 5	41 ± 9	68 ± 7	38 ± 17	30 ± 1	32 ± 5	28 ± 10
BZP-4(*)	< 5	55 ± 5	93 ± 15	64 ± 6	64 ± 4	61 ± 9	57 ± 7
PBSA	< 5	85 ± 16	72 ± 20	14 ± 11	26 ± 10	32 ± 3	28 ± 8
Benzothiazoles							
Benzothiazole	17 ± 8	59 ± 15	48 ± 35	77 ± 8	59 ± 7	69 ± 20	63 ± 7
MTBT	11 ± 17	50 ± 13	66 ± 13	82 ± 8	63 ± 10	71 ± 14	63 ± 12
BTSA	< 5	12 ± 11	22 ± 21	11 ± 7	14 ± 1	13 ± 1	8 ± 3
OHBT(*)	61 ± 6	71 ± 8	53 ± 6	14 ± 2	15 ± 2	14 ± 3	11 ± 1
Morpholinyl-BT	49 ± 5	61 ± 8	67 ± 1	40 ± 2	37 ± 1	35 ± 2	32 ± 1

[a] (50/50, v/v)

Table SI 2-3. Recoveries of biocides, UV-filters and benzothiazoles measured with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in raw and treated wastewater spiked with 4 and 2 µg L^{-1}, respectively. The range indicate the 95% confidence intervals (n = 4). (*) Analytes determined in negative ionization mode. ND: not determined.

	ESI				APCI			
	WWTP effluent		WWTP influent		WWTP effluent		WWTP influent	
Recovery [%]	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery
Biocides								
Diuron[a/a]	34 ± 1	98 ± 5	40 ± 1	102 ± 2	105 ± 11	86 ± 19	112 ± 3	98 ± 13
Isoproturon[b/b]	33 ± 1	100 ± 2	38 ± 1	103 ± 3	116 ± 6	88 ± 4	115 ± 5	95 ± 11
Mecoprop(*)[k/k]	34 ± 1	110 ± 6	54 ± 1	107 ± 5	117 ± 7	95 ± 4	110 ± 9	95 ± 7
Propiconazole[c/c]	63 ± 2	96 ± 6	52 ± 1	98 ± 4	124 ± 9	100 ± 10	117 ± 17	102 ± 8
Tebuconazole[d/i]	67 ± 1	105 ± 4	61 ± 2	97 ± 4	116 ± 6	106 ± 4	112 ± 11	111 ± 13
Imazalil[e/e]	30 ± 1	97 ± 5	30 ± 1	95 ± 5	143 ± 5	103 ± 10	100 ± 15	97 ± 10
Climbazole[j/ND]	69 ± 3	100 ± 4	72 ± 2	104 ± 10	ND	ND	ND	ND
Ketoconazole[f/ND]	62 ± 5	92 ± 8	60 ± 2	93 ± 6	ND	ND	ND	ND
Carbendazim[g/ND]	15 ± 1	114 ± 3	18 ± 1	123± 9	ND	ND	ND	ND
Thiabendazole[h/h]	16 ± 2	111 ± 5	26 ± 1	105 ± 4	85 ± 4	91 ± 3	91 ± 4	96 ± 10
Terbuthylazine[i/i]	51 ± 1	89 ± 1	48 ± 1	90 ± 4	106 ± 4	97 ± 8	100 ± 9	99 ± 12
Terbutryn[e/b]	35 ± 4	102 ± 14	30 ± 3	78 ± 13	141 ± 5	111 ± 11	119 ± 16	101 ± 25
Irgarol[e/b]	37 ± 1	109 ± 6	33 ± 1	91 ± 9	146 ± 3	111 ± 4	129 ± 20	106 ± 27
M1[e/b]	30 ± 2	97 ± 6	38 ± 2	128 ± 6	140 ± 9	109 ± 10	128 ± 3	108 ± 12
Dimethomorph[i/b]	61± 3	101 ± 6	65 ± 3	115 ± 5	150 ± 6	110 ± 6	129 ± 7	102 ± 12
Fenpropimorph[b/n]	39 ± 2	122 ± 13	39 ± 2	104 ± 10	99 ± 6	102 ± 7	76 ± 15	77 ± 16
Tridemorph[e/d]	15± 2	106 ± 11	12 ± 1	82 ± 9	41 ± 8	93 ± 15	53 ± 11	116 ± 10
BIT[e/n]	17 ± 1	76 ± 3	25 ± 1	102 ± 4	62 ± 7	73 ± 7	71 ± 7	81 ± 7
OIT[b/n]	46 ± 1	113 ± 5	37 ± 1	85 ± 4	93 ± 4	92 ± 4	95 ± 6	95 ± 6
DCOIT[e/n]	ND	ND	ND	ND	ND	ND	ND	ND
DMST[a/n]	25 ± 2	89 ± 5	28 ± 1	89 ± 2	89 ± 7	90 ± 7	98 ± 9	95 ± 12
DMSA(*)[k/n]	18 ± 1	92 ± 5	28 ± 1	88 ± 5	104 ± 8	116 ± 10	83 ± 12	89 ± 15
IPBC[j/ND]	42 ± 1	98 ± 4	42 ± 1	107 ± 3	ND	ND	ND	ND
Triclosan[l/l]	38 ± 5	101 ± 4	46 ± 3	99 ± 3	240 ± 69	109 ± 23	273 ± 66	103 ± 7
Triclocarban[m/m]	46 ± 11	107 ± 9	58 ± 7	107 ± 6	477 ± 185	112 ± 13	613 ± 168	109 ± 7
Chlorophene[a/n]	15 ± 3	99 ± 31	19 ± 2	121 ± 4	114 ± 21	108 ± 19	101 ± 33	96 ± 30
UV-filters								
BZP-1(*)[a/a]	17 ± 1	111 ± 13	28 ± 2	246 ± 17	121 ± 6	95 ± 10	113 ± 10	103 ± 8
BZP-2(*)[m/k]	11 ± 1	87 ± 13	16 ± 1	97 ± 6	190 ± 11	119 ± 6	161 ± 18	107 ± 12
BZP-3[j/n]	50 ± 2	106 ± 6	44 ± 3	100 ± 8	245 ± 57	74 ± 19	240 ± 32	72 ± 10
BZP-4(*)[n/ND]	93 ± 4	110 ± 5	92 ± 3	108 ± 3	ND	ND	ND	ND
PBSA[a/-]	32 ± 7	80 ± 13	36 ± 2	91 ± 4	> 1000	ND	> 1000	ND
Benzothiazoles								
Benzothiazole[d/n]	76 ± 2	133 ± 6	81 ± 9	149 ± 20	104 ± 16	88 ± 14	97 ± 27	82 ± 25
MTBT[n/n]	92 ± 5	87 ± 6	98 ± 5	99 ± 6	231±23	105 ± 11	218 ± 1	98 ± 6
BTSA[a/ND]	33 ± 7	78 ± 12	42 ± 3	93 ± 4	ND	ND	ND	ND
OHBT(*)[k/n]	15 ± 1	97 ± 4	21 ± 1	87 ± 4	79 ± 14	96 ± 17	74 ± 12	91 ± 14
Morpholinyl-BT[e/n]	24 ± 1	95 ± 7	27 ± 1	101 ± 6	100 ± 4	99 ± 4	100 ± 7	99 ± 7

Indices (a-n) indicate the surrogate standards used for calculation of the analyte concentration by internal standard calibration for the measurement with ESI (first index) and APCI (second index). [a] diuron-d_6, [b] isoproturon-d_6, [c] propiconazole-d_5, [d] tebuconazole-d_6, [e] imazalil-d_5, [f] ketoconazole-d_8, [g] carbendazim-d_4, [h] thiabendazole-d_6, [i] terbuthylazine-d_5, [j] terbutryn-d_5, [k] mecoprop-d_3, [l] triclosan-$^{13}C_{12}$, [m] triclocarban-$^{13}C_6$, [n] no surrogate.

Table SI 2-4. Recoveries of biocides, UV-filters and benzothiazoles measured with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in activated sludge spiked with 2 µg g_{TSS}^{-1}. The range indicate the 95% confidence intervals (n = 4). (*) Analytes determined in negative ionization mode. ND: not determined.

	ESI		APCI	
Recovery [%]	Absolute recovery	Relative recovery	Absolute recovery	Relative recovery
Biocides				
Diuron[a/a]	32 ± 4	77 ± 14	128 ± 23	103 ± 35
Isoproturon[b/b]	45 ± 7	98 ± 21	124 ± 7	107 ± 23
Mecoprop(*)[k/k]	118 ± 26	105 ± 21	102 ± 39	98 ± 23
Propiconazole[c/c]	88 ± 12	98 ± 15	167 ± 40	104 ± 34
Tebuconazole[d/d]	94 ± 13	110 ± 18	131 ± 38	112 ± 16
Imazalil[e/e]	38 ± 8	102 ± 21	156 ± 68	91 ± 48
Climbazole[e/c]	50 ± 8	113 ± 12	207 ± 68	91 ± 34
Ketoconazole[e/b]	41 ± 9	110 ± 17	109 ± 28	96 ± 35
Carbendazim[g/ND]	25 ± 6	127 ± 41	ND	ND
Thiabendazole[h/h]	15 ± 2	109 ± 23	93 ± 14	86 ± 12
Terbuthylazine[i/i]	57 ± 8	93 ± 11	149 ± 36	100 ± 19
Terbutryn[j/j]	45 ± 4	95 ± 13	147 ± 32	92 ± 25
Irgarol[l/j]	39 ± 7	95 ± 24	159 ± 25	97 ± 22
M1[j/j]	27 ± 6	79 ± 21	156 ± 17	97 ± 11
Dimethomorph[n/n]	107 ± 15	78 ± 11	181 ± 29	112 ± 19
Fenpropimorph[e/i]	36 ± 5	94 ± 11	113 ± 33	84 ± 31
Tridemorph[e/d]	14 ± 3	78 ± 13	77 ± 26	109 ± 27
BIT[g/b]	20 ± 5	95 ± 24	62 ± 18	83 ± 23
OIT[h/b]	22 ± 5	90 ± 24	40 ± 13	43 ± 18
DCOIT[e/n]	< 2	ND	< 2	ND
DMST[d/n]	59 ± 13	84 ± 22	94 ± 20	93 ± 20
DMSA(*)[n/a]	84 ± 12	79 ± 12	97 ± 30	97 ± 16
IPBC[j/ND]	< 2	ND	ND	ND
Triclosan(*)[l/l]	52 ± 23	102 ± 21	78 ± 42	100 ± 61
Triclocarban(*)[m/m]	159 ± 51	98 ± 13	162 ± 81	103 ± 15
Chlorophene(*)[l/l]	84 ± 22	81 ± 11	73 ± 23	100 ± 33
UV-filter				
BZP-1(*)[m/k]	48 ± 10	74 ± 10	100 ± 44	90 ± 27
BZP-2(*)[m/n]	28 ± 8	90 ± 10	150 ±51	90 ± 28
BZP-3[i/i]	52 ± 16	89 ± 20	120 ± 51	80 ± 23
BZP-4(*)[l/ND]	84 ± 18	92 ± 12	ND	ND
PBSA[i/ND]	68 ± 9	82 ± 25	ND	ND
Benzothiazoles				
Benzothiazole[e/b]	ND	ND	75 ± 23	76 ± 34
MTBT[e/b]	43 ± 19	72 ± 25	86 ± 21	84 ± 34
BTSA[h/ND]	23 ± 8	87 ± 9	ND	ND
OHBT(*)[l/k]	52 ± 11	86 ± 9	79 ± 22	75 ± 10
Morpholinyl-BT[i/b]	45 ± 12	80 ± 18	99 ± 15	94 ± 32

Indices (a-n) indicate the surrogate standards used for calculation of the analyte concentration by internal standard calibration for the measurement with ESI (first index) and APCI (second index). [a] diuron-d_6, [b] isoproturon-d_6, [c] propiconazole-d_5, [d] tebuconazole-d_6, [e] imazalil-d_5, [f] ketoconazole-d_8, [g] carbendazim-d_4, [h] thiabendazole-d_6, [i] terbuthylazine-d_5, [j] terbutryn-d_5, [k] mecoprop-d_3, [l] triclosan-$^{13}C_{12}$, [m] triclocarban-$^{13}C_6$, [n] no surrogate.

Table SI 2-5. Signal to noise ratios of the analytes in the investigated matrices together with the respective concentrations determined with ESI and APCI. For analytes with background concentrations > LOQ for the ESI method, the signal to noise ratios were determined from non-spiked extracts, whereas for analytes with background concentrations < LOQ spiked sample extracts were used. (*) Analytes determined in the negative ionization mode. ND: not determined.

Compounds	River water			WWTP effluent			WWTP influent			Activated sludge		
	Conc. [ng L^{-1}]	Signal to noise transition 1/transition 2		Conc. [ng L^{-1}]	Signal to noise transition 1/transition 2		Conc. [ng L^{-1}]	Signal to noise transition 1/transition 2		Conc. [ng g_{TSS}^{-1}]	Signal to noise transition 1/transition 2	
Biocides		ESI	APCI		ESI	APCI		ESI	APCI		ESI	APCI
Diuron	14	333/126	62/28	180	964/523	176/183	97	744/111	25/37	14	201/32	5/5
Isoproturon	3.6	400/17	90/2	50	1036/197	397/10	11	250/7	20/2	5.0	413/18	76/3
Mecoprop(*)	58	500/101	980/63	69	310/38	222/55	32	80/5	27/9	10	32/6	52/18
Propiconazole	2.4	43/19	29/14	10	61/49	22/19	10	10/6	8/7	7.0	30/15	8/6
Tebuconazole	2.6	112/56	94/30	6.5	59/16	57/14	11	18/11	7/3	5.2	28/3	11/5
Imazalil	1.2	15/6	3/2	5.6	20/7	3/2	20	12/5	4/4	20	35/21	3/3
Climbazole	21	183/312	90/39	440	343/530	174/144	1025	133/88	121/108	ND	ND	ND
Ketoconazole	5	95/26	17/15	20	82/40	34/20	50	17/13	15/9	ND	ND	ND
Carbendazim	41	855/30	ND	86	563/35	ND	135	45/19	ND	16	246/43	ND
Thiabendazole	8.3	113/27	66/18	14	222/18	47/16	14	80/8	19/11	8.0	55/16	17/7
Terbuthylazine	6.0	170/250	140/68	33	495/64	227/87	9.0	91/19	17/6	2.5	73/16	26/9
Terbutryn	23	1466/345	289/112	122	2130/183	613/61	136	829/148	205/58	40	645/193	151/67
Irgarol	5.2	61/105	142/29	6.3	150/26	20/7	6.7	77/12	3/2	4.8	141/16	12/2
M1	5.2	81/43	60/29	10	121/46	37/13	5.0	10/5	5/4	2.5	24/19	7/8
Dimethomorph	1.2	68/27	73/15	9.0	266/60	85/32	18	79/27	67/58	5	132/49	73/28
Fenpropimorph	0.5	36/20	16/5	2.5	90/53	20/8	5	34/23	9/4	6.7	64/62	21/15
Tridemorph	2	25/14	5/3	10	27/21	10/4	20	15/10	<2/<2	20	48/26	3/8
BIT	11	18/13	<2/<2	60	17/10	6/<2	105	10/5	<2/<2	120	135/46	10/3
OIT	0.5	75/10	4/<2	5.0	277/11	111/7	10	89/10	48/<2	25	231/21	177/10
DCOIT	1	72/291	8/11	5	93/150	13/13	10	26/19	12/10	ND	ND	ND
DMST	2.0	33/4	3/<2	17	112/48	6/<2	26	12/4	<2/<2	10	45/15	6/<2
DMSA(*)	11	21/14	21/2	50	50/8	30/3	46	16/5	16/<2	25	51/7	28/3
IPBC	5	33/7	ND	20	36/16	ND	50	17/9	ND	ND	ND	ND
Triclosan	7.9	63/59	9/5	13	43/27	8/<2	800	320/223	204/74	2500	1770/610	150/202
Triclocarban	1.6	149/26	12/9	4.0	103/74	14/28	11	92/18	46/18	113	210/144	96/88
Chlorophene	2.1	13/4	<2/<2	5.0	13/12	<2/<2	243	132/74	34/21	500	167/109	19/30
UV-filters												
BZP-1(*)	0.9	12/8	7/8	16	35/27	19/29	95	116/104	13/24	5.8	39/24	10/8
BZP-2(*)	ND	ND	ND	12	17/8	28/30	6.5	13/5	29/12	8.5	25/19	18/15
BZP-3	5	75/64	78/8	20	61/37	30/24	490	97/361	146/27	116	63/53	20/6
BZP-4(*)	150	167/89	ND	105	62/21	ND	5100	3420/184	ND	30	80/52	ND
PBSA	624	2053/478	ND	1900	3329/739	ND	4400	3670/410	ND	130	546/27	ND
Benzothiazoles												
Benzothiazole	70	69/20	80/41	50	19/8	58/28	430	35/10	113/34	290	32/15	ND
MTBT	115	62/53	207/135	254	101/65	432/316	418	38/43	152/312	135	75/39	ND
BTSA	735	587/270	ND	388	145/158	ND	1410	366/106	ND	80	129/58	ND
OHBT(*)	88	43/10	42/8	610	39/24	100/25	775	35/20	231/15	140	39/7	49/5
Morpholinyl-BT	2.4	30/15	25/18	8.7	74/31	31/24	12	12/14	9/9	5.6	19/18	8/7

Table SI 2-6. Summary of validation data (calibration, instrumental precision, recoveries and LOQs of the analytical method) using the ESI interface

	Calibration[a]		Instr. precision[b] (%RSD)		Relative recovery (%)				LOQ [ng L^{-1}], for sludge: [ng g_{TSS}^{-1}]			
	R^2	Range	Intra-day	Inter-day	River water	Effluent	Influent	Sludge	River water	Effluent	Influent	Sludge
Biocides												
Diuron	0.999	0.5-2000	4	4	104 ± 5	100 ± 3	99 ± 4	88 ± 9	0.5	2.5	5	2.5
Isoproturon	0.993	0.5-1000	2	1	101 ± 2	96 ± 2	96 ± 2	111 ± 11	1	5	10	5
Mecoprop(*)	0.999	2-2000	5	3	106 ± 6	110 ± 17	106 ± 7	112 ± 13	2	10	20	10
Propiconazole	0.999	0.5-2000	2	2	96 ± 3	91 ± 5	93 ± 7	105 ± 12	0.5	2.5	10	5
Tebuconazole	0.999	0.5-1000	2	5	103 ± 3	97 ± 3	91 ± 4	115 ± 14	0.5	2.5	5	5
Imazalil	0.997	1-2000	2	1	101 ± 2	92 ± 4	89 ± 3	106 ± 14	1	5	20	5
Climbazole	0.994	1-2000	5	2	93 ± 16	95 ± 4	95 ± 16	136 ± 28	1	5	10	5
Ketoconazole	0.996	5-2000	3	2	115 ± 24	97 ± 8	93 ± 10	119 ± 11	5	25	50	25
Carbendazim	0.997	0.5-400	4	1	116 ± 4	117 ± 10	122 ± 5	114 ± 23	0.5	2.5	5	5
Thiabendazole	0.999	0.5-1000	3	3	101 ± 4	95 ± 7	90 ± 7	118 ± 19	0.5	2.5	5	2.5
Terbuthylazine	0.996	0.5-2000	2	1	94 ± 2	86 ± 1	89 ± 3	107 ± 12	0.5	2.5	5	2.5
Terbutryn	0.999	0.5-1000	2	2	100 ± 17	91 ± 17	71 ± 7	104 ± 31	0.5	2.5	5	2.5
Irgarol	0.998	0.5-400	3	1	108 ± 7	101 ± 10	82 ± 4	101 ± 8	0.5	2.5	5	2.5
M1	0.998	0.5-2000	1	1	84 ± 3	85 ± 3	108 ± 1	84 ± 16	0.5	2.5	5	2.5
Dimethomorph	0.999	1-2000	2	11	109 ± 12	98 ± 7	124 ± 6	90 ± 5	1	5	10	5
Fenpropimorph	0.999	0.5-1000	1	1	118 ± 4	98 ± 9	94 ± 10	101 ± 8	0.5	2.5	5	2.5
Tridemorph	0.999	2-2000	4	7	125 ± 39	104 ± 21	86 ± 16	87 ± 5	2	10	20	25
BIT	0.999	10-2000	5	8	90 ± 8	73 ± 7	110 ± 6	96 ± 28	10	50	100	50
OIT	0.999	0.5-400	1	8	129 ± 5	103 ± 3	79 ± 1	110 ± 33	1	5	10	10
DCOIT	0.999	1-200	9	3	198 ± 32	69 ± 13	78 ± 19	ND	1	5	10	
DMST	0.999	2-2000	6	13	94 ± 5	90 ± 4	92 ± 9	84 ± 10	2	10	20	10
DMSA(*)	0.999	5-2000	4	4	76 ± 2	93 ± 20	83 ± 5	91 ± 10	5	25	50	25
IPBC	0.999	5-2000	2	8	97 ± 4	93 ± 4	101 ± 1	ND	5	25	50	
Triclosan	0.999	2-2000	3	2	102 ± 2	98 ± 7	95 ± 3	101 ± 57	2	10	20	10
Triclocarban	0.996	0.5-2000	1	3	105 ± 5	100 ± 5	102 ± 6	108 ± 11	0.5	2.5	5	2.5
Chlorophene	0.998	1-2000	2	6	120 ± 14	90 ± 16	108 ± 35	96 ± 18	2	5	10	10
UV-filters												
BZP-1(*)	0.994	0.5-1000			98 ±11	93 ± 8	180 ± 14	74 ± 9	0.5	2.5	5	2.5
BZP-2(*)	0.999	0.5-2000			53 ± 7	93 ± 15	111 ± 6	99 ± 11	0.5	2.5	5	2.5
BZP-3	0.997	5-2000	3	18	130 ± 31	102 ± 17	100 ± 12	104 ± 14	5	25	50	25
BZP-4(*)	0.996	1-2000	8	11	81 ± 7	105 ± 11	105 ± 3	114 ± 28	1	5	10	5
PBSA	0.998	1-2000	5	5	100 ± 10	66 ± 32	96 ± 19	118 ± 19	1	5	10	5
Benzothiazoles												
Benzothiazole	0.996	10-2000	3	9	97 ± 6	109 ± 13	124 ± 21	89 ± 29	20	100	200	100
MTBT	0.996	5-2000	1	6	98 ± 8	86 ± 11	87 ± 10	90 ± 5	5	25	50	25
BTSA	0.998	2-2000	5	4	62 ± 9	42 ± 57	98 ± 12	99 ± 25	2	10	20	10
OHBT(*)	0.999	5-2000	5	5	70 ± 5	102 ± 42	84 ± 12	105 ± 24	20	100	200	100
Morpholinyl-BT	0.999	0.5-2000	2	2	84 ± 5	89 ± 6	94 ± 5	92 ± 16	0.5	2.5	5	2.5

[a] For calibration a quadratic fitting ($y = ax^2 + bx + c$) with a weighing factor of 1/x was used.

[b] Determined by the repeated injection of a groundwater extract spiked before extraction (100 ng L^{-1}) on the same day (intra-day precision, n = 5) and on three different days (inter-day precision, n = 3).

7.2 SI of chapter 3

Outline

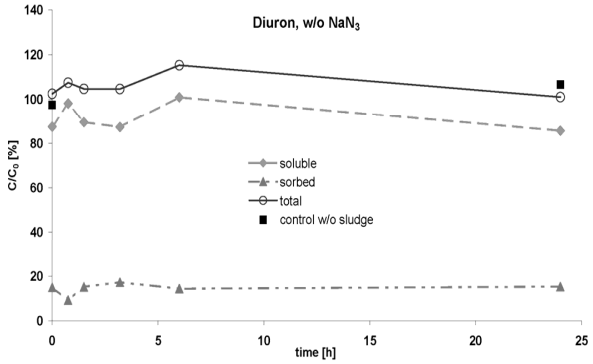
Table SI 3-1. Retention time, precursor ion, product ions and MS parameters used for LC-MS/MS detection of triazines and phenyl urea herbicides which have not been reported in Wick et al. (2010).

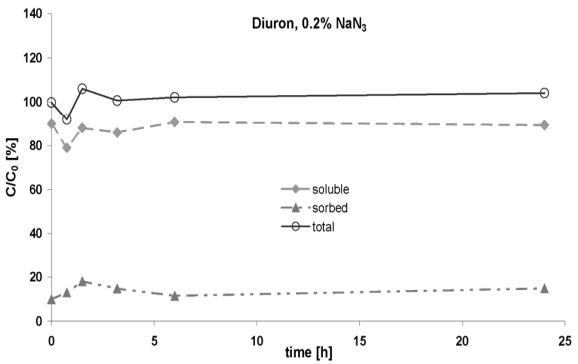
Compound	Retention time [min]	MRM 1	MRM 2	DP[a] [V]	CE[b] (MRM1/MRM2) [eV]	CXP[c] (MRM1/MRM2) [V]
Fluometuron	11.7	233 → 72	233 → 46	80	38/35	10/10
Chloroxuron	14.6	291 →72	291 → 218	90	42/35	10/10
Linuron	13.9	249 → 160	249 → 182	80	23/22	10/10
Monolinuron	11.9	215 → 126	215 → 99	60	24/46	10/10
Neburon	15.7	275 → 88	275 → 114	85	23/20	10/10
Monuron	10.5	199 → 72	199 → 126	90	39/36	10/10
Methabenzthiazuron	11.3	222 → 165	222 → 150	50	29/45	14/14
Atrazine	11.5	216 → 174	216 → 132	70	27/34	19/9
Atrazine-d_5	11.4	221 → 179	223 → 181	60	23/23	10/10
Simazine	10.2	202 → 132	202 → 124	85	26/25	10/10
Cyanazine	10.3	241 → 214	241 → 104	120	23/42	10/10
Propazine	12.9	230 → 146	330 → 188	35	31/25	15/15
Ametryn	12.2	228 → 186	228 → 68	85	26/54	12/12
Prometryn	13.5	242 → 158	242 → 200	85	33/27	12/12
Prometon	11.1	226 → 142	226 → 184	90	33/32	10/10

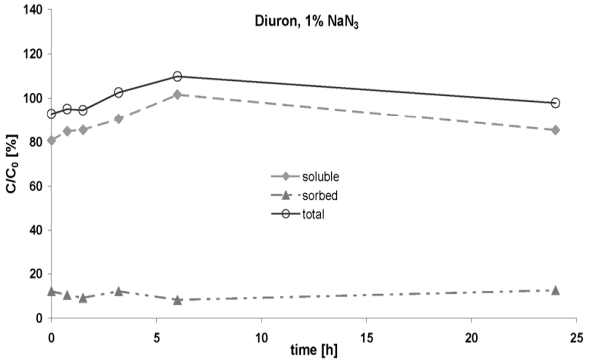
[a] DP = Declustering potential, [b] CE = Collision energy, [c] CXP = Collision cell exit potential,

Table SI 3-2. Relative recovery [%] (results of duplicate measurements) and limit of quantification (LOQ) determined for the aqueous phase and the freeze-dried secondary sludge within the isotherm experiments. In those cases where the relative recoveries were <70% or >130%, concentrations were corrected with the corresponding relative recoveries.

	Aqueous phase		Solid phase		Surrogate standard used
	Rel. Recovery [%]	LOQ [ng L^{-1}]	Rel. Recovery [%]	LOQ [ng $g_{dw\ sludge}^{-1}$]	
Phenylurea herbicides					
Diuron	79	2.5	90	2.5	Diuron-d_6
Isoproturon	91	5.0	98	5.0	Isoproturon-d_6
Fluometuron	91	2.5	93	2.5	Diuron-d_6
Chloroxuron	79	2.5	49	2.5	Diuron-d_6
Linuron	88	2.5	53	2.5	Diuron-d_6
Monolinuron	97	2.5	85	2.5	Diuron-d_6
Neburon	85	2.5	59	2.5	Diuron-d_6
Monuron	88	5.0	108	5.0	Diuron-d_6
Methabenzthiazuron	90	2.5	111	2.5	Diuron-d_6
Triazines					
Atrazine	94	2.5	93	2.5	Atrazine-d_5
Simazine	98	2.5	113	2.5	Atrazine-d_5
Cyanazine	53	5.0	97	5.0	Terbuthylazine-d_5
Propazine	97	2.5	103	2.5	Terbuthylazine-d_5
Terbuthylazine	89	2.5	98	2.5	Terbuthylazine-d_5
Terbutryn	83	2.5	79	2.5	Terbutryn-d_5
Irgarol	91	2.5	98	2.5	Terbutryn-d_5
M1	89	5.0	117	5.0	Terbutryn-d_5
Ametryn	108	2.5	124	2.5	Terbuthylazine-d_5
Prometryn	121	2.5	132	2.5	Terbuthylazine-d_5
Prometon	118	2.5	122	2.5	Terbuthylazine-d_5
Biocides					
Mecoprop	111	10	101	10	Mecoprop-d_3
Propiconazole	84	2.5	96	5.0	Propiconazole-d_5
Tebuconazole	99	2.5	110	5.0	Tebuconazole-d_6
Imazalil	94	5.0	88	5.0	Imazalil-d_5
Carbendazim	87	2.5	88	5.0	Carbendazim-d_4
Thiabendazole	92	2.5	107	2.5	Thiabendazole-d_6
Dimethomorph	93	5.0	95	5.0	Terbuthylazine-d_5
Fenpropimorph	84	2.5	106	2.5	Imazalil-d_5
Tridemorph	64	20	93	50	Imazalil-d_5
BIT	92	100	82	100	Propiconazole-d_5
OIT	102	5	54	10	Propiconazole-d_5
DMST	90	10	86	10	Isoproturon-d_6
DMSA	111	25	119	25	Mecoprop-d_3
Triclosan	96	10	100	10	Triclosan-$^{13}C_6$
Triclocarban	107	2.5	99	2.5	Triclocarban-$^{13}C_6$
Chlorophene	95	5.0	106	10	Triclosan-$^{13}C_6$
UV-filters					
BZP-1	105	2.5	83	2.5	Diuron-d_6
BZP-2	113	2.5	99	2.5	Diuron-d_6
BZP-3	90	25	108	25	Thiabendazole-d_6
BZP-4	106	5.0	103	5.0	Mecoprop-d_3
PBSA	110	5.0	118	5.0	Terbutryn-d_5


Diuron, w/o NaN3
C/C0 [%]
time [h]
soluble
sorbed
total
control w/o sludge


Diuron, 0.2% NaN3
C/C0 [%]
time [h]
soluble
sorbed
total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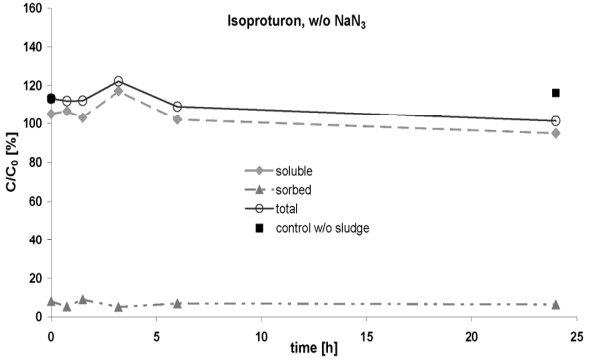
C/C0 [%]
time [h]
soluble
sorbed
total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time [h]
soluble
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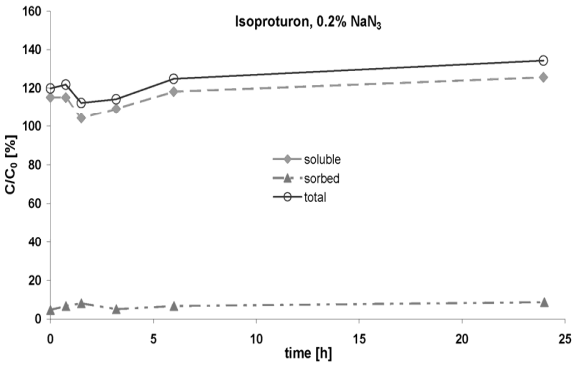
total
control w/o sludge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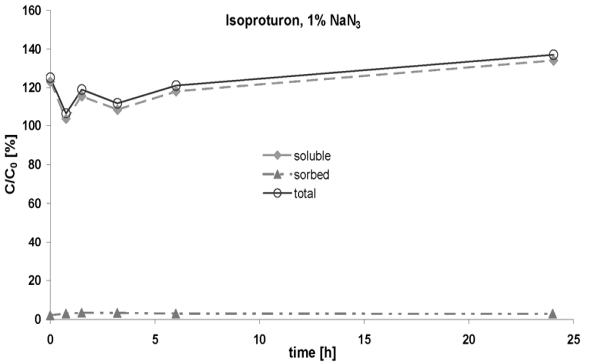
sorbed
total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sorbed
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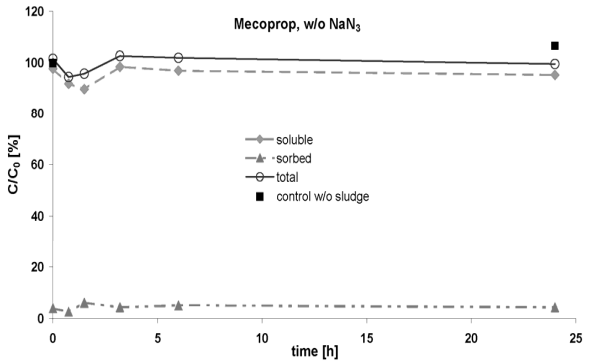
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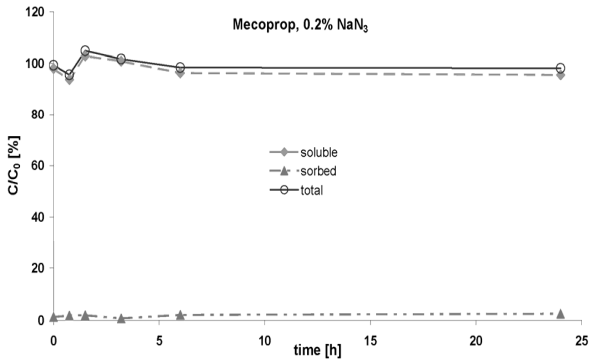
C/C0 [%]
time [h]
soluble
sorbed
total
control w/o sludge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time [h]
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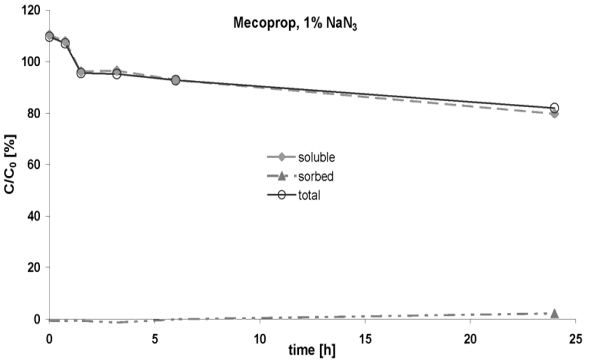
sorbed
total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time [h]
soluble
sorbed
total

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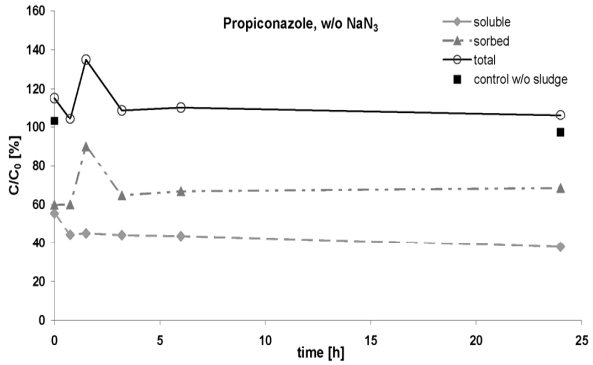
Propiconazole, w/o NaN3
soluble
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total
control w/o sludge
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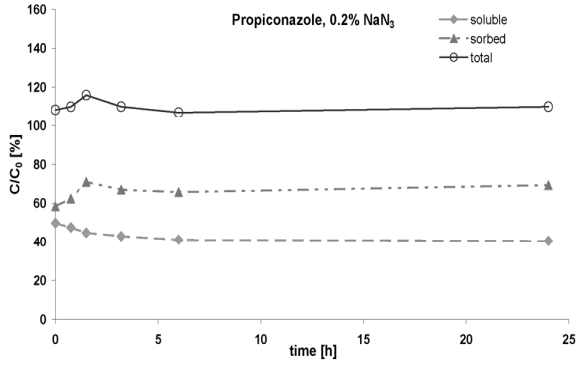
time [h]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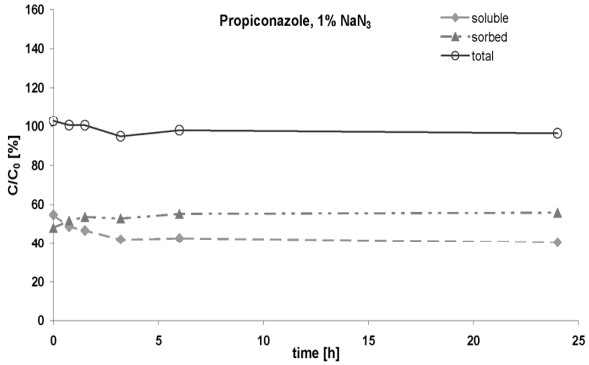
soluble
sorbed
total
C/C0 [%]
time [h]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sorbed
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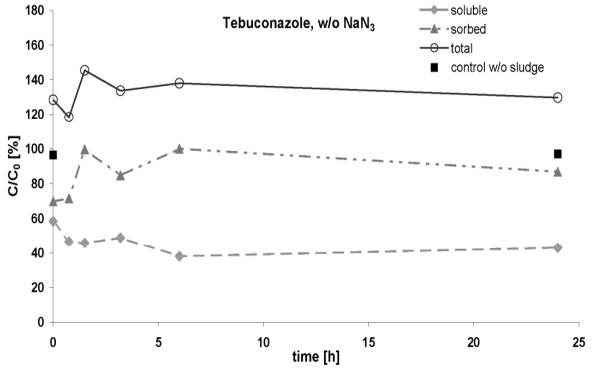
time [h]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sorbed
total
control w/o sludge
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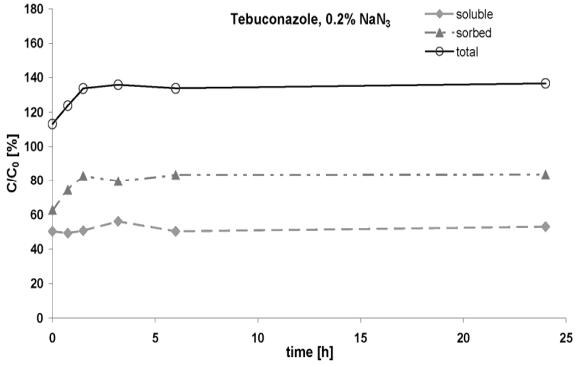
time [h]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sorbed
total
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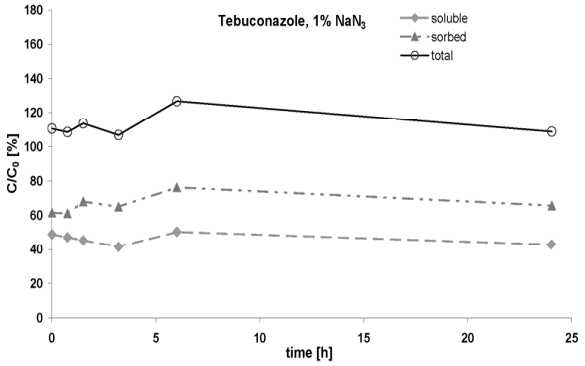
time [h]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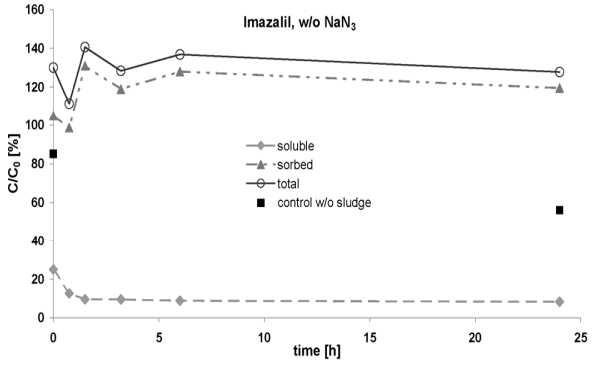
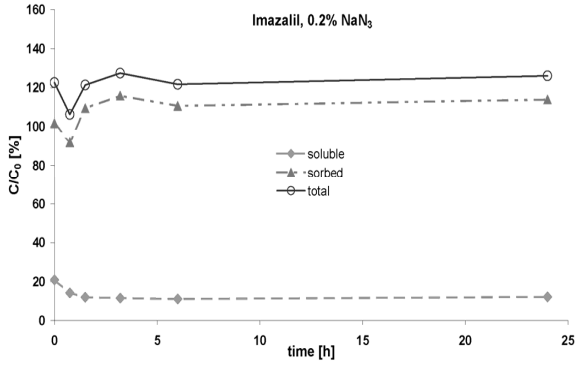
soluble
sorbed
total
C/C0 [%]
time [h]


Imazalil, w/o NaN3
soluble
sorbed
total
control w/o sludge
C/C0 [%]
time [h]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sorbed
total
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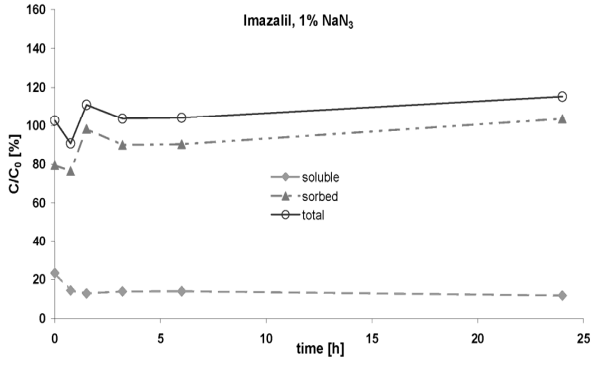
time [h]


Imazalil, 1% NaN3
soluble
sorbed
total
C/C0 [%]
time [h]

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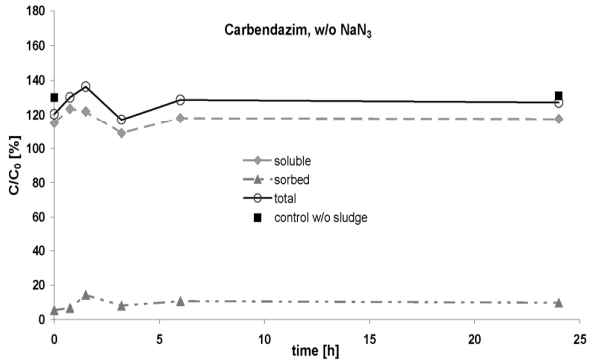
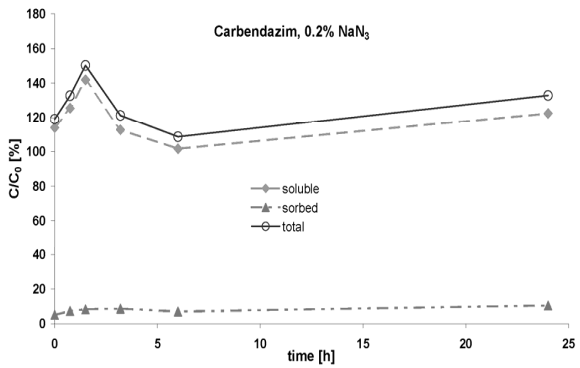

Carbendazim, w/o NaN3
C/C0 [%]
time [h]
soluble
sorbed
total
control w/o sludge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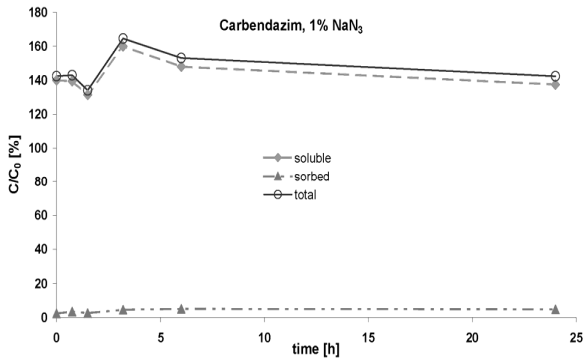
total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C/C0 [%]
time [h]
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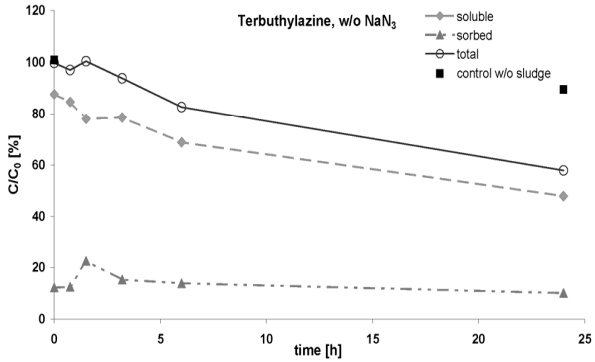
sorbed
total


Terbuthylazine, w/o NaN3
C/C0 [%]
time [h]
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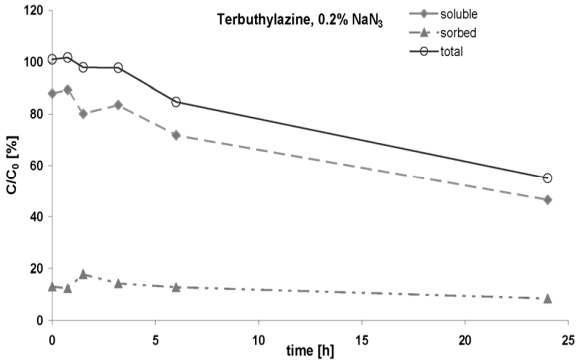
sorbed
total
control w/o sludge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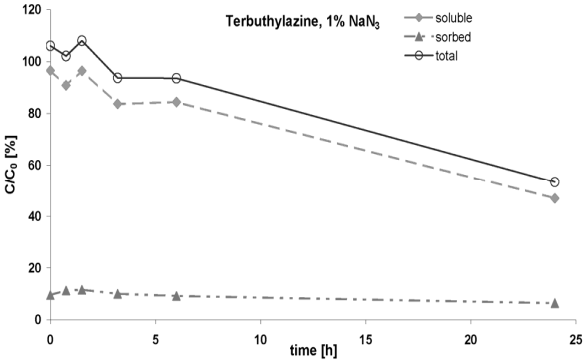
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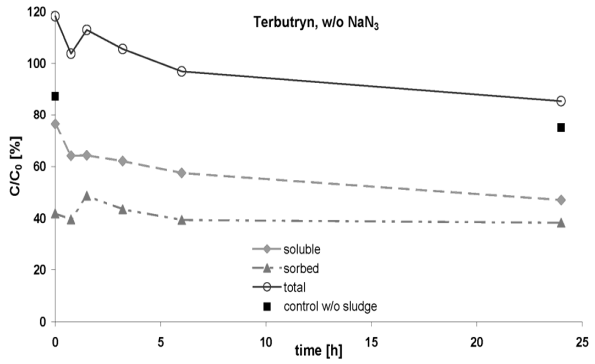
total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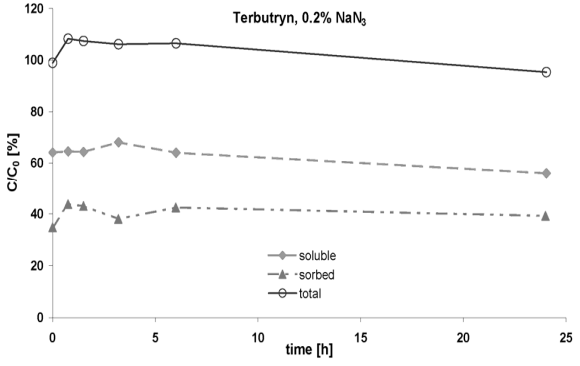
control w/o sludge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time [h]
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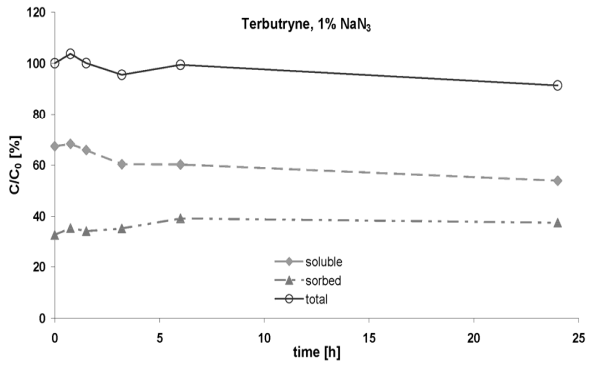
total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C/C0 [%]
time [h]
soluble
sorbed
total

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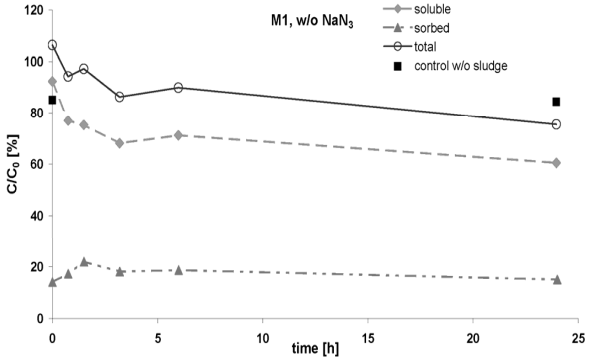
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sorbed
total
control w/o sludge
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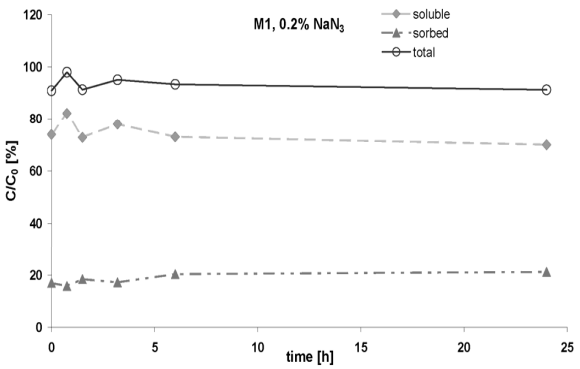
time [h]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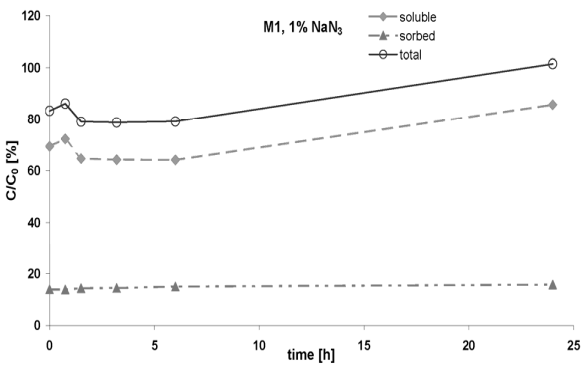
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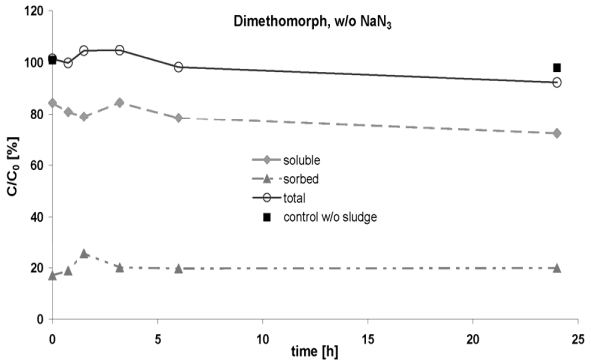
time [h]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sorbed
total
control w/o sludge
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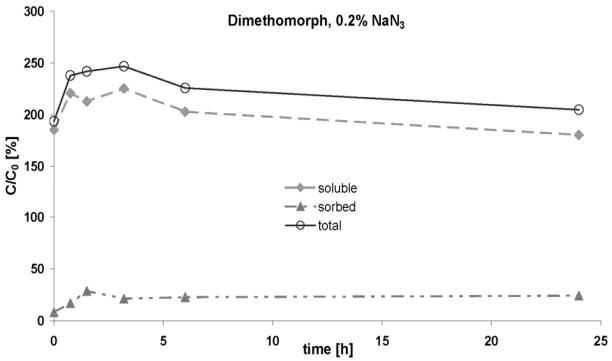
time [h]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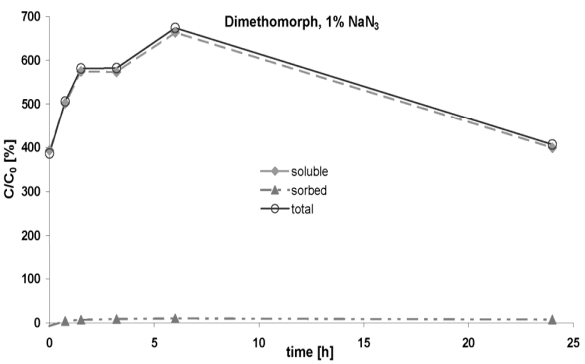
time [h]


Dimethomorph, 1% NaN3
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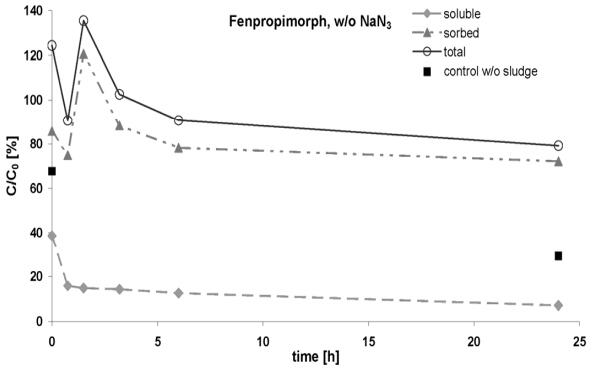
time [h]


Fenpropimorph, w/o NaN3
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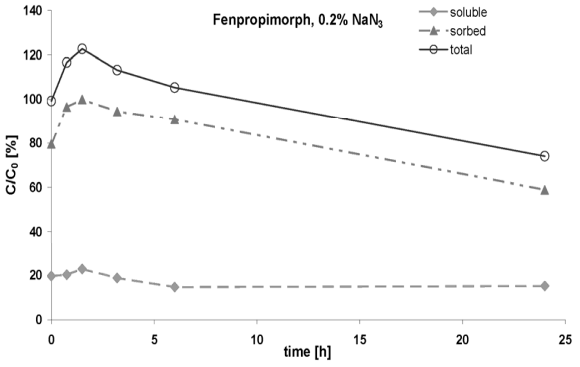
time [h]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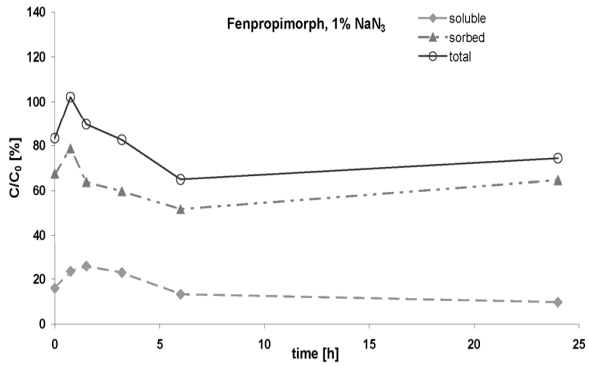
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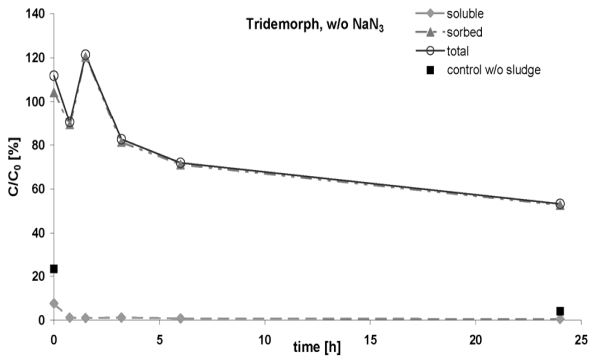
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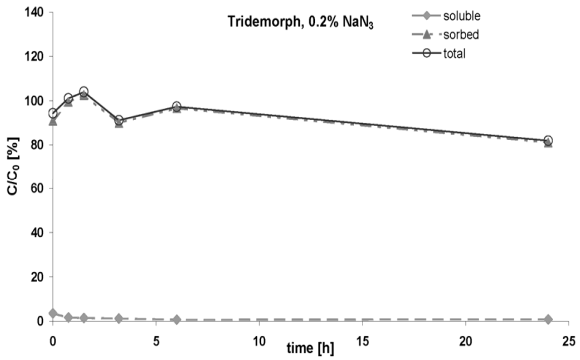
time [h]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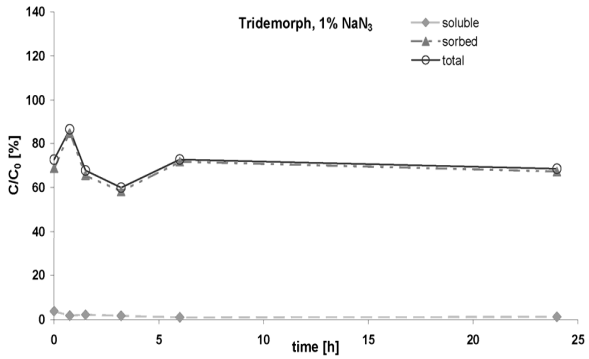
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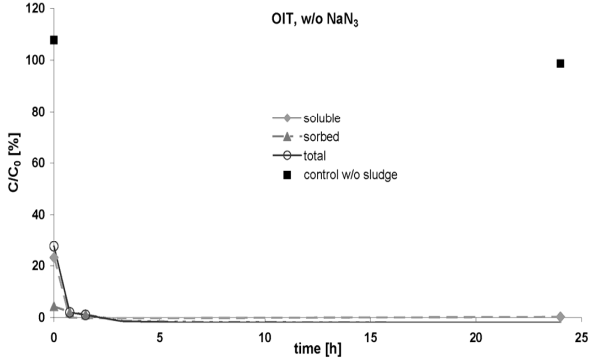
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total
control w/o sludge
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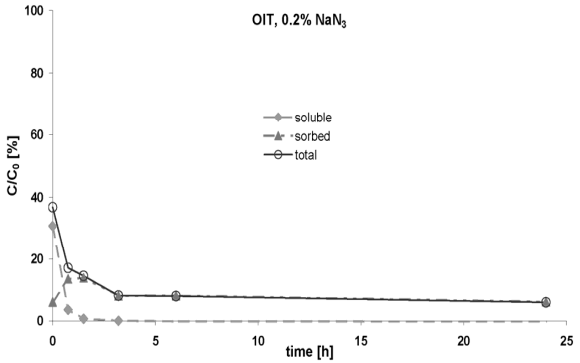
time [h]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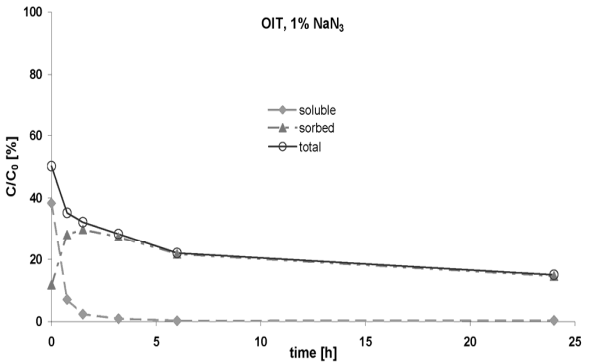
time [h]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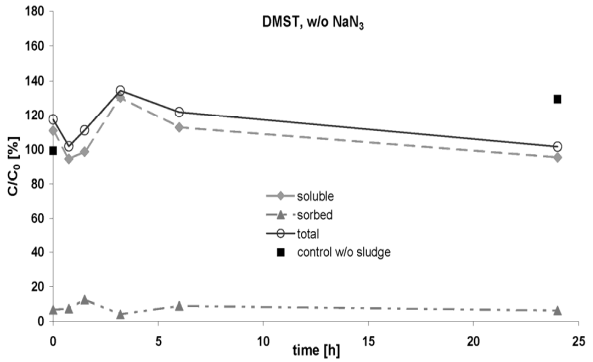
time [h]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sorbed
total
control w/o sludge
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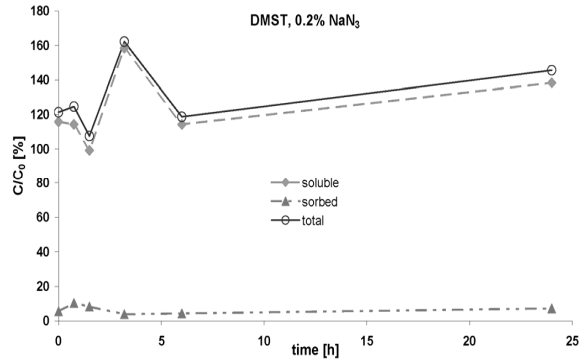
time [h]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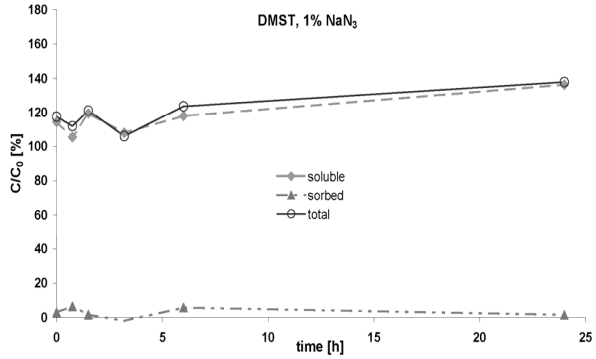
sorbed
total
C/C0 [%]
time [h]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soluble
sorbed
total
C/C0 [%]
time [h]

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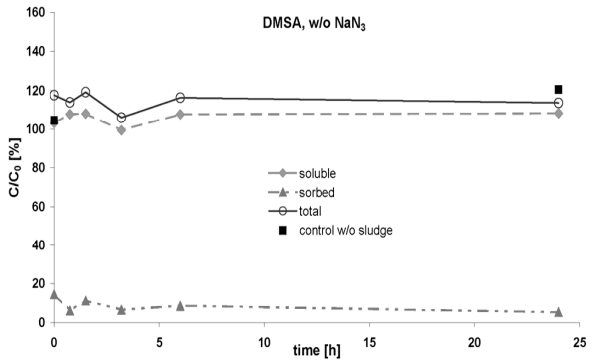
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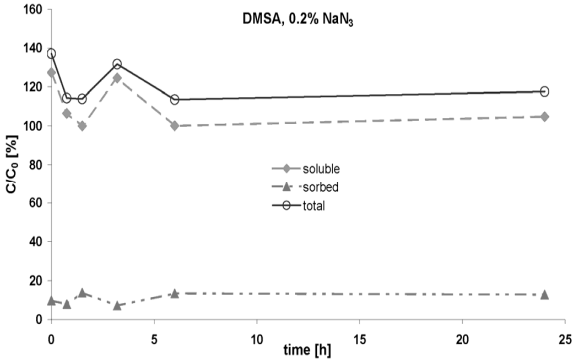
time [h]
soluble
sorbed
total
control w/o sludge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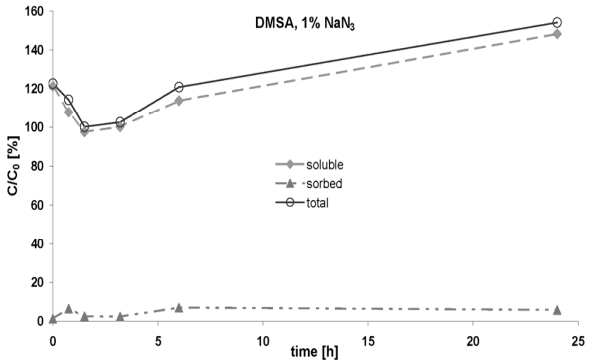
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C/C0 [%]
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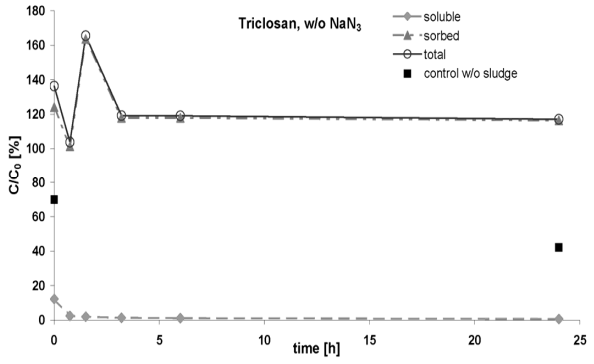
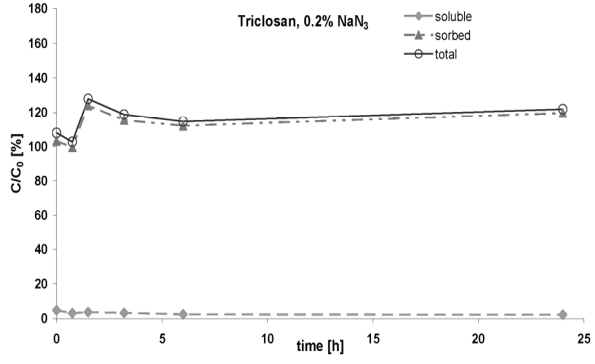

Triclosan, w/o NaN3
C/C0 [%]
time [h]
soluble
sorbed
total
control w/o sludge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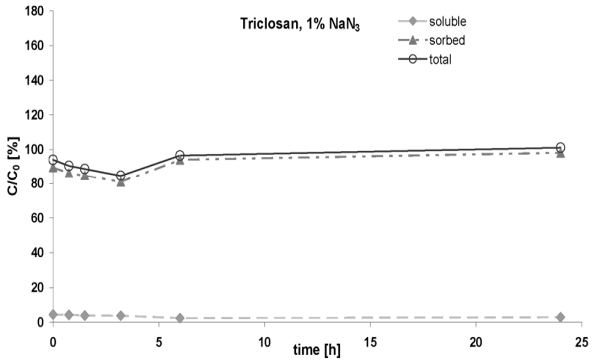
sorbed
total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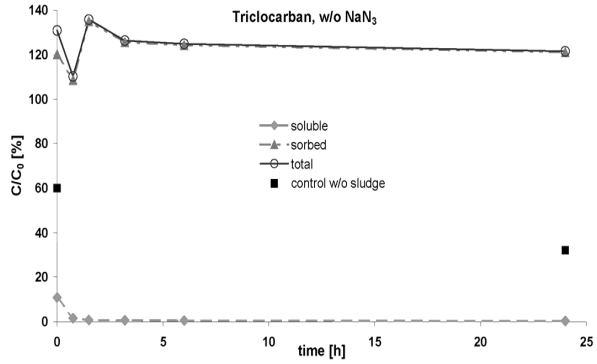
sorbed
total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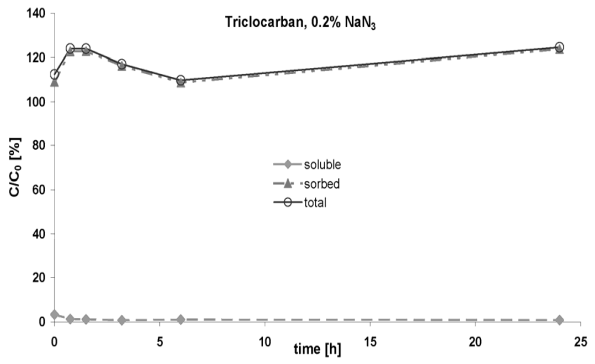
C/C0 [%]
time [h]
soluble
sorbed
total
control w/o sludge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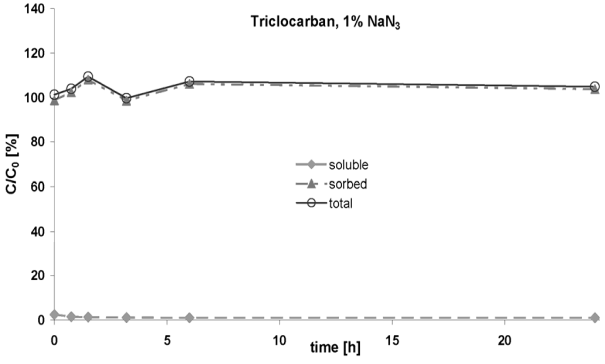
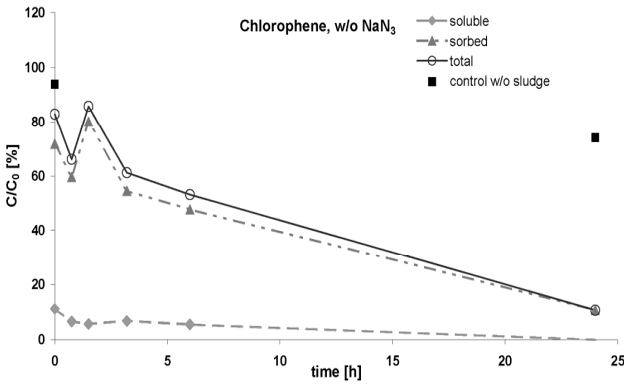
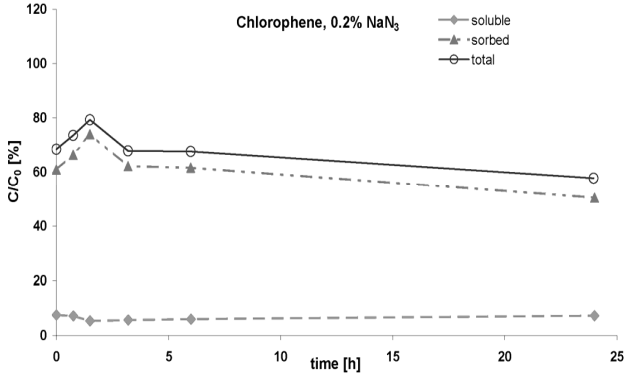
C/C0 [%]
time [h]
soluble
sorbed
total


Triclocarban, 1% NaN3
C/C0 [%]
time [h]
soluble
sorbed
total

continues


Chlorophene, w/o NaN3
soluble
sorbed
total
control w/o sludge
C/C0 [%]
time [h]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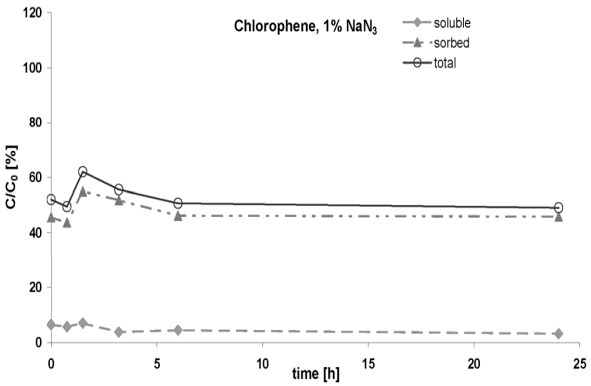
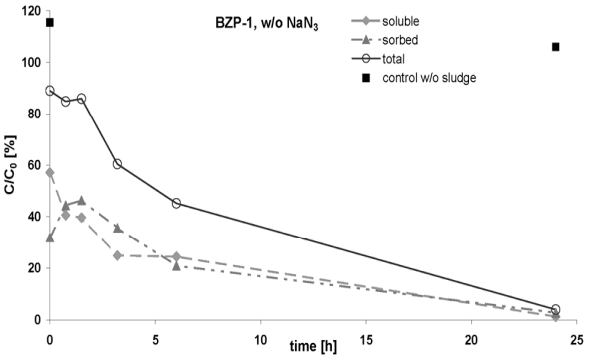
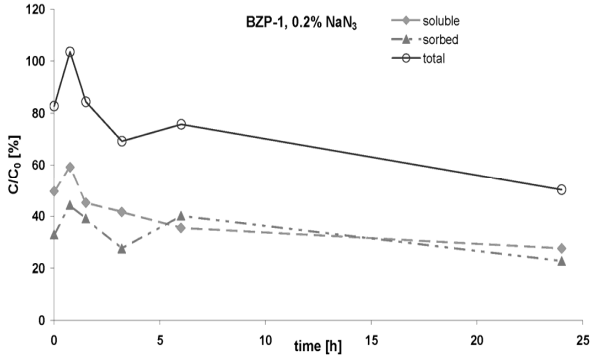
soluble
sorbed
total
C/C0 [%]
time [h]


Chlorophene, 1% NaN3
soluble
sorbed
total
C/C0 [%]
time [h]


BZP-1, w/o NaN3
soluble
sorbed
total
control w/o sludge
C/C0 [%]
time [h]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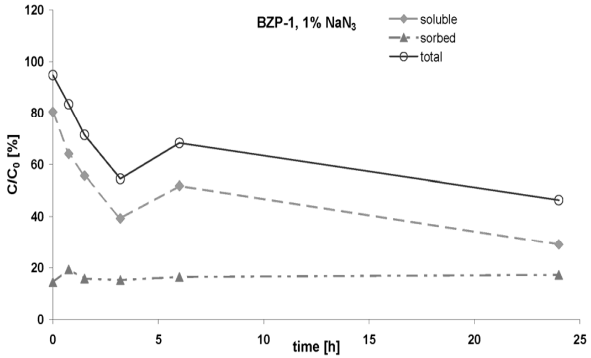
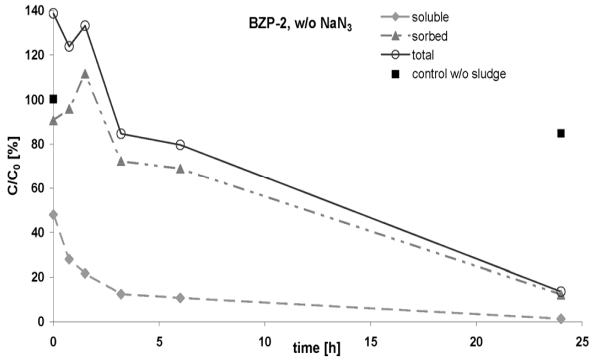
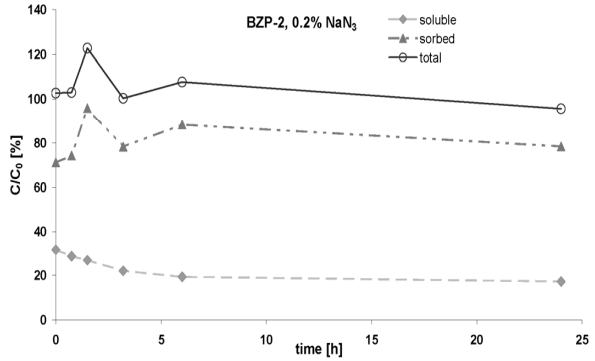
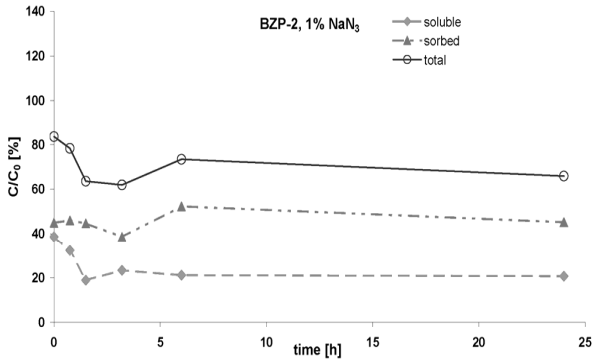
soluble
sorbed
total
C/C0 [%]
time [h]


BZP-1, 1% NaN3
soluble
sorbed
total
C/C0 [%]
time [h]


BZP-2, w/o NaN3
soluble
sorbed
total
control w/o sludge
C/C0 [%]
time [h]


BZP-2, 0.2% NaN3
soluble
sorbed
total
C/C0 [%]
time [h]


BZP-2, 1% NaN3
soluble
sorbed
total
C/C0 [%]
time [h]

continues

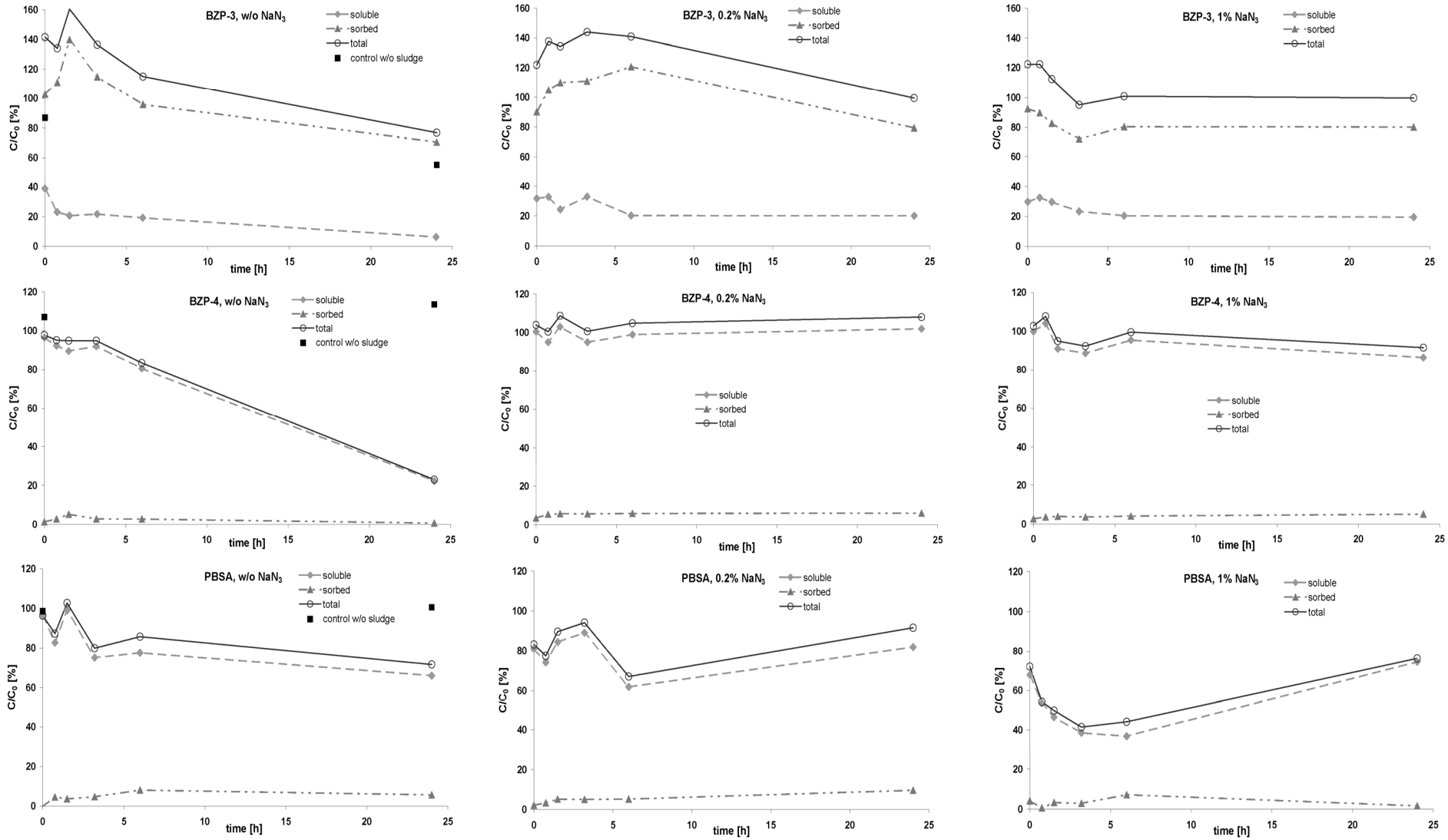


Figure SI 3-1. Ratio [%] of sorbed, soluble and the total concentration to the spike concentration of 10 µg L^{-1} over time for biocides und UV-filters with and without addition of NaN_3 for microbial activity inhibition. The natural background concentrations as well as the water content of the sludge were assessed and considered for the calculation.

7.3 SI of chapter 4

Outline

Table SI 4-1. Wastewater characterization along the treatment processes as average values for the different weekly sampling campaigns. ND: not determined.

Parameter		Sampling campaign	Spot 1	Spot 2	Spot 3	Spot 4
pH	-	March	7.9	7.8	7.5	7.9
		May	7.9	7.8	7.5	8.0
		July	7.9	7.9	7.6	7.9
TSS	[g m^{-3}]	March	244	52	6	3
		May	238	55	6	4
		July	159	42	8	7
COD	[g m^{-3}]	March	427	144	24	25
		May	492	165	19	18
		July	300	100	16	26
BOD	[g m^{-3}]	March	157	45	<4	<4
		May	167	48	<4	<4
		July	109	34	<4	<4
TOC	[g m^{-3}]	March	161	40	7	7
		May	218	48	7	7
		July	98	29	6	6
NH_4-N	[g m^{-3}]	March	27	22	ND	ND
		May	33	26	ND	ND
		July	24	21	ND	ND
NO_3-N	[g m^{-3}]	March	ND	ND	18	9
		May	ND	ND	19	9
		July	ND	ND	18	8
PO_4-P	[g m^{-3}]	March	4.3	2.8	0.60	0.67
		May	5.3	3.4	0.70	0.56
		July	4.2	2.1	0.58	0.46

Table SI 4-2. Sorption coefficients $K_{d,sec}$ and recoveries of the spiked psycho-active drugs (15 µg L^{-1}) determined in sorption batch experiments with activated sludge (0.25 g_{TOC} g_{TSS}^{-1}). The $K_{d,sec}$ values were determined separately for every sampling time point (*n*=3) and as an average of all samplings (*n*=9). The range indicates the 95% confidence interval for *n*=3 and *n*=9, respectively.

Sampling time		2.5 h (*n* = 3)		5 h (*n* = 3)		14h (*n* = 3)		All time points (n = 9)		
Compound	**Log K_{OW}[a]**	**$K_{d,sec}$ [L Kg_{TSS}^{-1}]**	**recovery [%]**	**$K_{d,sec}$ [L Kg_{TSS}^{-1}]**	**recovery [%]**	**$K_{d,sec}$ [L Kg_{TSS}^{-1}]**	**recovery [%]**	**K_d [L Kg_{TSS}^{-1}]**	**K_{OC} [L Kg_{TOC}^{-1}]**	**Log K_d**
Analgesics										
Morphine	-0.1	11 ± 5	43.0 ± 10	13 ± 8	29 ± 12	16 ± 5	18 ± 4	12 ± 2	49 ± 7	1.08
Codeine	0.6-1.2	14 ± 2	73 ± 2	14 ± 3	63 ± 8	13 ± 2	52 ± 7	14 ± 1	55 ± 3	1.15
Dihydrocodeine	n.a.	12 ± 2	97 +/- 4	11 ± 2	93 ± 5	12 ± 3	91 ± 3	12 ± 1	48 ± 3	1.08
Hydrocodon	2.2	16 ± 3	101 ± 16	16 ± 2	96 ± 2	20 ± 9	94 ± 6	17 ± 2	71 ± 9	1.23
Oxycodon	0.7	12 ± 4	92 ± 18	13 ± 1	85 ± 5	16 ± 6	83 ± 11	14 ± 2	55 ± 7	1.15
Tramadol	3.0	9 ± 10	96 ± 40	14 ± 7	75 ± 11	15 ± 8	83 ± 10	13 ± 3	53 ± 12	1.11
Methadone	3.9	73 ± 12	88 ± 8	80 ± 11	77 ± 5	74 ± 11	87 ± 5	76 ± 4	308 ± 16	1.88
Tranquilizers										
Diazepam	2.7	54 ± 3	98 ± 3	52 ± 3	96 ± 2	53 ± 6	97 ± 4	53 ± 1	216 ± 5	1.72
Nordiazepam	2.9	65 ± 4	102 ± 4	65 ± 8	99 ± 6	64 ± 7	100 ± 4	65 ± 2	263 ± 7	1.81
Oxazepam	2.2	47 ± 3	99 ± 2	45 ± 4	98 ± 4	48 ± 5	100 ± 4	47 ± 1	191 ± 6	1.67
Antiepileptics										
Carbamazepine	2.45	17 ± 4	104 ± 16	18 ± 4	97 ± 4	16 ± 4	103 ± 8	17 ± 1	68 ± 4	1.23
DH-CBZ	n.a.	12 ± 2	103 ± 7	12 ± 2	99 ± 2	11 ± 2	102 ± 10	12 ± 1	48 ± 2	1.08
DHH	n.a.	8 ± 3	103 ± 14	7 ± 4	94 ± 27	5 ± 2	101 ± 7	7 ± 1	28 ± 5	0.85
Primidone	0.9	8 ± 3	96 ± 4	7 ± 2	94 ± 6	6 ± 2	94 ± 6	7 ± 1	28 ± 4	0.85
Antidepressants										
Doxepin	2.4	151 ± 99	95 ± 27	154 ± 39	78 ± 11	107 ± 30	79 ± 11	139 ± 23	566 ± 93	2.14

K_{OW}: octanol-water partition coefficient, K_{OC}: organic-carbon-normalized K_d, n.a.: not available
[a] Moffat et al., 2004

7.4 SI of chapter 5

Outline

Table SI 5-1. Absolute recoveries [%] of codeine and its TPs: Influence of enrichment conditions (n = 2). TPs for which reference standards were available are printed in bold.

	Groundwater				WWTP effluent		WWTP influent	
SPE cartridge	MCX		HLB		MCX			
pH	pH 3	pH 3	pH 2	pH 7	pH 3	pH 3	pH 3	pH 3
Elution solvent	ACN/ NH_4OH[a]	MeOH/ NH_4OH[b]	ACN	ACN	ACN/ NH_4OH[a]	MeOH/ NH_4OH[b]	ACN/ NH_4OH[a]	MeOH/ NH_4OH[b]
TP 264	52	73	30	34	6	45	11	68
TP 298	ND	ND	ND	ND	112	12	90	17
Codeine	97	ND	67	94	85	71	84	69
TP 300(1)	91	85	82	92	88	81	86	79
TP 300(2)	ND	ND	ND	ND	ND	ND	ND	ND
TP 300(3)	91	62	43	38	ND	ND	ND	ND
TP 302	95	83	67	31	79	71	78	70
TP 314	90	86	111	102	110	40	84	48
TP 316(1)	93	88	84	102	94	85	97	72
TP 316(2)	ND	ND	ND	ND	ND	ND	ND	ND
TP 318	109	99	51	94	ND	ND	ND	ND
TP 320	5	100	77	31	ND	ND	ND	ND
TP 330	52	99	85	116	ND	ND	ND	ND
TP 332(1)	66	86	89	89	94	83	87	86
TP 332(2)	ND	ND	ND	ND	ND	ND	ND	ND
TP 334	96	90	43	58	ND	ND	ND	ND
TP 346	11	68	24	21	10	29	14	32
TP 348	16	88	74	58	ND	ND	ND	ND
TP 364	< 5	15	20	5	ND	ND	ND	ND

[a] final method 1, [b] final method 2

Acidification after sampling

Measurements of standard solutions of TP 298 (codeinone), TP 314 and TP 316 prepared in ultrapure water, acidified ultrapure water (0.2% formic acid, ~ pH 2) and basified ultrapure water (ammonium hydroxide, pH 10) revealed the dependency of the compound stability from pH. While the addition of formic acid had no influence on the peak intensities, an increase of the pH supported the water addition at the β-position of the α,β-unsaturated keto group of these TPs. Therefore, all samples and standard solutions were acidified with formic acid to a final concentration of 0.2%.

Table SI 5-2. Retention time, precursor ion, product ions and MS parameters used for LC-MS/MS detection of opium alkaloids (codeine, dihydrocodeine, hydrocodone, oxycodone and morphine) and TPs of codeine and dihydrocodeine. LC-MS/MS chromatograms of selected compounds are shown in Figure SI 5-1.

compound	retention time [min]	q_1 transition	q_2 transition	DP[a] [V]	CE[b] (q_1/q_2) [eV]	CXP[c] (q_1/q_2) [V]
codeine	19.9	300 → 215	300 → 165	70	37/53	16/12
TP 264	9.0	264 → 218	264 → 44	55	29/63	11/5
Morphine	8.5	286 → 201	286 → 152	85	35/77	6/14
TP 298	23.1	298 → 241	298 → 58	60	32/50	19/9
TP 300(1)	16.0	300 → 199	300 → 243	70	35/27	15/14
TP 300(2)	22.0	300 → 199	300 → 243	70	35/27	15/14
TP 300(3)	21.4	300 → 240	300 → 167	60	35/45	10/10
Hydrocodone	22.6	300 → 199	300 → 128	90	41/77	6/10
TP 302	13.2	302 → 284	302 → 240	45	19/35	10/10
Dihydrocodeine	19.3	302 → 128	302 → 201	70	85/39	12/16
Isodihydrocodeine	22.2	302 → 128	302 → 201	70	85/39	12/16
TP 314	22.9	314 → 296	314 → 254	50	26/35	6/5
TP 316(1)	18.2	316 → 298	316 → 254	50	23/35	6/5
Oxycodone	21.8	316 → 256	316 → 212	56	35/59	8/14
TP 316(2)	22.2	316 → 241	316 → 58	60	32/50	19/9
TP 318	14.3	318 → 199	318 → 215	60	40/40	10/10
TP 320	20.3	320 → 204	320 → 216	85	35/35	10/10
TP 330	10.0	330 → 199	330 → 227	70	45/40	10/13
TP 332(1)	19.5	332 → 314	332 → 270	60	30/33	8/6
TP 332(2)	10.2	332 → 239	332 → 314	80	40/29	12/9
TP 334	13.7	334 →316	334 → 254	60	40/45	10/10
TP 346	8.8	346 → 58	346 → 271	70	50/27	9/15
TP 348	18.0	348 → 330	348 → 148	60	40/50	10/10
TP 364	10.8	364 → 320	364 → 276	60	40/45	10/10

[a] DP = Declustering potential, [b] CE = Collision energy, [c] CXP = Collision cell exit potential,

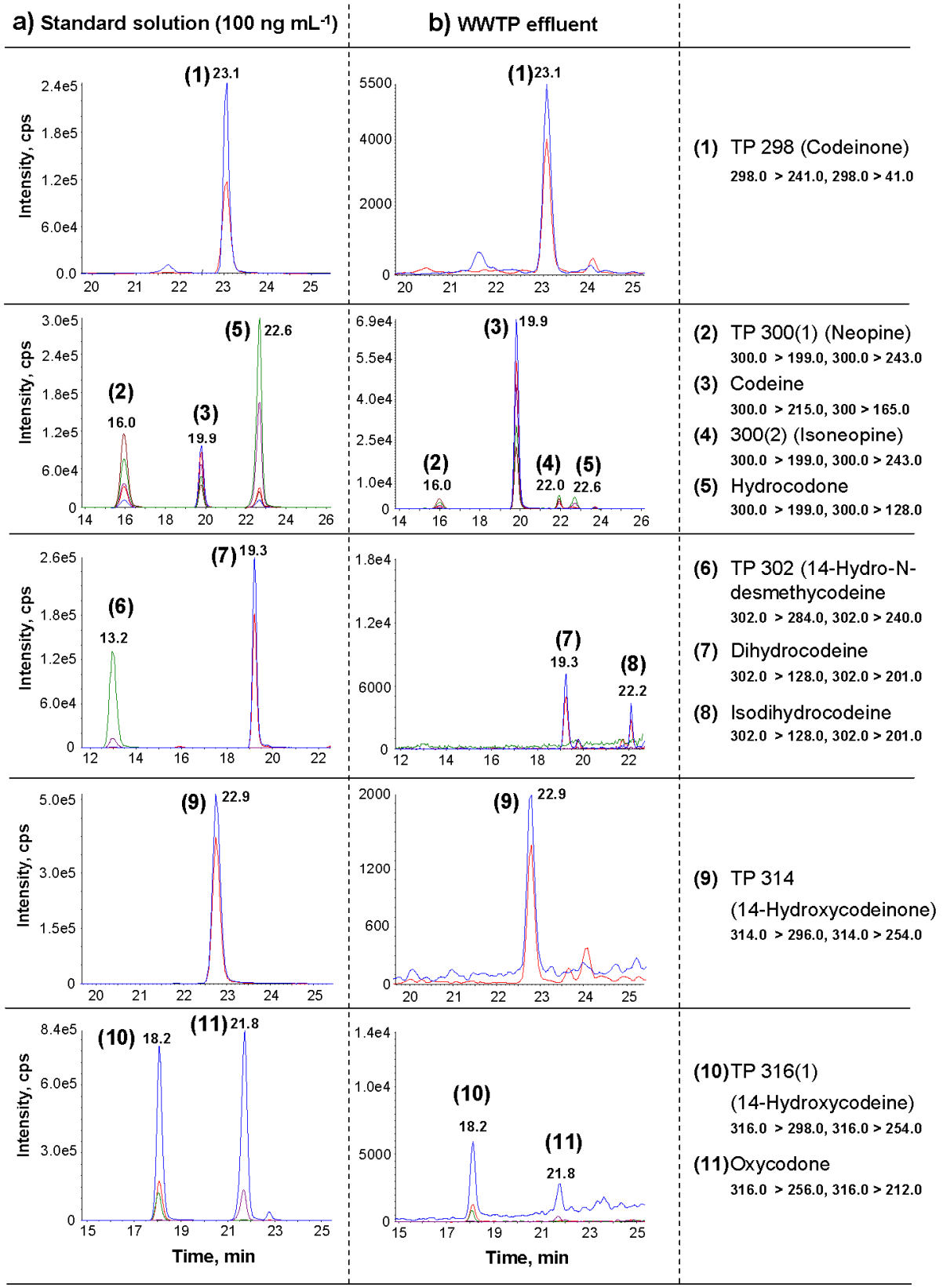
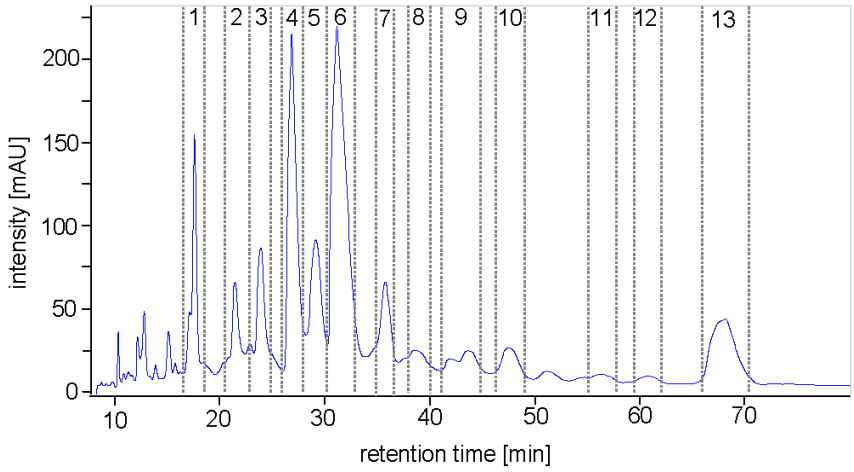


Figure SI 5-1. Selected LC-MS/MS chromatograms of opium alkaloids and TPs of codeine and dihydrocodeine for a) a standard solution (100 ng L^{-1}) and b) an extract from the effluent of a WWTP. The analytes were selected according to their occurrence in WWTPs. The chromatograms show that any interferences of analytes exhibiting the same precursor and product ions can be excluded due to their sufficient chromatographic separation. For details about the MS parameters see Table SI 5-2.

Transformation of codeine at elevated concentrations and extended incubation periods

Concentrations of codeine up to 200 mg L^{-1} and longer incubation periods of up to 35 d were used in order to isolate high quantities of TPs for identification and preparation of analytical reference standards. The addition of an additional carbon source (peptone) did not increase the transformation rate, which indicated that the carbon source was not growth limiting. The same major TPs as for the concentrations in the µg L^{-1} range and no significant inhibition of transformation was detected. Hence, the results obtained from the batch experiments performed at elevated codeine concentrations were qualitatively similar to those performed at environmental relevant concentrations. However, negative effects of elevated codeine concentrations on the microbial activity cannot be excluded, since the transformation kinetics was only performed at environmental relevant concentrations.



HPLC fraction	nominal mass	denotation	HPLC fraction	nominal mass	denotation
1	263	TP 264	7	347	TP 348
2	363	TP 364	8	331	TP 332
3a	301	TP 302	9a	315	
3b	317	TP 318	9b	319	TP 320
3c	333	TP 334	10	335	
4	299	TP 300(1)	11	299	TP 300(3)
5	345	TP 346	12	299	TP 300(2)
6	315	TP 316	13	313	TP 314

Figure SI 5-2. HPLC-UV chromatogram (213 nm) of diluted activated sludge after 27 d of incubation. The sludge was spiked with 200 mg L^{-1} codeine and incubated under aerobic conditions. The sample was filtered through a 1 µm glass fibre filter and a 0.45 µm cellulose acetate filter and concentrated about ten times by freeze-drying. From this sample, 13 fractions were collected for isolation of TPs using an automated sample collector. In total, 14 TPs (bold printed) were isolated from these fractions for NMR analysis and preparation of analytical standards.

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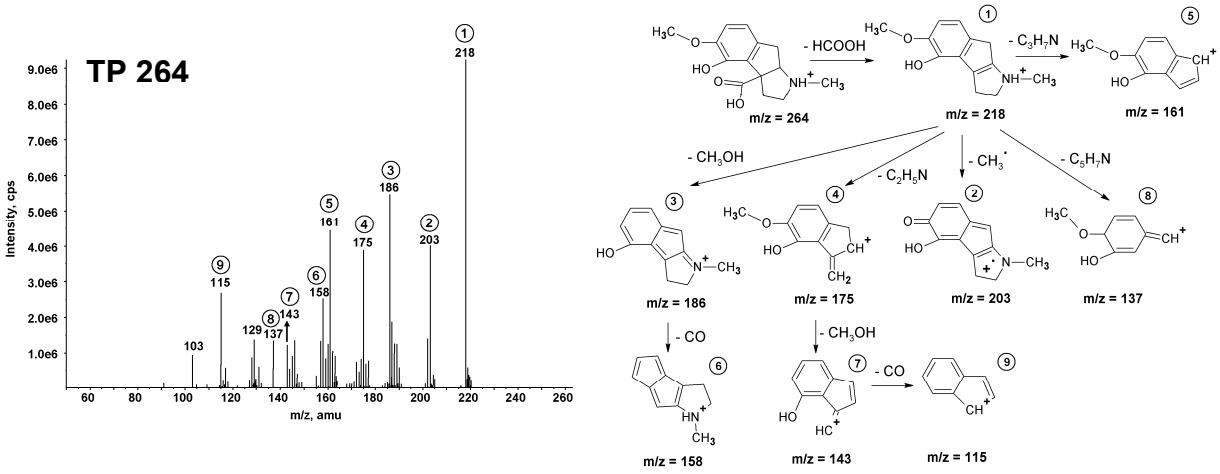
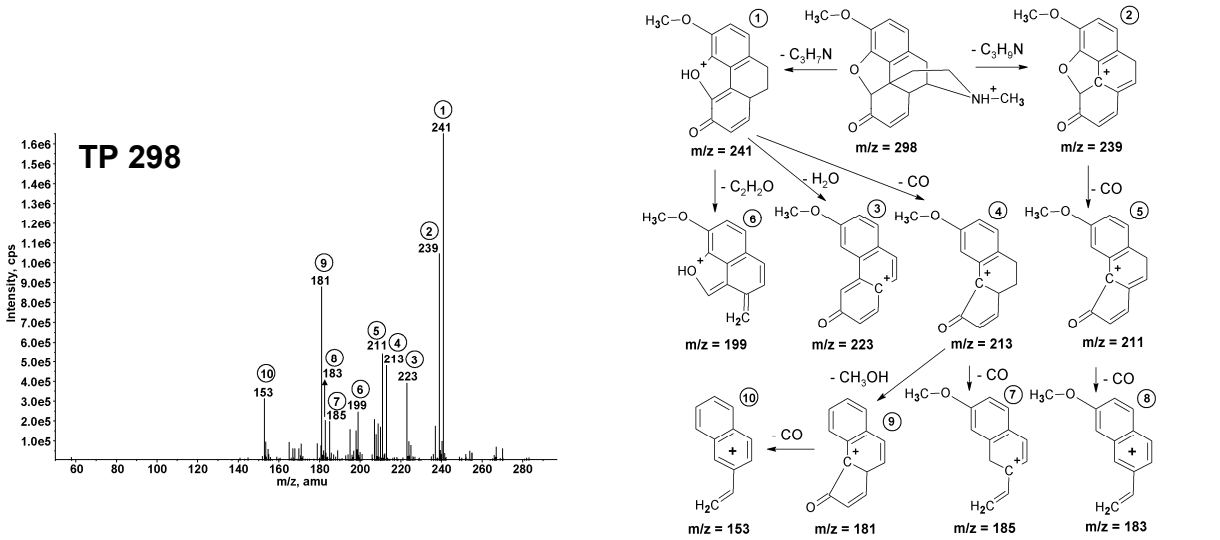
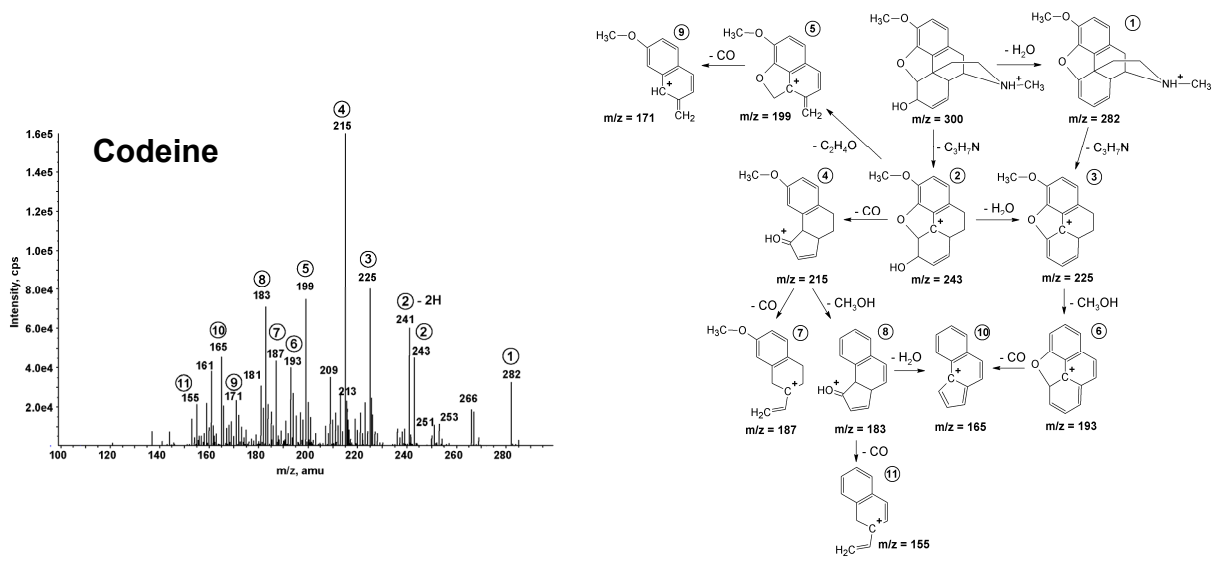
Proposed MS fragmentation pathways of codeine and codeine TPs







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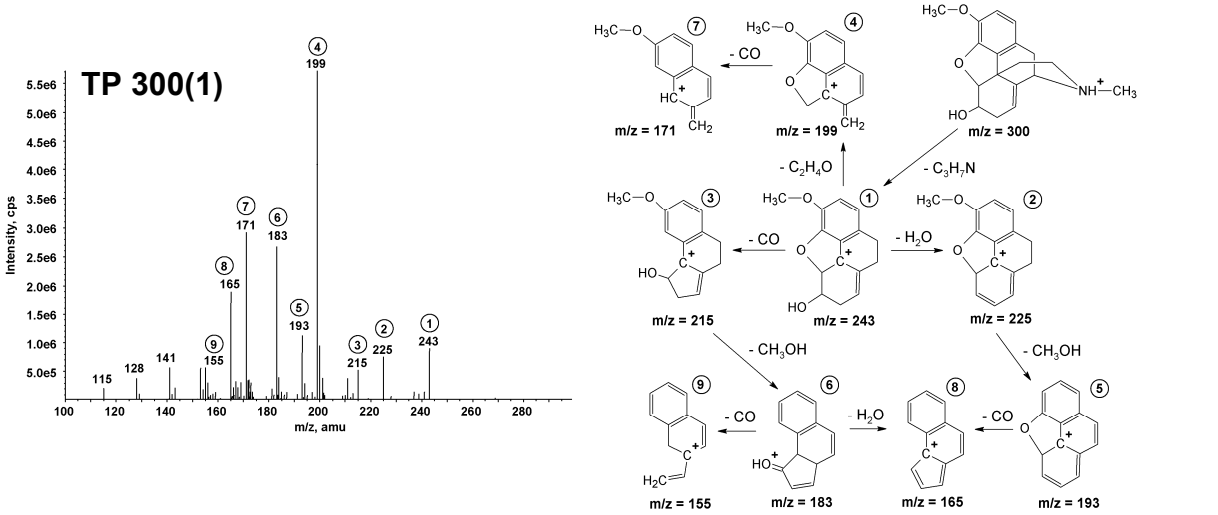
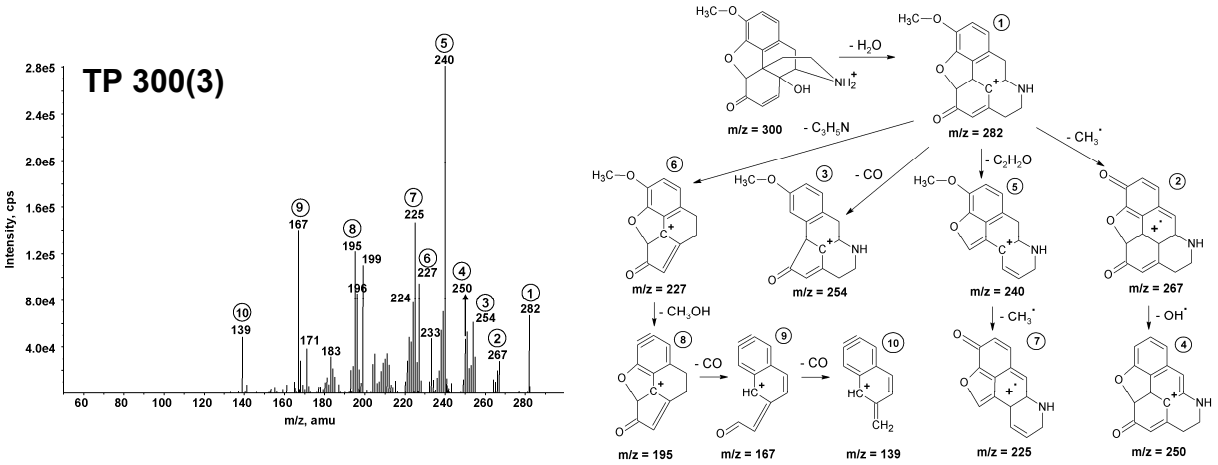
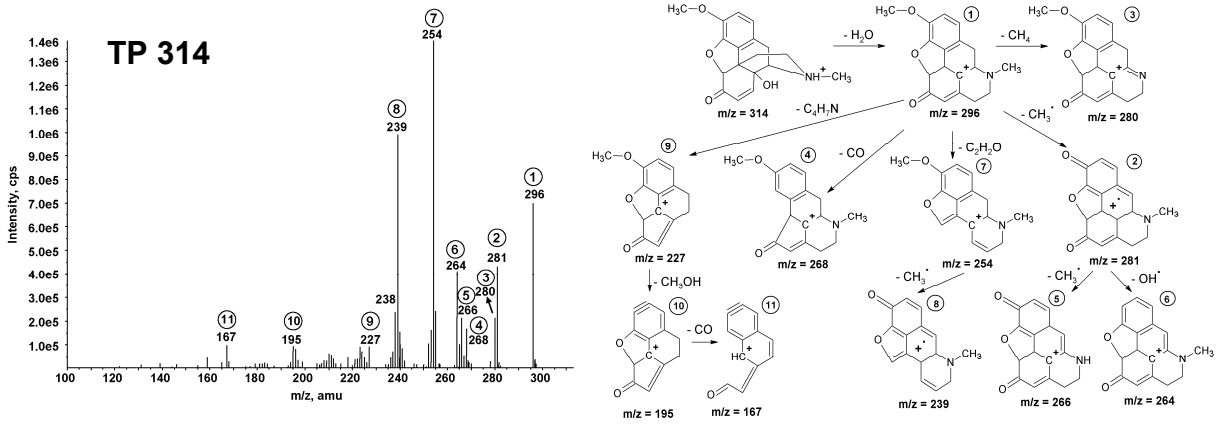
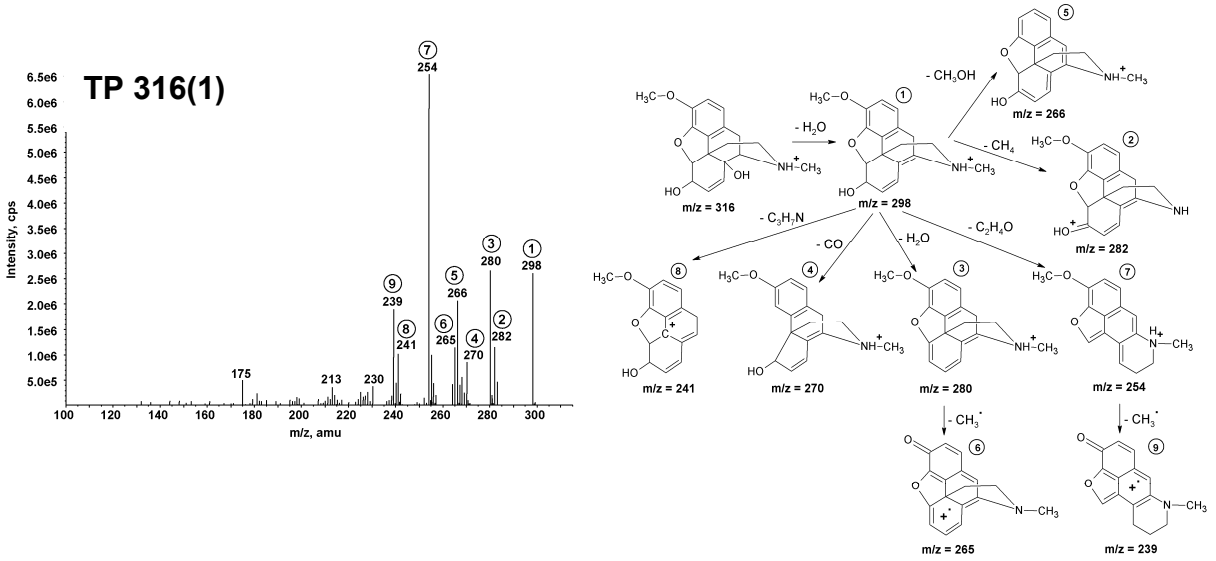
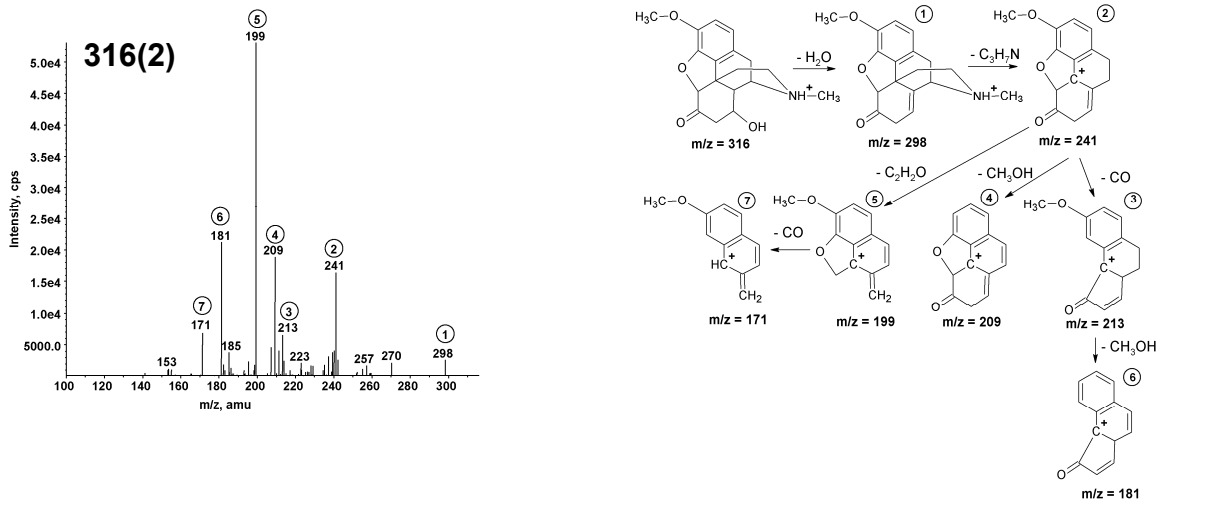

TP 300(1)
Intensity, cps
m/z, amu
m/z = 171
m/z = 199
m/z = 300
m/z = 215
m/z = 243
m/z = 225
m/z = 155
m/z = 183
m/z = 165
m/z = 193
- CO
- C_2H_4O
- C_3H_7N
- H_2O
- CH_3OH


TP 300(3)
Intensity, cps
m/z, amu
m/z = 300
m/z = 282
m/z = 227
m/z = 254
m/z = 240
m/z = 267
m/z = 195
m/z = 167
m/z = 139
m/z = 225
m/z = 250
- H_2O
- C_3H_5N
- $CH_3^{\bullet}$
- C_2H_2O
- CO
- CH_3OH
- $OH^{\bullet}$


TP 314
Intensity, cps
m/z, amu
m/z = 314
m/z = 296
m/z = 280
m/z = 227
m/z = 268
m/z = 254
m/z = 281
m/z = 195
m/z = 167
m/z = 239
m/z = 266
m/z = 264
- H_2O
- CH_4
- C_4H_7N
- $CH_3^{\bullet}$
- C_2H_2O
- CO
- CH_3OH
- $OH^{\bullet}$

continues


TP 316(1)
Intensity, cps
m/z, amu
m/z = 316
m/z = 298
m/z = 266
m/z = 282
m/z = 241
m/z = 270
m/z = 280
m/z = 254
m/z = 265
m/z = 239


316(2)
Intensity, cps
m/z, amu
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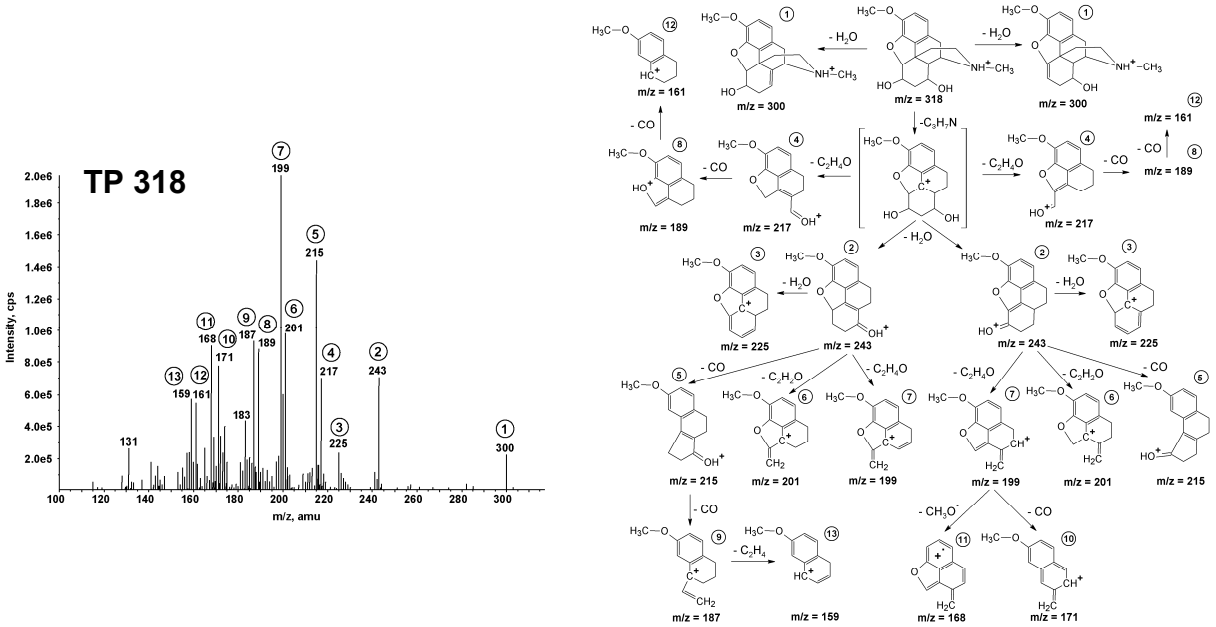
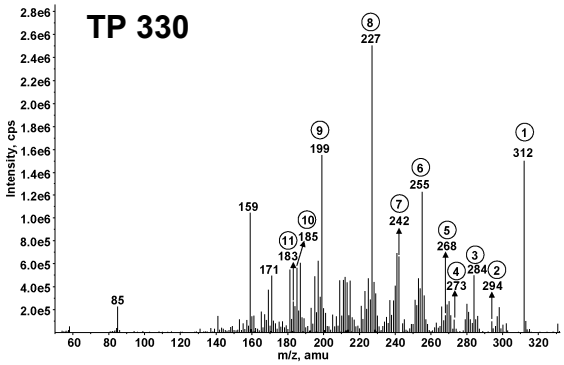
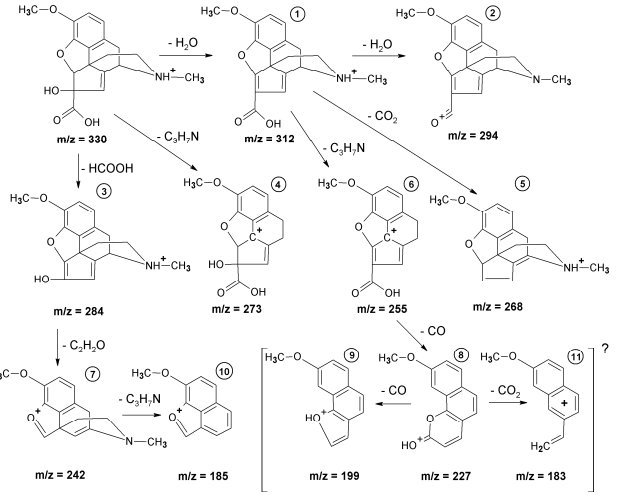
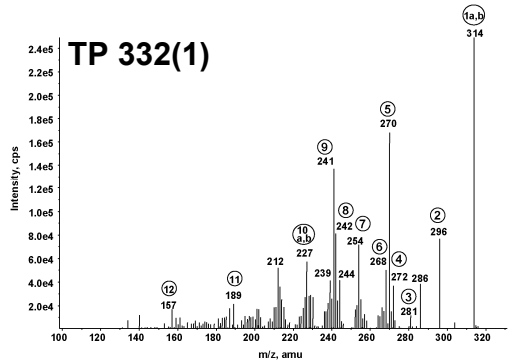
m/z = 298
m/z = 241
m/z = 171
m/z = 199
m/z = 209
m/z = 213
m/z = 181

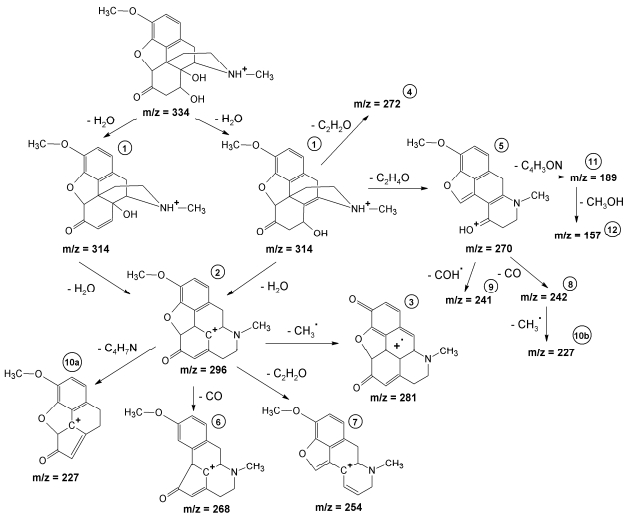

TP 318
Intensity, cps
m/z, amu
m/z = 161
m/z = 300
m/z = 318
m/z = 189
m/z = 217
m/z = 225
m/z = 243
m/z = 215
m/z = 201
m/z = 199
m/z = 187
m/z = 159
m/z = 168
m/z = 171

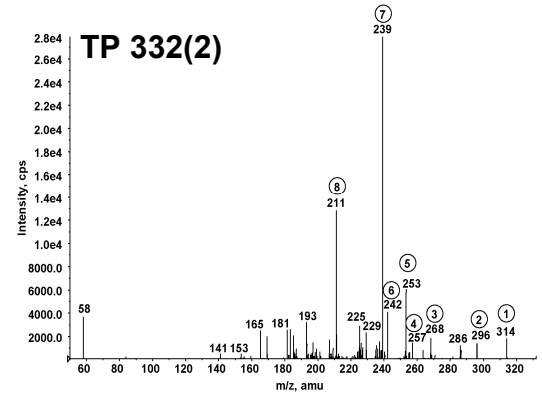
continues


TP 330
Intensity, cps
m/z, amu
85
159
171
183
185
199
227
242
255
268
273
284
294
312


m/z = 330
m/z = 312
m/z = 294
m/z = 284
m/z = 273
m/z = 255
m/z = 268
m/z = 242
m/z = 185
m/z = 199
m/z = 227
m/z = 183
- H2O
- CO2
- C3H7N
- HCOOH
- C2H2O
- CO


TP 332(1)
Intensity, cps
m/z, amu
157
189
212
227
239
241
242
244
254
270
272
281
286
296
314


m/z = 334
m/z = 314
m/z = 272
m/z = 270
m/z = 189
m/z = 157
m/z = 241
m/z = 242
m/z = 227
m/z = 296
m/z = 281
m/z = 268
m/z = 254
- H2O
- C2H2O
- C2H4O
- C4H3ON
- CH3OH
- COH
- CO
- CH3
- C2H4NF
- C2H2O


TP 332(2)
Intensity, cps
m/z, amu
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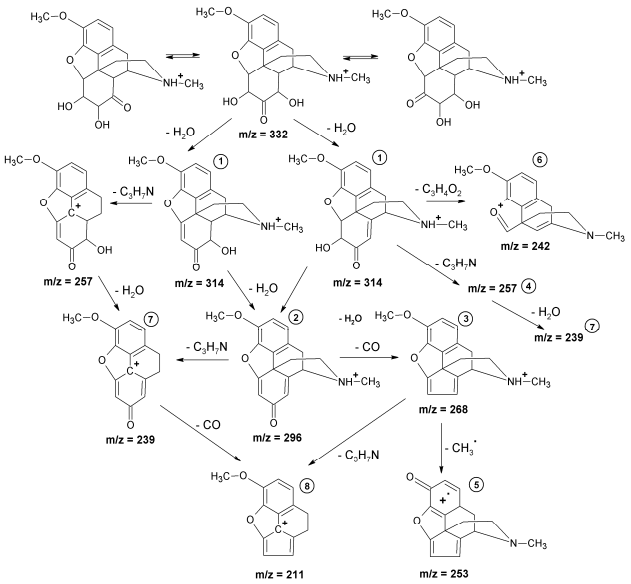
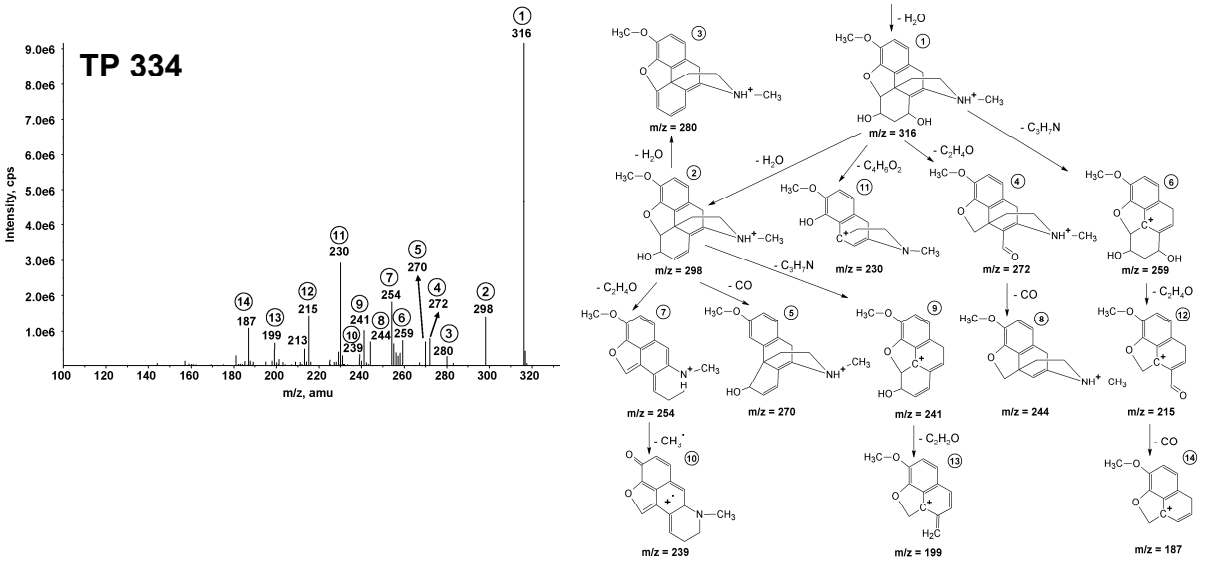
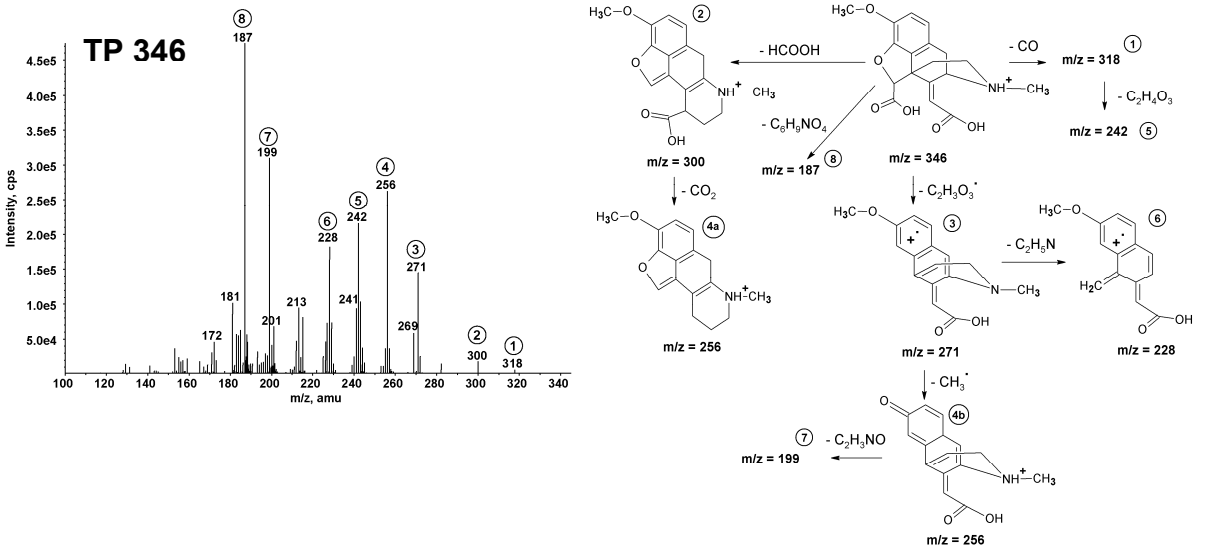
141
153
165
181
193
211
225
229
239
242
253
257
268
286
296
314


m/z = 332
m/z = 257
m/z = 314
m/z = 242
m/z = 239
m/z = 296
m/z = 268
m/z = 211
m/z = 253
- H2O
- C3H7N
- C3H4O2
- CO
- CH3

continues


TP 334
Intensity, cps
m/z, amu
m/z = 334
m/z = 316
m/z = 280
m/z = 298
m/z = 230
m/z = 272
m/z = 259
m/z = 254
m/z = 270
m/z = 241
m/z = 244
m/z = 215
m/z = 239
m/z = 199
m/z = 187


TP 346
Intensity, cps
m/z, amu
m/z = 346
m/z = 318
m/z = 300
m/z = 187
m/z = 242
m/z = 256
m/z = 271
m/z = 228
m/z = 199
m/z = 256

continues

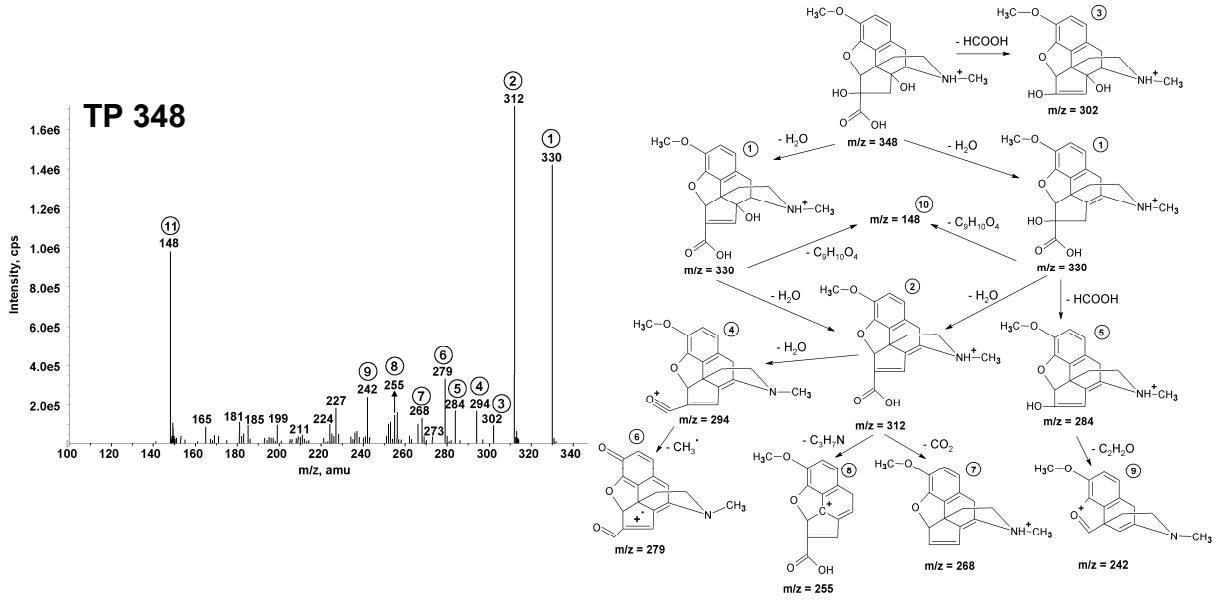


Figure SI 5-3. MS^2 spectra of codeine and codeine TPs determined by Qq-LIT-MS (CE 40 eV). The corresponding fragmentation pathways were proposed based on MS^3 and precursor ion scans using Qq-LIT-MS as well as MS^n experiments using LTQ-Orbitrap MS. The accurate masses and elemental compositions of the precursor and product ions determined by LTQ-Orbitrap-MS are listed in Table SI 5-3.

Results from HRMS: Lists of precursor and product ions

Table SI 5-3. List of precursor $[M+H]^+$ and product ions from MS and MS^n spectra obtained from codeine and codeine TPs using LTQ-Orbitrap-MS with electrospray ionization in the positive ionization mode.

Compound	Precursor ion	Product ions		Proposed fragmentation
	Measured MW (Error in ppm) Elemental comp.	Measured MW (Error in ppm)	Elemental composition	
TP 264	264.12312 (0.32) $C_{14}H_{18}NO_4$	218.11752 (-0.2)	$C_{13}H_{16}NO_2$	$[MH\text{-}HCOOH]^+$
		203.09393 (-0.7)	$C_{12}H_{13}NO_2$	$[MH\text{-}HCOOH\text{-}CH_3]^{+\bullet}$
		186.09116 (-1.0)	$C_{12}H_{12}NO$	$[MH\text{-}HCOOH\text{-}CH_3OH]^+$
		175.07518 (-1.0)	$C_{11}H_{11}O_2$	$[MH\text{-}HCOOH\text{-}C_2H_5N]^+$
		161.05956 (-0.9)	$C_{10}H_9O_2$	$[MH\text{-}HCOOH\text{-}C_3H_7N]^+$
		158.09629 (-0.9)	$C_{11}H_{12}N$	$[MH\text{-}HCOOH\text{-}CH_3OH\text{-}CO]^+$
		143.04899 (-1.0)	$C_{10}H_7O$	$[MH\text{-}HCOOH\text{-}C_2H_5N\text{-}CH_3OH]^+$
		137.05959 (-0.9)	$C_8H_9O_2$	$[MH\text{-}HCOOH\text{-}C_5H_7N]^+$
		115.05408 (-1.3)	C_9H_7	$[MH\text{-}HCOOH\text{-}C_2H_5N\text{-}CH_3OH\text{-}CO]^+$
TP 298	298.14376 (-0.1) $C_{18}H_{20}NO_3$	241.08595 (0.1)	$C_{15}H_{13}O_3$	$[MH\text{-}C_3H_7N]^+$
		239.07034 (0.3)	$C_{15}H_{11}O_3$	$[MH\text{-}C_3H_9N]^+$
		223.07540 (0.2)	$C_{15}H_{11}O_2$	$[MH\text{-}C_3H_7N\text{-}H_2O]^+$
		213.09105 (0.2)	$C_{14}H_{13}O_2$	$[MH\text{-}C_3H_7N\text{-}CO]^+$
		211.07543 (0.3)	$C_{14}H_{11}O_2$	$[MH\text{-}C_3H_9N\text{-}CO]^+$
		199.07542 (0.3)	$C_{13}H_{11}O_2$	$[MH\text{-}C_3H_7N\text{-}C_2H_2O]^+$
		185.09610 (0.1)	$C_{13}H_{13}O$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CO]^+$
		183.08045 (0.1)	$C_{13}H_{11}O$	$[MH\text{-}C_3H_9N\text{-}CO\text{-}CO]^+$
		181.06484 (0.3)	$C_{13}H_9O$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CH_3OH]^+$
		153.06990 (0.2)	$C_{12}H_9$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CH_3OH\text{-}CO]^+$
Codeine	300.15907 (-1.2) $C_{18}H_{22}NO_3$	282.14856 (-1.0)	$C_{18}H_{20}NO_2$	$[MH\text{-}H_2O]^+$
		243.10136 (-0.9)	$C_{15}H_{15}O_3$	$[MH\text{-}C_3H_7N]^+$
		241.08578 (-0.6)	$C_{15}H_{13}O_3$	$[MH\text{-}C_3H_9N]^+$
		225.09092 (-0.4)	$C_{15}H_{13}O_2$	$[MH\text{-}C_3H_7N\text{-}H_2O]^+$
		215.10653 (-0.6)	$C_{14}H_{15}O_2$	$[MH\text{-}C_3H_7N\text{-}CO]^+$
		199.07532 (-0.2)	$C_{13}H_{11}O_2$	$[MH\text{-}C_3H_7N\text{-}C_2H_4O]^+$
		193.06473 (-0.3)	$C_{14}H_9O$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}CH_3OH]^+$
		187.11164 (-0.5)	$C_{13}H_{15}O$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CO]^+$
		183.08037 (-0.4)	$C_{13}H_{11}O$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CH_3OH]^+$
		171.08033 (-0.7)	$C_{12}H_{11}O$	$[MH\text{-}C_3H_7N\text{-}C_2H_4O\text{-}CO]^+$
		165.06977 (-0.7)	$C_{13}H_9$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CH_3OH\text{-}H_2O]^+$ $[MH\text{-}C_3H_7N\text{-}H_2O\text{-}CH_3OH\text{-}CO]^+$
		155.08540 (-0.8)	$C_{12}H_{11}$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CH_3OH\text{-}CO]^+$
TP 300(1)	300.15924 (-0.6) $C_{18}H_{22}NO_3$	243.10144 (-0.5)	$C_{15}H_{15}O_3$	$[MH\text{-}C_3H_7N]^+$
		225.09097(-0.2)	$C_{15}H_{13}O_2$	$[MH\text{-}C_3H_7N\text{-}H_2O]^+$
		215.10658 (-0.4)	$C_{14}H_{15}O_2$	$[MH\text{-}C_3H_7N\text{-}CO]^+$
		193.06477 (-0.1)	$C_{14}H_9O$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}CH_3OH]^+$
		183.08039 (-0.3)	$C_{13}H_{11}O$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CH_3OH]^+$
		171.08040 (-0.2)	$C_{12}H_{11}O$	$[MH\text{-}C_3H_7N\text{-}C_2H_4O\text{-}CO]^+$
		165.06982 (-0.4)	$C_{13}H_9$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CH_3OH\text{-}H_2O]^+$ $[MH\text{-}C_3H_7N\text{-}H_2O\text{-}CH_3OH\text{-}CO]^+$
		155.08547 (-0.4)	$C_{12}H_{11}$	$[MH\text{-}C_3H_7N\text{-}CO\text{-}CH_3OH\text{-}CO]^+$

continues

TP 300(3)	300.12262 (-1.4) $C_{17}H_{18}NO_4$	282.11207 (-1.4)	$C_{17}H_{16}NO_3$	$[MH\text{-}H_2O]^+$
		267.08862 (-1.4)	$C_{16}H_{13}NO_3$	$[MH\text{-}H_2O\text{-}CH_3]^{+\bullet}$
		254.11724 (-1.3)	$C_{16}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}CO]^+$
		250.08593 (-1.3)	$C_{16}H_{12}NO_2$	$[MH\text{-}H_2O\text{-}CH_3\text{-}OH]^+$
		240.10157 (-1.4)	$C_{15}H_{14}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_2O]^+$
		227.07003 (-1.1)	$C_{14}H_{11}O_3$	$[MH\text{-}H_2O\text{-}C_3H_5N]^+$
		225.07816 (-1.2)	$C_{14}H_{11}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_2O\text{-}CH_3]^{+\bullet}$
		195.04389 (-0.9)	$C_{13}H_7O_2$	$[MH\text{-}H_2O\text{-}C_3H_5N\text{-}CH_3OH]^+$
		167.04898 (-1.0)	$C_{12}H_7O$	$[MH\text{-}H_2O\text{-}C_3H_5N\text{-}CH_3OH\text{-}CO]^+$
TP 302	302.13883 (-0.5) $C_{17}H_{20}NO_4$	284.12811 (-0.1)	$C_{17}H_{18}NO_3$	$[MH\text{-}H_2O]^+$
		266.11765 (0.3)	$C_{17}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}H_2O]^+$
		256.13336 (0.6)	$C_{16}H_{18}NO_2$	$[MH\text{-}H_2O\text{-}CO]^+$
		251.09415 (0.3)	$C_{16}H_{13}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}CH_3]^{+\bullet}$
		241.08603 (0.5)	$C_{15}H_{13}O_3$	$[MH\text{-}H_2O\text{-}C_2H_5N]^+$
		240.10205 (0.6)	$C_{15}H_{14}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_4O]^+$
		225.07840 (-0.1)	$C_{14}H_{11}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_4O\text{-}CH_3]^{+\bullet}$
		224.10712 (0.6)	$C_{15}H_{14}NO$	$[MH\text{-}H_2O\text{-}CO\text{-}CH_3OH]^+$
		213.09119 (0.9)	$C_{14}H_{13}O_2$	$[MH\text{-}H_2O\text{-}CO\text{-}C_2H_5N]^+$
		181.06490 (0.6)	$C_{13}H_9O$	$[MH\text{-}H_2O\text{-}CO\text{-}C_2H_5N\text{-}CH_3OH]^+$
		153.06977 (-0.7)	$C_{12}H_9$	$[MH\text{-}H_2O\text{-}CO\text{-}C_2H_5N\text{-}CH_3OH\text{-}CO]^+$
TP 314	314.13841 (-0.9) $C_{18}H_{20}NO_4$	296.12787 (-0.9)	$C_{18}H_{18}NO_3$	$[MH\text{-}H_2O]^+$
		281.10441 (-0.8)	$C_{17}H_{15}NO_3$	$[MH\text{-}H_2O\text{-}CH_3]^{+\bullet}$
		280.09671 (-0.4)	$C_{17}H_{14}NO_3$	$[MH\text{-}H_2O\text{-}CH_4]^+$
		268.13303 (-0.6)	$C_{17}H_{18}NO_2$	$[MH\text{-}H_2O\text{-}CO]^+$
		266.08981 (-1.3)	$C_{16}H_{12}NO_3$	$[MH\text{-}H_2O\text{-}CH_3\text{-}CH_3]^+$
		264.10165 (-1.0)	$C_{17}H_{14}NO_2$	$[MH\text{-}H_2O\text{-}CH_3\text{-}OH]^+$
		254.11757 (0.1)	$C_{16}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_2O]^+$
		239.09392 (-0.7)	$C_{15}H_{13}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_2O\text{-}CH_3]^{+\bullet}$
		227.07007 (-0.9)	$C_{14}H_{11}O_3$	$[MH\text{-}H_2O\text{-}C_4H_7N]^+$
		195.04387 (-1.0)	$C_{13}H_7O_2$	$[MH\text{-}H_2O\text{-}C_4H_7N\text{-}CH_3OH]^+$
		167.04895 (-1.2)	$C_{12}H_7O$	$[MH\text{-}H_2O\text{-}C_4H_7N\text{-}CH_3OH\text{-}CO]^+$
TP 316(1)	316.15399 (-1.1) $C_{18}H_{22}NO_4$	298.14343 (-1.1)	$C_{18}H_{20}NO_3$	$[MH\text{-}H_2O]^+$
		282.11212 (-1.2)	$C_{17}H_{16}NO_3$	$[MH\text{-}H_2O\text{-}CH_4]^+$
		280.13284 (-1.3)	$C_{18}H_{18}NO_2$	$[MH\text{-}H_2O\text{-}H_2O]^+$
		270.14859 (-1.0)	$C_{17}H_{20}NO_2$	$[MH\text{-}H_2O\text{-}CO]^+$
		266.11731 (-0.9)	$C_{17}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}CH_3OH]^+$
		265.10950 (-0.8)	$C_{17}H_{15}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}CH_3]^{+\bullet}$
		254.11734 (-0.9)	$C_{16}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_4O]^+$
		241.08565 (-1.1)	$C_{15}H_{13}O_3$	$[MH\text{-}H_2O\text{-}C_3H_7N]^+$
		239.09380 (-1.2)	$C_{15}H_{13}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_4O\text{-}CH_3]^{+\bullet}$
TP 316(2)	316.15420 (-0.4) $C_{18}H_{22}NO_4$	298.14369 (-0.3)	$C_{18}H_{20}NO_3$	$[MH\text{-}H_2O]^+$
		241.08599 (0.3)	$C_{15}H_{13}O_3$	$[MH\text{-}H_2O\text{-}C_3H_7N]^+$
		213.09105 (0.2)	$C_{14}H_{13}O_2$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}CO]^+$
		209.05976 (0.3)	$C_{14}H_9O_2$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}CH_3OH]^+$
		199.07541 (0.3)	$C_{13}H_{11}O_2$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}C_2H_2O]^+$
		181.06483 (0.2)	$C_{13}H_9O$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}CO\text{-}CH_3OH]^+$
		171.08045 (0.1)	$C_{12}H_{11}O$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}C_2H_2O\text{-}CO]^+$

continues

TP 318	318.16954 (-1.2) $C_{18}H_{24}NO_4$	300.15417 (-0.8)	$C_{18}H_{22}NO_3$	$[MH\text{-}H_2O]^+$
		243.10135 (-0.8)	$C_{15}H_{15}O_3$	$[MH\text{-}C_3H_7N\text{-}H_2O]^+$
		225.09084 (-0.8)	$C_{15}H_{13}O_2$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}H_2O]^+$
		217.08573 (-0.9)	$C_{13}H_{13}O_3$	$[MH\text{-}C_3H_7N\text{-}C_2H_4O]^+$
		215.10650 (-0.7)	$C_{14}H_{15}O_2$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}CO]^+$
		201.09087 (-0.7)	$C_{13}H_{13}O_2$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}C_2H_2O]^+$
		199.07526 (-0.5)	$C_{13}H_{11}O_2$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}C_2H_4O]^+$
		189.09089 (-0.6)	$C_{12}H_{13}O_2$	$[MH\text{-}C_3H_7N\text{-}C_2H_4O\text{-}CO]^+$
		187.11162 (-0.7)	$C_{13}H_{15}O$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}CO\text{-}CO]^+$
		171.08033 (-0.6)	$C_{12}H_{11}O$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}C_2H_4O\text{-}CO]^+$
		168.05682 (-0.9)	$C_{12}H_7O$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}C_2H_4O\text{-}CH_3O]^{+\bullet}$
		161.09597 (-0.7)	$C_{11}H_{13}O$	$[MH\text{-}C_3H_7N\text{-}C_2H_4O\text{-}CO\text{-}CO]^+$
		159.08033 (-0.7)	$C_{11}H_{11}O$	$[MH\text{-}C_3H_7N\text{-}H_2O\text{-}CO\text{-}CO\text{-}C_2H_4]^+$
TP 320	320.14849 (-2.4) $C_{17}H_{22}NO_5$	302.13808 (-2.0)	$C_{17}H_{20}NO_4$	$[MH\text{-}H_2O]^+$
		289.10649 (-1.9)	$C_{16}H_{17}O_5$	$[MH\text{-}CH_3NH_2]^+$
		274.14328 (-1.8)	$C_{16}H_{20}NO_3$	$[MH\text{-}H_2O\text{-}CO]^+$
		259.09597 (-2.0)	$C_{15}H_{15}O_4$	$[MH\text{-}C_2H_7NO]^+$
		218.11715 (-1.9)	$C_{13}H_{16}NO_2$	$[MH\text{-}C_4H_6O_3]^+$
		217.10932 (-1.9)	$C_{13}H_{15}NO_2$	$[MH\text{-}H_2O\text{-}CO\text{-}C_3H_5O]^{+\bullet}$
		216.07771 (-1.8)	$C_{13}H_{12}O_3$	$[MH\text{-}H_2O\text{-}C_4H_{10}NO_2]^+$
		204.10156 (-1.7)	$C_{12}H_{14}NO_2$	$[MH\text{-}H_2O\text{-}CO\text{-}C_4H_6O]^+$
		177.09071 (-1.7)	$C_{11}H_{13}O_2$	$[MH\text{-}H_2O\text{-}C_6H_9NO_3]^+$
		145.06453 (-1.8)	$C_{10}H_9O$	$[MH\text{-}H_2O\text{-}C_6H_9NO_3\text{-}CH_3OH]^+$
TP 330	330.13351 (-0.1) $C_{18}H_{20}NO_5$	312.12295 (-0.1)	$C_{18}H_{18}NO_4$	$[MH\text{-}H_2O]^+$
		294.11230 (-0.2)	$C_{18}H_{16}NO_3$	$[MH\text{-}H_2O\text{-}H_2O]^+$
		284.12800 (-0.1)	$C_{17}H_{18}NO_3$	$[MH\text{-}HCOOH]^+$
		273.07558 (-0.1)	$C_{15}H_{13}O_5$	$[MH\text{-}C_3H_7N]^+$
		268.13298 (-0.2)	$C_{17}H_{18}NO_2$	$[MH\text{-}H_2O\text{-}CO_2]^+$
		255.06516 (-0.1)	$C_{15}H_{11}O_4$	$[MH\text{-}H_2O\text{-}C_3H_7N]^+$
		242.11753 (-0.1)	$C_{15}H_{16}NO_2$	$[MH\text{-}HCOOH\text{-}C_2H_2O]^+$
		227.07034 (0.3)	$C_{14}H_{11}O_3$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}CO]^+$
		199.07539 (0.1)	$C_{13}H_{11}O_2$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}CO\text{-}CO]^+$
		185.05969 (-0.1)	$C_{12}H_9O_2$	$[MH\text{-}HCOOH\text{-}C_2H_2O\text{-}C_3H_7N]^+$
		183.08040 (-0.2)	$C_{13}H_{11}O$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}CO\text{-}CO_2]^+$
TP332(1)	332.14929 (0.1) $C_{18}H_{22}NO_5$	314.13834 (-1.1)	$C_{18}H_{20}NO_4$	$[MH\text{-}H_2O]^+$
		296.12772 (-1.4)	$C_{18}H_{18}NO_3$	$[MH\text{-}H_2O\text{-}H_2O]^+$
		281.10431 (-1.2)	$C_{17}H_{15}NO_3$	$[MH\text{-}H_2O\text{-}H_2O\text{-}CH_3]^{+\bullet}$
		272.12781 (-1.1)	$C_{16}H_{18}NO_3$	$[MH\text{-}H_2O\text{-}C_2H_2O]^+$
		270.11221 (-1.0)	$C_{16}H_{16}NO_3$	$[MH\text{-}H_2O\text{-}C_2H_4O]^+$
		268.13300 (-0.8)	$C_{17}H_{18}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}CO]^+$
		254.11743 (-0.5)	$C_{16}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}C_2H_2O]^+$
		242.11756 (-1.0)	$C_{15}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_4O\text{-}CO]^+$
		241.10950 (-1.0)	$C_{15}H_{15}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_4O\text{-}COH]^{+\bullet}$
		227.09390 (-0.8)	$C_{14}H_{13}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_4O\text{-}CO\text{-}CH_3]^{+\bullet}$
		227.06985 (-1.8)	$C_{14}H_{11}O_3$	$[MH\text{-}H_2O\text{-}H_2O\text{-}C_4H_7N]^+$
		189.09081 (-1.0)	$C_{12}H_{13}O_2$	$[MH\text{-}H_2O\text{-}C_2H_4O\text{-}C_4H_3NO]^+$
		157.06462 (-1.1)	$C_{11}H_9O$	$[MH\text{-}H_2O\text{-}C_2H_4O\text{-}C_4H_3NO\text{-}CH_3OH]^+$
TP 332(2)	332.14924 (-0.1) $C_{18}H_{22}NO_5$	314.13867 (-0.1)	$C_{18}H_{20}NO_4$	$[MH\text{-}H_2O]^+$
		296.12806 (-0.2)	$C_{18}H_{18}NO_3$	$[MH\text{-}H_2O\text{-}H_2O]^+$
		268.13316 (-0.2)	$C_{17}H_{18}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}CO]^+$
		257.08082 (-0.1)	$C_{15}H_{13}O_4$	$[MH\text{-}H_2O\text{-}C_3H_7N]^+$
		253.10971 (-0.1)	$C_{16}H_{15}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}CO\text{-}CH_3]^{+\bullet}$
		242.11756 (0.1)	$C_{15}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}C_3H_4O_2]^+$
		239.07036 (0.4)	$C_{15}H_{11}O_3$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}H_2O]^+$
		211.07546 (0.5)	$C_{14}H_{11}O_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}CO\text{-}C_3H_7N]^+$
				$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}H_2O\text{-}CO]^+$

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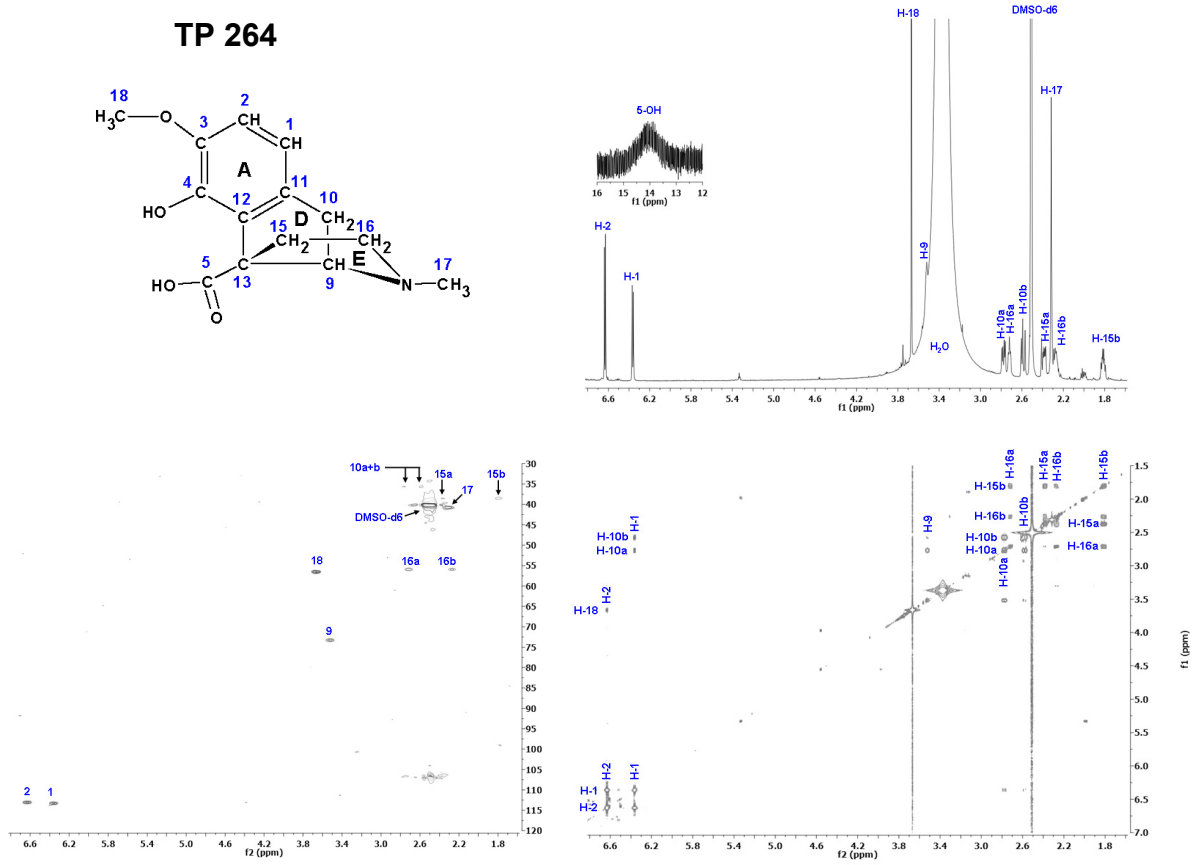
TP 334	334.16502 (0.4) $C_{18}H_{24}NO_5$	316.15405 (-0.9)	$C_{18}H_{22}NO_4$	$[MH\text{-}H_2O]^+$
		298.14358 (-0.6)	$C_{18}H_{20}NO_3$	$[MH\text{-}H_2O\text{-}H_2O]^+$
		280.13304 (-0.6)	$C_{18}H_{18}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}H_2O]^+$
		272.12800 (-0.4)	$C_{16}H_{18}NO_3$	$[MH\text{-}H_2O\text{-}C_2H_4O]^+$
		270.14875 (-0.4)	$C_{17}H_{20}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}CO]^+$
		259.09628 (-0.8)	$C_{15}H_{15}O_4$	$[MH\text{-}H_2O\text{-}C_3H_7N]^+$
		254.11746 (-0.4)	$C_{16}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}C_2H_4O]^+$
		244.13305 (-0.7)	$C_{15}H_{18}NO_2$	$[MH\text{-}H_2O\text{-}C_2H_4O\text{-}CO]^+$
		241.08585 (-0.3)	$C_{15}H_{13}O_3$	$[MH\text{-}H_2O\text{-}H_2O\text{-}C_3H_7N]^+$
		239.09379 (-1.2)	$C_{15}H_{13}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}C_2H_4O\text{-}CH_3]^{+\bullet}$
		230.11741 (-0.6)	$C_{14}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}C_4H_6O_2]^+$
		215.07025 (-0.1)	$C_{13}H_{11}O_3$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}C_2H_4O]^+$
		199.07532 (-0.2)	$C_{13}H_{11}O_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}C_3H_7N\text{-}C_2H_2O]^+$
		187.07530 (-0.3)	$C_{12}H_{11}O_2$	$[MH\text{-}H_2O\text{-}C_3H_7N\text{-}C_2H_4O\text{-}CO]^+$
TP 346	346.12810 (-1.2) $C_{18}H_{20}NO_6$	318.13320 (-1.2)	$C_{17}H_{20}NO_5$	$[MH\text{-}CO]^+$
		300.12269 (-1.2)	$C_{17}H_{18}NO_4$	$[MH\text{-}HCOOH]^+$
		271.12007 (-0.8)	$C_{16}H_{17}NO_3$	$[MH\text{-}C_2H_3O_3]^{+\bullet}$
		256.09658 (-0.9)	$C_{15}H_{14}NO_3$	$[MH\text{-}C_2H_3O_3\text{-}CH_3]^+$
		256.12396 (-1.0)	$C_{16}H_{18}NO_2$	$[MH\text{-}HCOOH\text{-}CO_2]^+$
		242.11733 (-0.9)	$C_{15}H_{16}NO_2$	$[MH\text{-}CO\text{-}C_2H_4O_3]^+$
		228.07791 (-0.8)	$C_{14}H_{12}O_3$	$[MH\text{-}C_2H_3O_3\text{-}C_2H_5N]^{+\bullet}$
		199.07522(-0.7)	$C_{13}H_{11}O_2$	$[MH\text{-}C_2H_3O_3\text{-}CH_3\text{-}C_2H_3NO]^+$
		187.07524(-0.6)	$C_{12}H_{11}O_2$	$[MH\text{-}C_6H_9NO_4]^+$
TP 348	348.14407 (-0.3) $C_{18}H_{22}NO_6$	330.13351(-0.3)	$C_{18}H_{20}NO_5$	$[MH\text{-}H_2O]^+$
		312.12293 (-0.3)	$C_{18}H_{18}NO_4$	$[MH\text{-}H_2O\text{-}H_2O]^+$
		302.13861 (-0.2)	$C_{17}H_{20}NO_4$	$[MH\text{-}HCOOH]^+$
		294.11245 (-0.1)	$C_{18}H_{16}NO_3$	$[MH\text{-}H_2O\text{-}H_2O\text{-}H_2O]^+$
		284.12806 (-0.2)	$C_{17}H_{18}NO_3$	$[MH\text{-}H_2O\text{-}HCOOH]^+$
		279.08890 (-0.4)	$C_{17}H_{13}NO_3$	$[MH\text{-}H_2O\text{-}H_2O\text{-}H_2O\text{-}CH_3]^{+\bullet}$
		268.13316 (-0.2)	$C_{17}H_{18}NO_2$	$[MH\text{-}H_2O\text{-}H_2O\text{-}CO_2]^+$
		255.06514 (-0.2)	$C_{15}H_{11}O_4$	$[MH\text{-}H_2O\text{-}H_2O\text{-}C_3H_7N]^+$
		242.11755 (0.1)	$C_{15}H_{16}NO_2$	$[MH\text{-}H_2O\text{-}HCOOH\text{-}C_2H_2O]^+$
		148.07567 (-0.1)	$C_9H_{10}NO$	$[MH\text{-}H_2O\text{-}C_9H_{10}O_4]^+$
TP 364	364.13879 (-0.8) $C_{18}H_{22}NO_7$	320.14877 (-1.5)	$C_{17}H_{22}NO_5$	$[MH\text{-}CO_2]^+$
		302.13840 (-0.9)	$C_{17}H_{20}NO_4$	$[MH\text{-}CO_2\text{-}H_2O]^+$
		276.12272 (-1.1)	$C_{15}H_{18}NO_4$	$[MH\text{-}CO_2\text{-}C_2H_4O]^+$
		274.14354 (-0.8)	$C_{16}H_{20}NO_3$	$[MH\text{-}CO_2\text{-}HCOOH]^+$
		260.12781 (-1.2)	$C_{15}H_{18}NO_3$	$[MH\text{-}CO_2\text{-}C_2H_4O_2]^+$
		245.08056 (-1.1)	$C_{14}H_{13}O_4$	$[MH\text{-}CO_2\text{-}C_3H_9NO]^+$
		243.10130 (-1.1)	$C_{15}H_{15}O_3$	$[MH\text{-}CO_2\text{-}C_2H_7NO_2]^+$
		230.11733 (-1.0)	$C_{14}H_{16}NO_2$	$[MH\text{-}CO2\text{-}C_2H_4O\text{-}CH_2O_2]^+$
		217.08575 (-0.8)	$C_{13}H_{13}O_3$	$[MH\text{-}CO2\text{-}C_4H_9NO_2]^+$

Table SI 5-4. List of precursor $[M-H]^-$ and product ions from MS and MS^n spectra obtained from codeine TPs using LTQ-Orbitrap-MS with electrospray ionization in the negative ionization mode.

Compound	Precursor ion	Product ions		Proposed fragmentation
	Measured MW (Error in ppm) Elemental comp.	Measured MW (Error in ppm)	Elemental composition	
TP 264	262.10875 (1.0) $C_{14}H_{16}NO_4$	218.11901 (1.6)	$C_{13}H_{16}NO_2$	$[M-H-CO_2]^-$
		203.09551 (1.6)	$C_{12}H_{13}NO_2$	$[M-H-CO_2-CH_3]^{-\bullet}$
		176.10843 (1.9)	$C_{11}H_{14}NO$	$[M-H-CO_2-C_2H_2O]^-$
		160.05331 (2.0)	$C_{10}H_8O_2$	$[M-H-CO_2-CH_3-C_2H_5N]^-$
TP 320	318.13439 (-1.0) $C_{17}H_{20}NO_5$	300.12363 (-1.7)	$C_{17}H_{18}NO_4$	$[M-H-H_2O]^-$
		274.14443 (-1.6)	$C_{16}H_{20}NO_3$	$[M-H-CO_2]^-$
		259.12109 (-1.2)	$C_{15}H_{17}NO_3$	$[M-H-CO_2-CH_3]^{-\bullet}$
		256.13406 (-0.9)	$C_{16}H_{18}NO_2$	$[M-H-H_2O-CO_2]^-$
		241.11053 (-1.3)	$C_{15}H_{15}NO_2$	$[M-H-H_2O-CO_2-CH_3]^{-\bullet}$
		218.11845 (-0.9)	$C_{13}H_{16}NO_2$	$[M-H-CO_2-C_3H_4O]^-$
		203.09499(-0.9)	$C_{12}H_{13}NO_2$	$[M-H-CO_2-CH_3-C_3H_5O]^{-\bullet}$
TP 346	344.11356 (-1.2) $C_{18}H_{18}NO_6$	300.12410 (3.6)	$C_{17}H_{18}NO_4$	$[M-H-CO_2]^-$
		285.10064 (-0.1)	$C_{16}H_{15}NO_4$	$[M-H-CO_2-CH_3]^{-\bullet}$
		256.13441 (0.4)	$C_{16}H_{18}NO2$	$[M-H-CO_2-CO_2]^-$
		241.11097 (0.6)	$C_{15}H_{15}NO_2$	$[M-H-CO_2-CO_2-CH_3]^{-\bullet}$
		226.08749 (0.6)	$C_{14}H_{12}NO_2$	$[M-H-CO_2-CO_2-CH_3-CH_3]^-$
		213.09229 (0.9)	$C_{14}H_{13}O_2$	$[M-H-CO_2-CO_2-C_2H_5N]^-$
TP 348	346.12972 (0.3) $C_{18}H_{20}NO_6$	302.13962 (-0.5)	$C_{17}H_{20}NO_4$	$[M-H-CO_2]^-$
		287.11624 (-0.2)	$C_{16}H_{17}NO_4$	$[M-H-CO_2-CH_3]^{-\bullet}$
		284.12930 (0.3)	$C_{17}H_{18}NO_3$	$[M-H-CO_2-H_2O]^-$
		272.09275 (-0.3)	$C_{15}H_{14}NO_4$	$[M-H-CO_2-CH_3-CH_3]^-$
		270.11348 (-0.3)	$C_{16}H_{16}NO_3$	$[M-H-CO_2-CH_3-OH]^-$
		269.10571 (-0.1)	$C_{16}H_{15}NO_3$	$[M-H-CO_2-CH_3-H_2O]^{-\bullet}$ $[M-H-CO_2-H_2O-CH_3]^{-\bullet}$
		242.11870 (0.2)	$C_{15}H_{16}NO_2$	$[M-H-CO_2-C_2H_4O_2]^-$
		229.05073 (0.4)	$C_{13}H_9O_4$	$[M-H-CO_2-CH_3-C_3H_8N]^{-\bullet}$
TP 364	362.12494 (1.1) $C_{18}H_{20}NO_7$	318.13474(0.1)	$C_{17}H_{20}NO_5$	$[M-H-CO_2]^-$
		303.11136(0.5)	$C_{16}H_{17}NO_5$	$[M-H-CO_2-CH_3]^{-\bullet}$
		300.12432(0.6)	$C_{17}H_{18}NO_4$	$[M-H-CO_2-H_2O]^-$
		274.14507(0.7)	$C_{16}H_{20}NO_3$	$[M-H-CO_2-CO_2]^-$
		272.12939(0.6)	$C_{16}H_{18}NO_3$	$[M-H-CO_2-HCOOH]^-$
		259.12155(0.2)	$C_{15}H_{17}NO_3$	$[M-H-CO_2-CO_2-CH_3]^{-\bullet}$
		246.15022(1.1)	$C_{15}H_{20}NO_2$	$[M-H-CO_2-C_2O_3]^-$
		231.12676(1.2)	$C_{14}H_{17}NO_2$	$[M-H-CO_2-C_2O_3-CH_3]^{-\bullet}$
		137.06118(2.8)	$C_8H_9O_2$	$[M-H-CO_2-CO_2-C_8H_{11}NO]^-$

NMR spectra of codeine TPs



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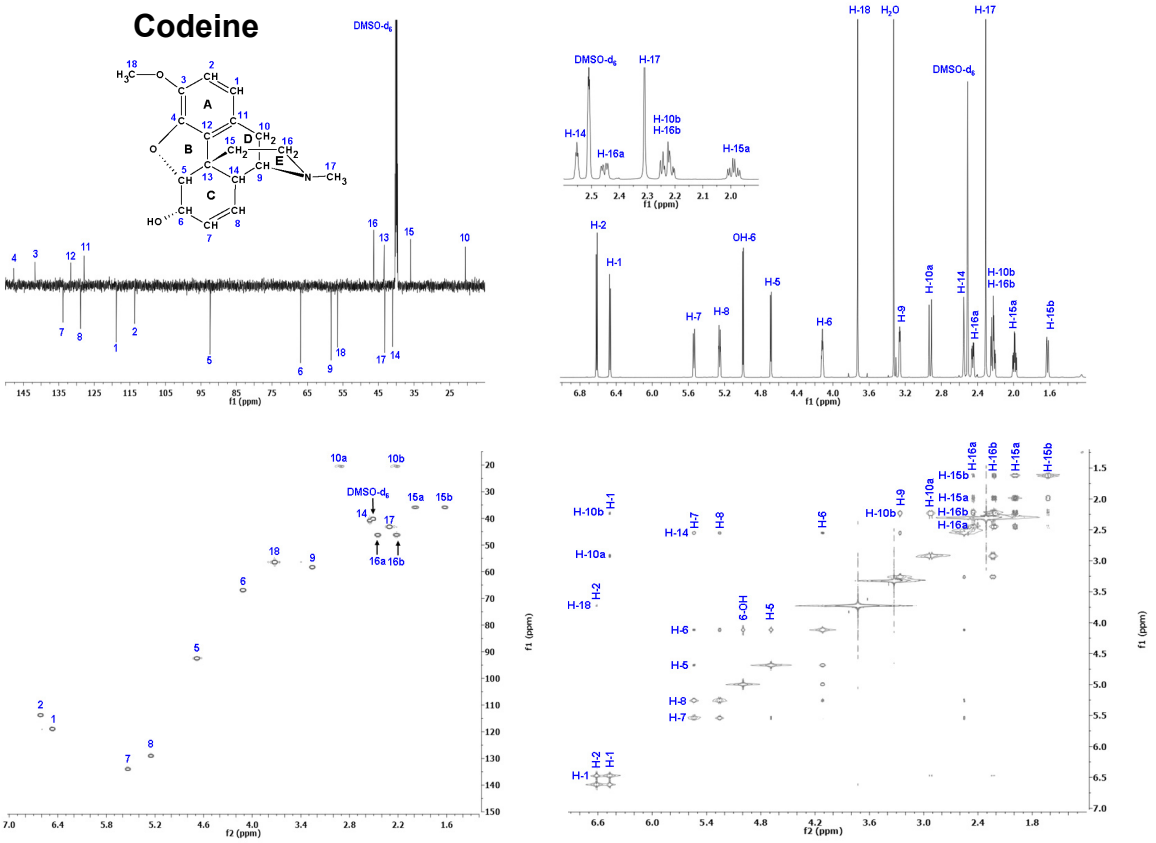
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H-17
H-14
H-16a
H-10b
H-16b
H-15a
H-2
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OH-6
H-5
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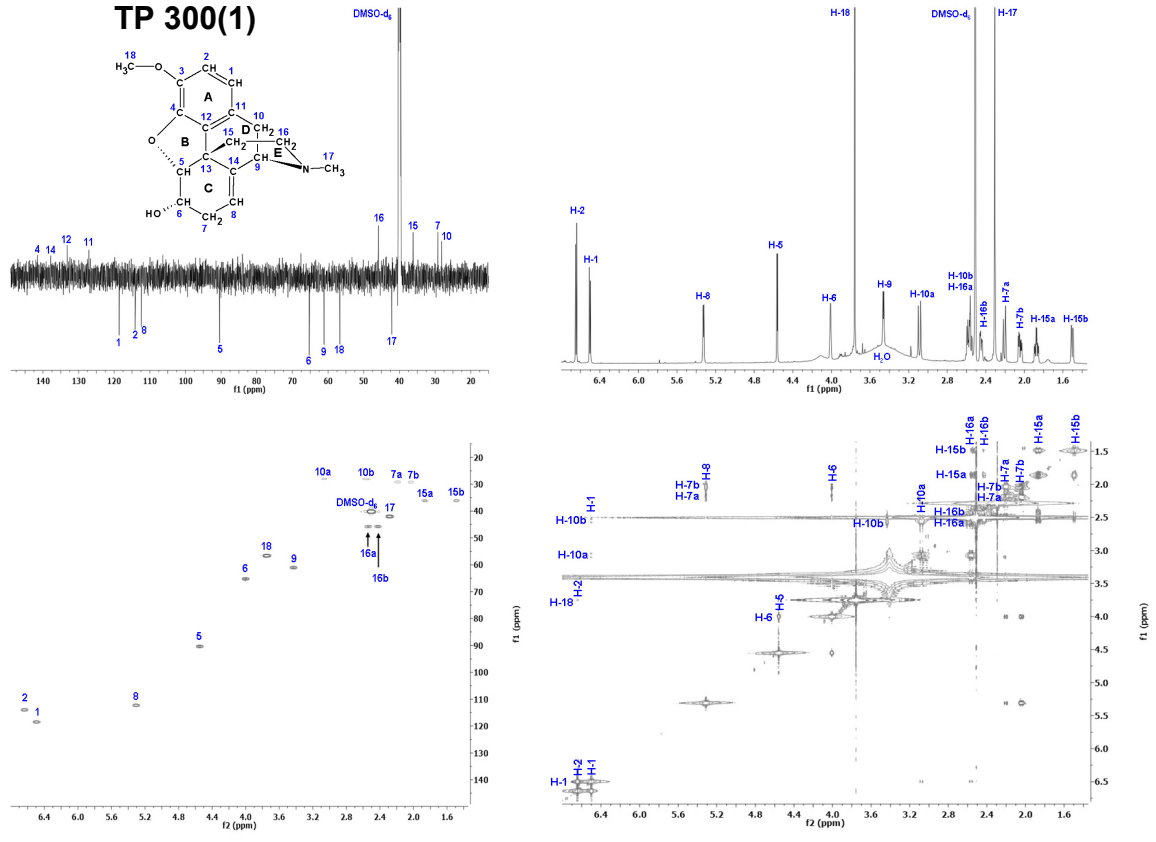
H-8
H-6
H-9
H-10a
H-15b
f1 (ppm)
f2 (ppm)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H-1
H-8
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H-6
H-9
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H-7b
H-15a
H-15b
H2O
f1 (ppm)
f2 (ppm)

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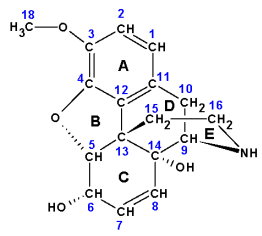
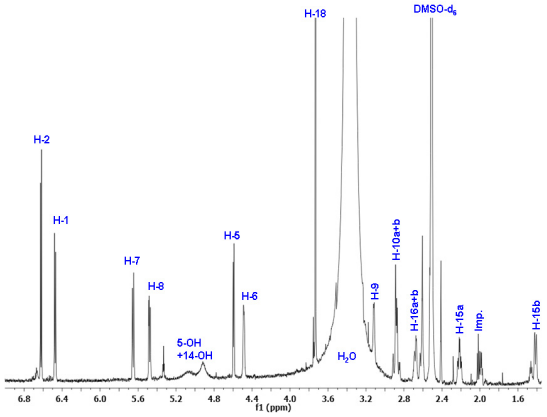
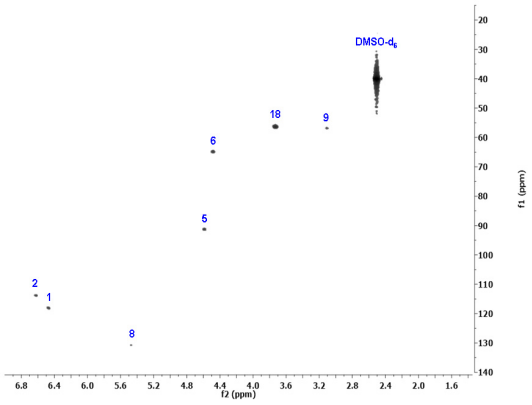
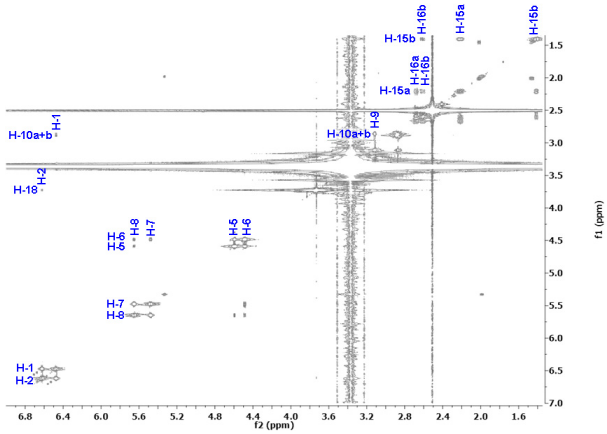
TP 302

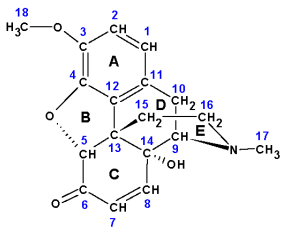


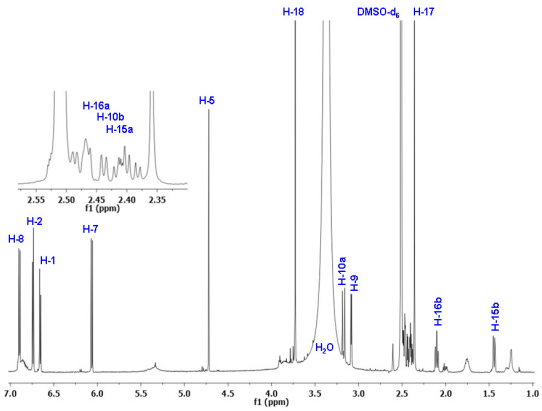


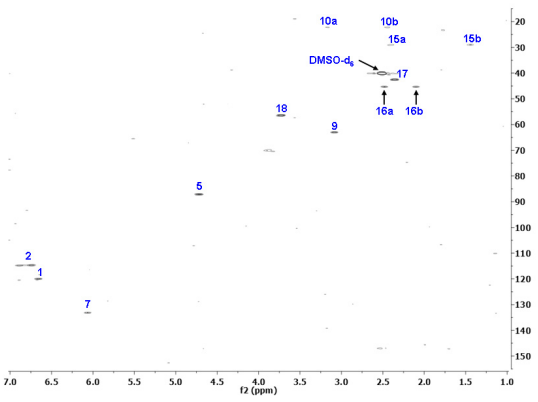


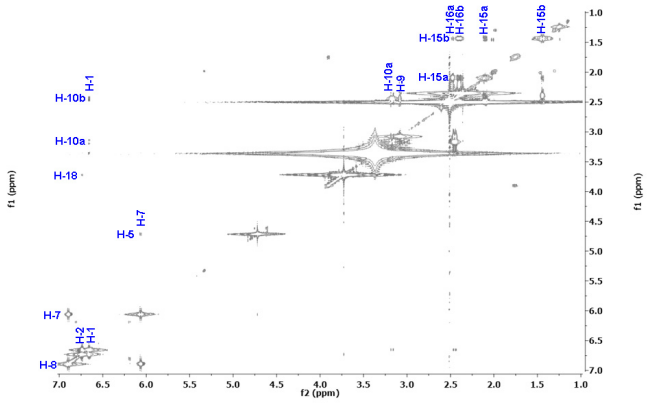


TP 314









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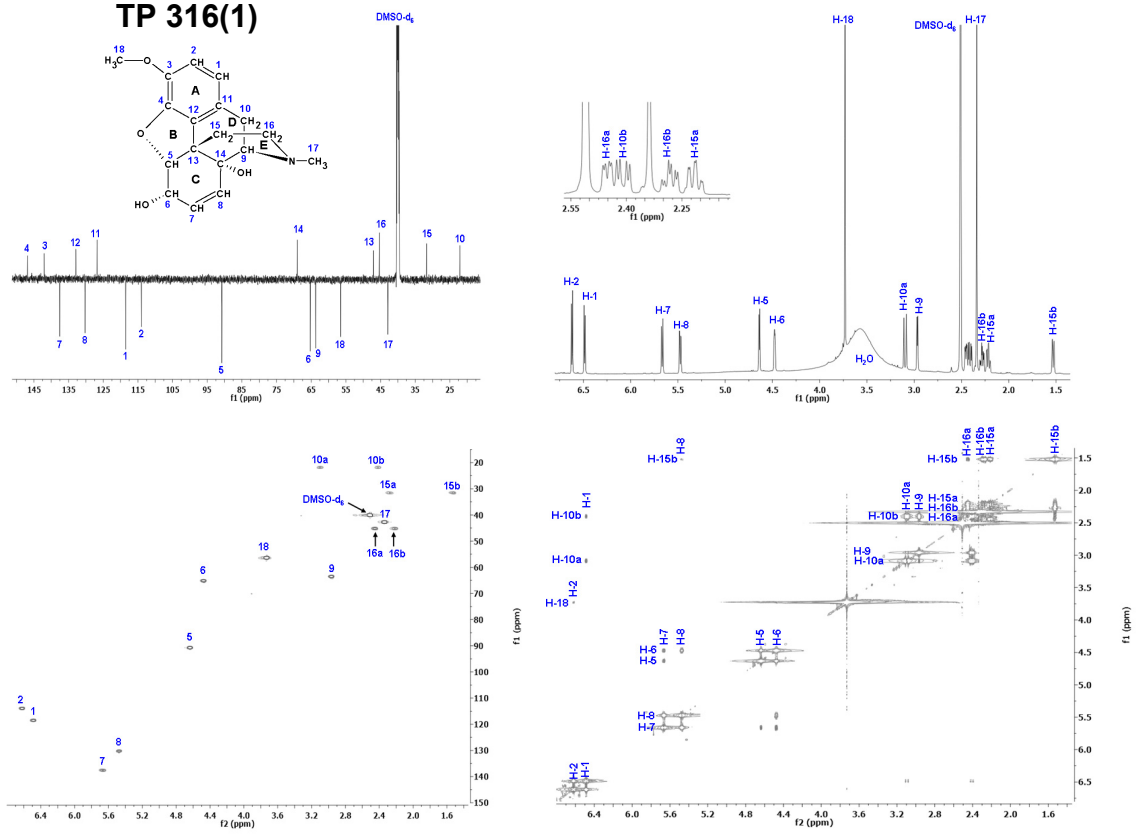
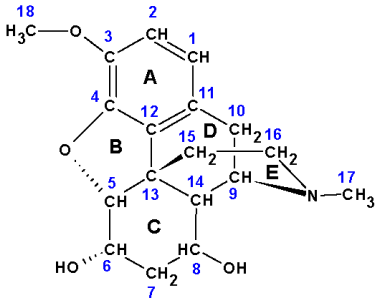
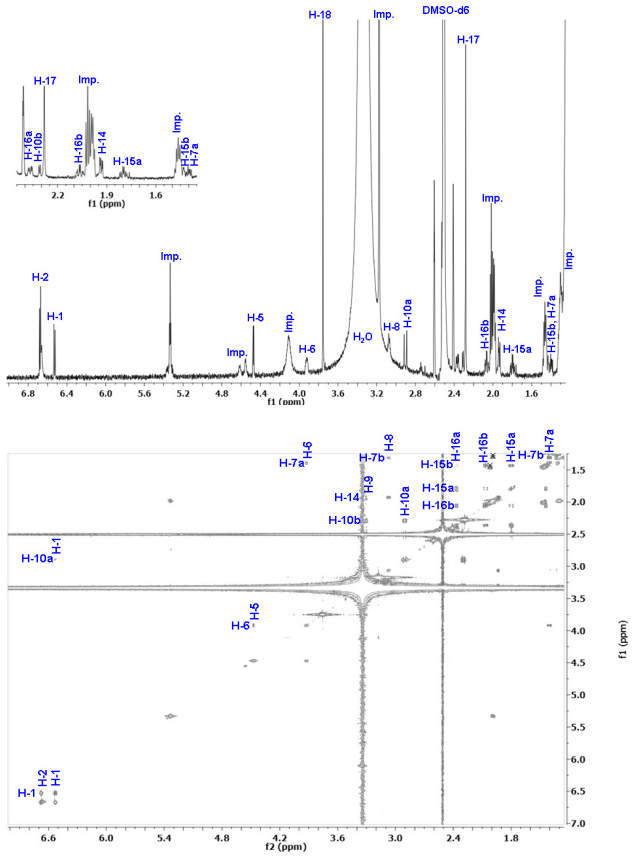
TP 316(1)



TP 318



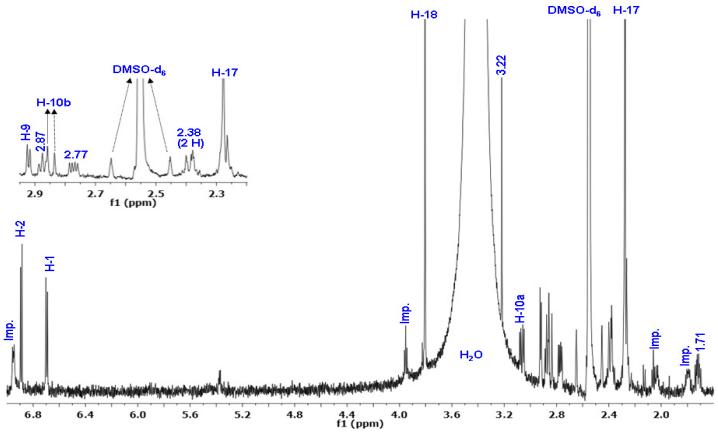


HSQC not available

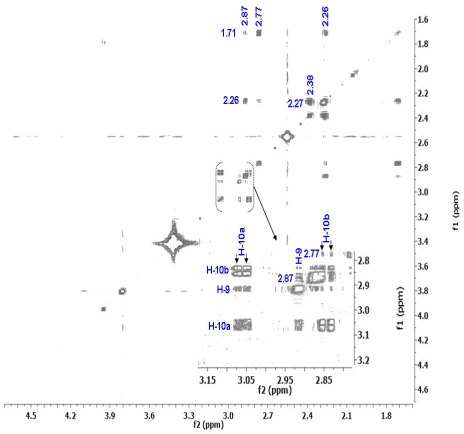
continues

TP 320

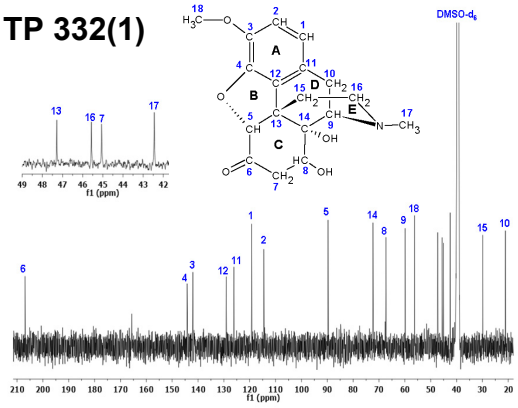
no final structure

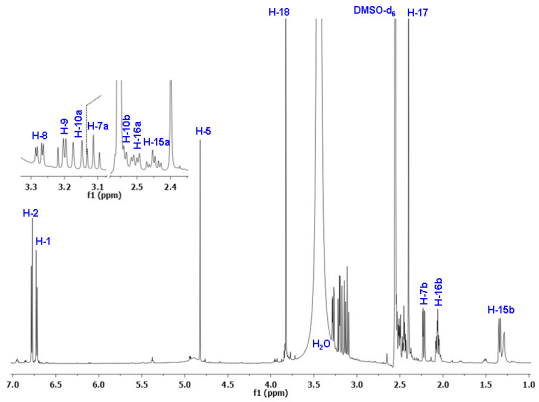


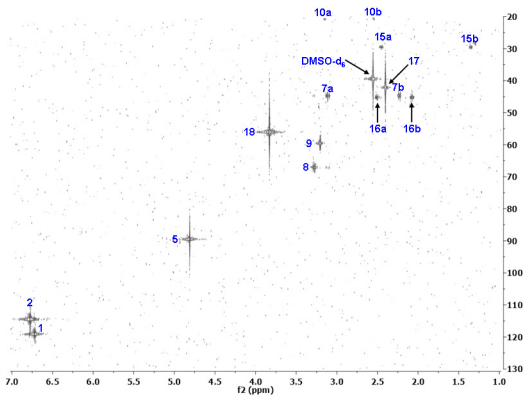
HSQC not available

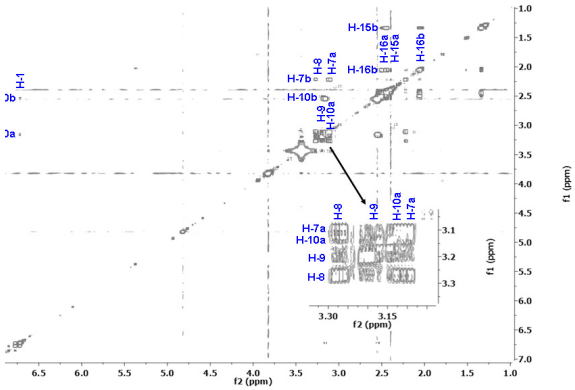


TP 332(1)









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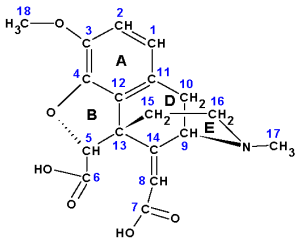
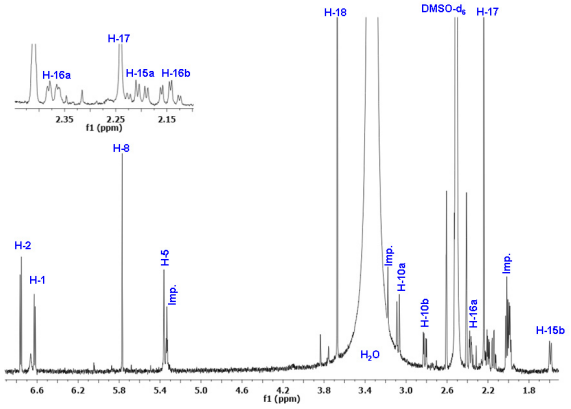
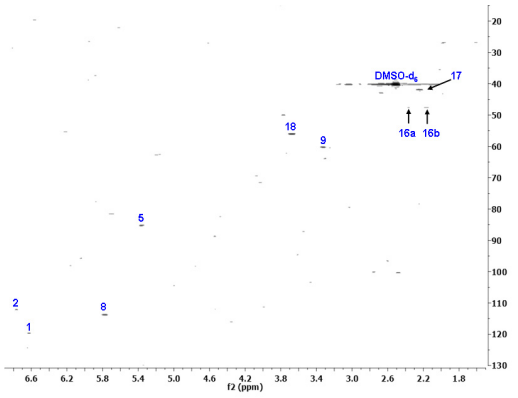
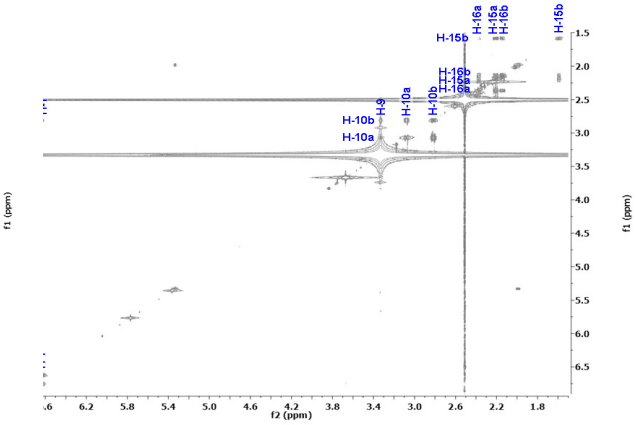
TP 346

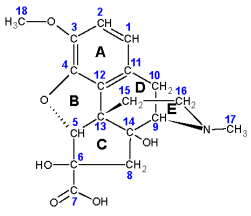


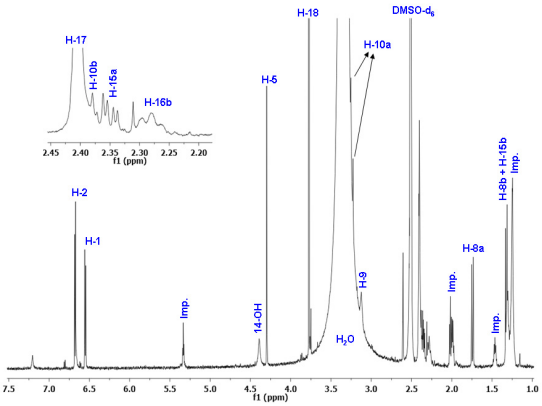




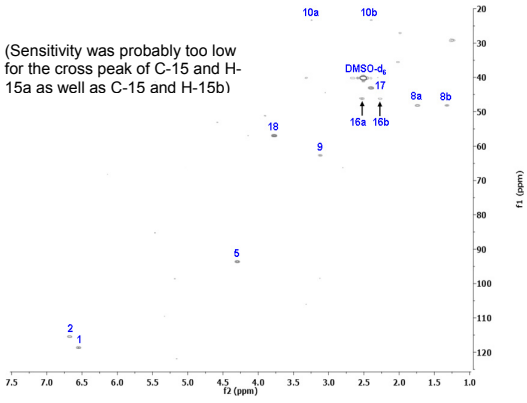


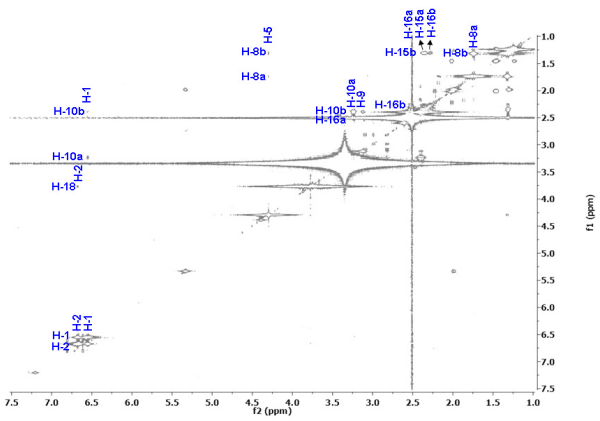
TP 348





(Sensitivity was probably too low for the cross peak of C-15 and H-15a as well as C-15 and H-15b)





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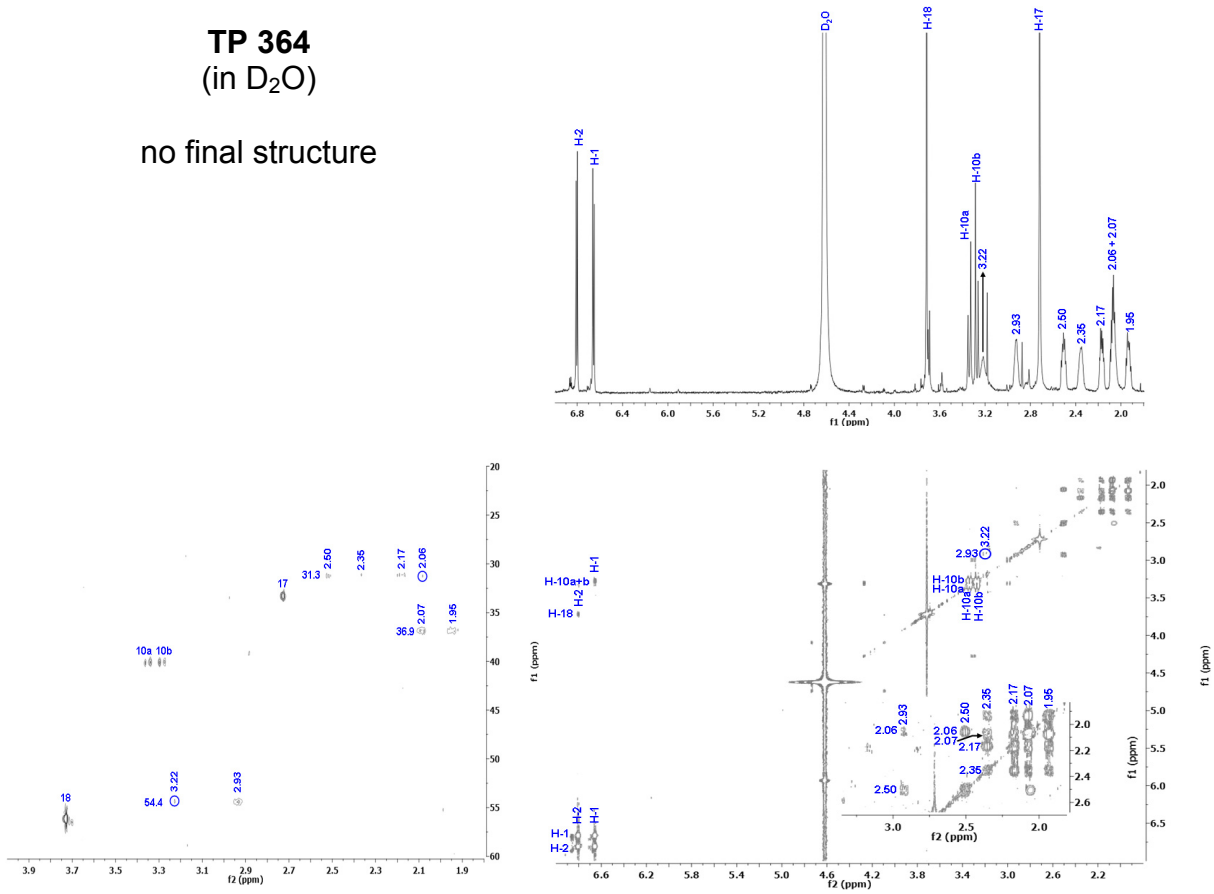


Figure SI 5-4. NMR results: top left: ^{13}C-NMR if available (spin-echo except for TP 332(1)), top right: ^{1}H-NMR, down left ^{13}C,^{1}H-HSQC, down right: ^{1}H,^{1}H-NMR. Measurements of ^{1}H-NMR and ^{13}C-NMR were performed at 700 and 176 MHz, respectively. All samples were measured at 298.3 K in DMSO-d_6 except for TP 364 which was measured in D_2O. The NMR data for TP 300(2) is not shown due to its similarity to the data of TP 300(1). For TP 300(3) and TP 334, the signal intensities were too low for data evaluation.

Assignment of the stereoisomers neopine and isoneopine

In literature (Gollwitzer et al., 1993) the chemical reduction of neopinone to neopine and isoneopine with sodium borhydride ($NaBH_4$) is described to be a non-stereoselective reaction leading to the formation of approximately 12% neopine and 88% isoneopine. Since in an ultrapure water sample spiked with 20 mg L^{-1} codeinone, the formation of neopinone was observed after sonication, this sample was used for a reduction experiment with $NaBH_4$. Briefly, 50 µL of an aqueous solution of 0.2% sodium borhydride ($NaBH_4$) and 0.05% NaOH was added to 400 µL of the ultrapure water sample containing the mixture of codeinone and neopinone. After a reaction time of 1 h, 8 µL of 1.75 M H_2SO_4 was added and the sample was measured with HPLC-UV at 280 nm. Neopinone was completely reduced to approximately 20% TP 300(1) and 80% TP300(2) as determined by HPLC-UV at 280 nm. Comparing this ratio with that from literature, TP 300(1) was identified being the (S)-6-OH-isomer neopine and TP 300(2) the (R)-6-OH-isomer isoneopine.

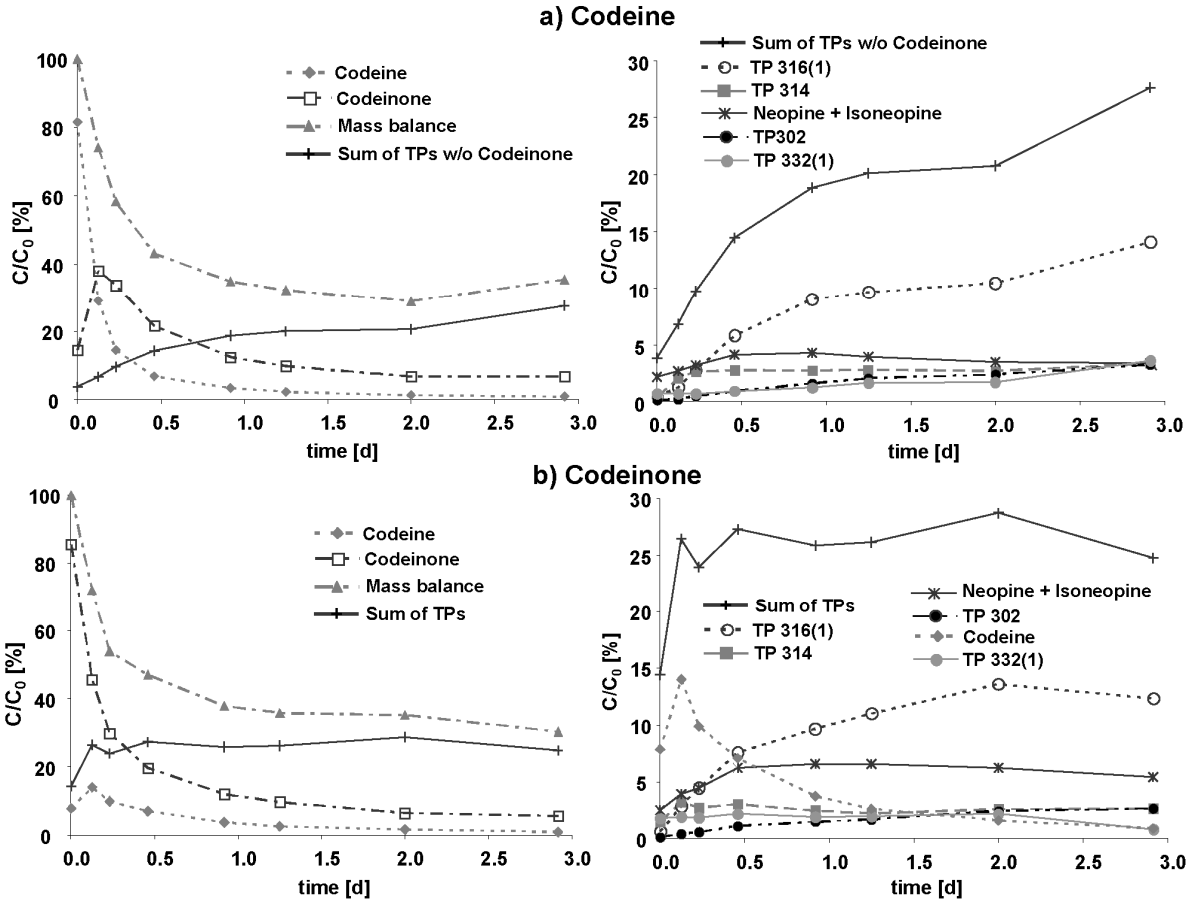


Figure SI 5-5. Mass balance [%] in incubation experiments of a) codeine and b) codeinone using undiluted activated sludge. Codeine and codeinone were spiked separately at a concentration of 2 µg L^{-1}. The mass balance includes all TPs for which analytical standards were available and which were applicable to SPE method 1 (codeine, codeinone, neopine, isoneopine, TP 316(1), TP 314, TP 302 and TP 332(1)). Calculations were done on a molar basis.

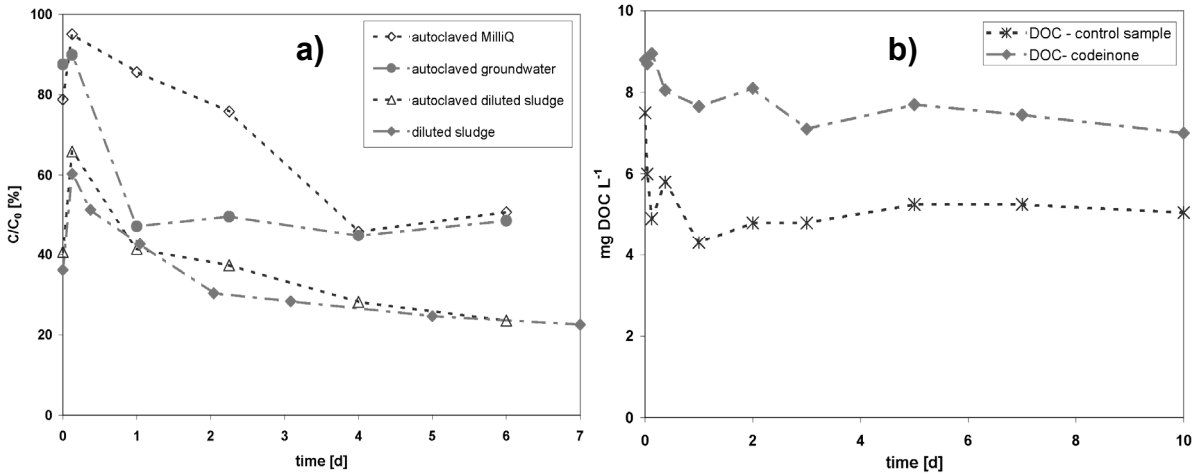


Figure SI 5-6. a) Mass balance [%] in incubation experiments of codeinone using sterile (autoclaved) and non-sterile activated sludge (both diluted 1:20 with groundwater, 0.2 g_{SS} L^{-1}) as well as autoclaved groundwater and ultrapure water. Codeinone was spiked at a concentration of 5 mg L^{-1}. The low mass balance at the beginning is attributed to the initially incomplete dissolution of spiked codeinone.
b) Average DOC concentration [mg L^{-1}] in non-sterile diluted sludge samples spiked with 5 mg L^{-1} codeinone and in non-spiked control samples (n = 2).

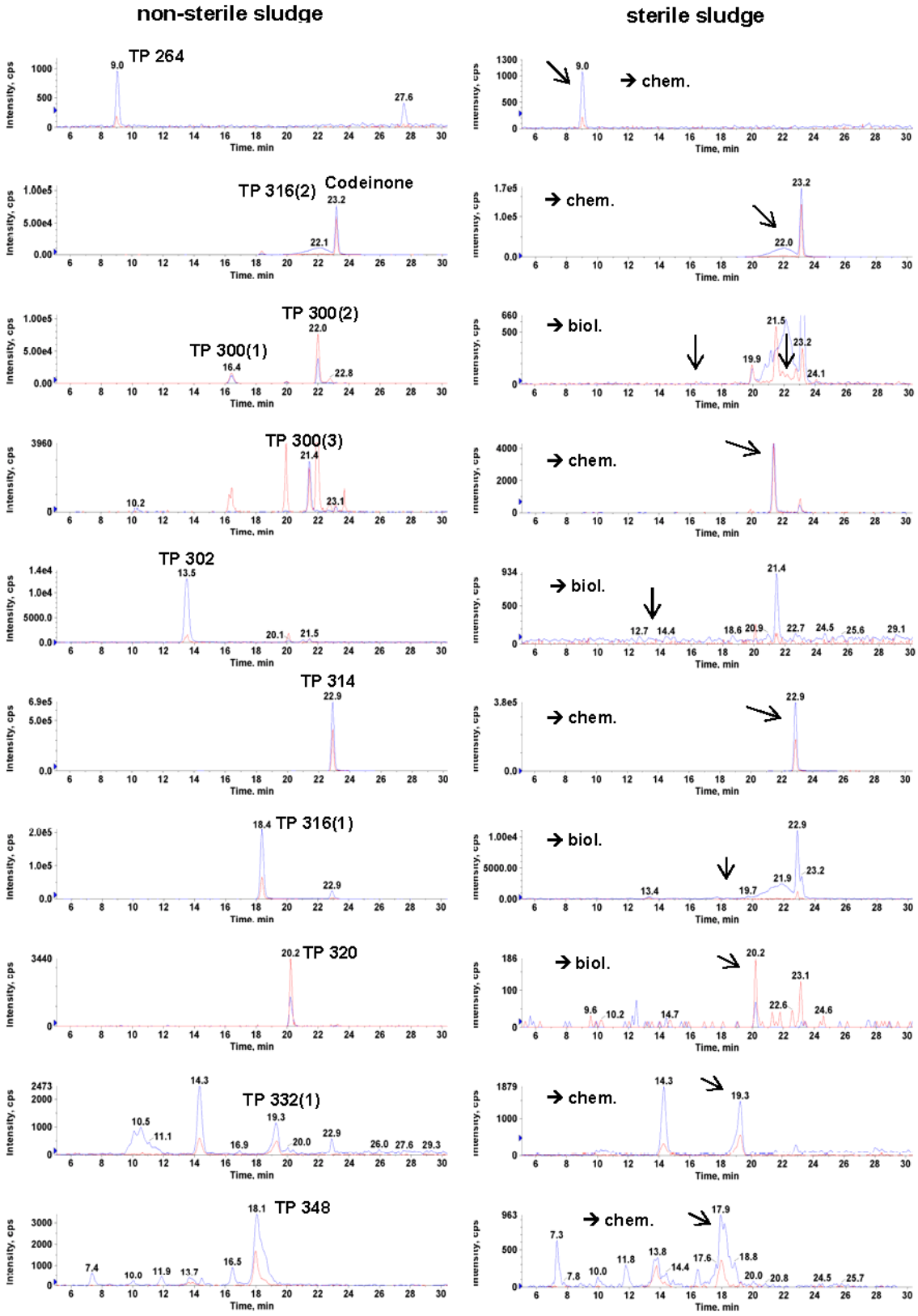
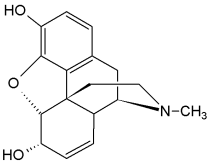
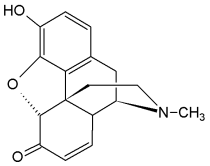
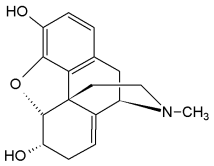
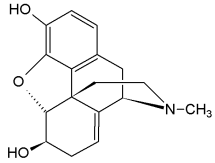
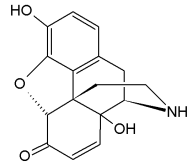
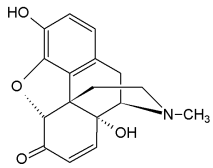
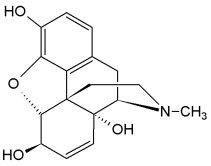
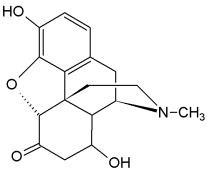
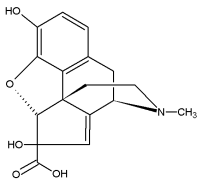
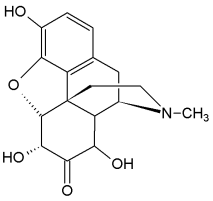
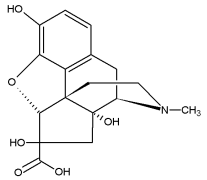
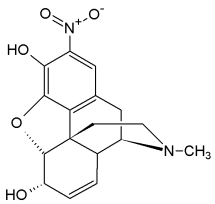


Figure SI 5-7: Occurrence of selected codeine TPs determined by LC tandem MS in the MRM mode after 4 d and 3 d of incubation in sterile (autoclaved) and non-sterile diluted activated sludge, respectively. The sludge was initially spiked with 5 mg L^{-1} codeinone and incubated under aerobic conditions. TPs determined in the sterile sludge indicated an abiotic (non-enzymatic) formation mechanism.

Table SI 5-5. List of identified and proposed TPs from morphine with chemical structures, retention times, maximum intensities, names of corresponding codeine TPs and precursor $[M+H]^+$ and characteristic product ions determined in batch samples using a LTQ-Orbitrap-MS screening with data dependent acquisition.

Compound structure	Formation details Retention time Max. intensity Corr. codeine TP	Precursor ion $[M+H]^+$ Measured MW (Error in ppm) Elemental comp.	Characteristic product ions Measured MW (Error in ppm)	Elemental composition	Fragmentation
 **Morphine**	8.9 min $1.4x10^7$ (t=0 h) parent compound	286.14374 (-0.1) $C_{17}H_{20}NO_3$	268.13324 (0.1)	$C_{17}H_{18}NO_2$	$[MH-H_2O]^+$
			229.08595 (0.1)	$C_{14}H_{13}O_3$	$[MH-C_3H_7N]^+$
			211.07541 (0.3)	$C_{14}H_{11}O_2$	$[MH-H_2O-C_3H_7N]^+$
			201.09108 (0.4)	$C_{13}H_{13}O_2$	$[MH-C_3H_7N-CO]^+$
			185.05974 (0.2)	$C_{12}H_9O_2$	$[MH-C_3H_7N-C_2H_4O]^+$
 **Morphinone**	11.4 min $7.7x10^6$ (t=5 h) Cod TP 298	284.12802 (-0.3) $C_{17}H_{18}NO_3$	227.07025 (-0.1)	$C_{14}H_{11}O_3$	$[MH-C_3H_7N]^+$
			225.05464 (0.1)	$C_{14}H_9O_3$	$[MH-C_3H_9N]^+$
			209.05969 (-0.1)	$C_{14}H_9O_2$	$[MH-C_3H_7N-H_2O]^+$
			199.07530 (-0.3)	$C_{13}H_{11}O_2$	$[MH-C_3H_7N-CO]^+$
			197.05966 (-0.2)	$C_{13}H_9O_2$	$[MH-C_3H_9N-CO]^+$
			181.06474 (-0.3)	$C_{13}H_9O$	$[MH-C_3H_7N-H_2O-CO]^+$
 **Neomorphine**	11.4 min $4.0x10^5$ (t=2 d) Cod TP 300(1)	286.14374 (-0.1) $C_{17}H_{20}NO_3$	268.13324 (0.1)	$C_{17}H_{18}NO_2$	$[MH-H_2O]^+$
			229.08588 (-0.2)	$C_{14}H_{13}O_3$	$[MH-C_3H_7N]^+$
			211.07541 (0.3)	$C_{14}H_{11}O_2$	$[MH-H_2O-C_3H_7N]^+$
			185.05969 (-0.1)	$C_{12}H_9O_2$	$[MH-C_3H_7N-C_2H_4O]^+$
 **Isoneomorphine**	11.9 min $4.0x10^4$ (t=2 d) Cod TP 300(2)	286.14417 (1.4) $C_{17}H_{20}NO_3$	similar to Neomorphine		
 **14-Hydroxy-N-desmethylmorphinone** (proposed)	8.0 min $3.4x10^4$ (t=2 d) Cod TP 300(3)	286.10757 (0.7) $C_{16}H_{16}NO_4$	268.09644 (-1.4)	$C_{16}H_{14}NO_3$	$[MH-H_2O]^+$
			240.10155 (-1.5)	$C_{15}H_{14}NO_2$	$[MH-H_2O-CO]^+$
			226.08606 (-0.9)	$C_{14}H_{12}NO_2$	$[MH-H_2O-C_2H_2O]^+$
			213.05447 (-0.7)	$C_{13}H_9O3$	$[MH-C_3H_5N]^+$
 **14-Hydroxymorphinone**	10.4 min $2.3x10^6$ (t=2 d) Cod TP 314	300.12308 (0.1) $C_{17}H_{18}NO_4$	282.11249 (0.1)	$C_{17}H_{16}NO_3$	$[MH-H_2O]^+$
			254.11758 (0.1)	$C_{16}H_{16}NO_2$	$[MH-H_2O-CO]^+$
			240.10193 (0.1)	$C_{15}H_{14}NO_2$	$[MH-H_2O-C_2H_2O]^+$
			223.07544 (0.4)	$C_{15}H_{11}O_2$	$[MH-H_2O-C_3H_9N]^{]+}$

continues

 **14-Hydroxymorphine**	7.6 min $3.4x10^5$ (t=4 d) Cod TP 316	302.13861 (-0.2) $C_{17}H_{20}NO_4$	284.12808 (-0.1)	$C_{17}H_{18}NO_3$	$[MH-H_2O]^+$
			266.11752 (-0.1)	$C_{17}H_{16}NO_2$	$[MH-H_2O-H_2O]^+$
			240.10185 (-0.2)	$C_{15}H_{14}NO_2$	$[MH-H_2O-C_2H_4O]^+$
			227.07019 (-0.4)	$C_{14}H_{11}O_3$	$[MH-H_2O-C_3H_7N]^+$
 **8-Hydroxy-7,8-dihydromorphinone**	8 -12 min $2.4x10^5$ (t=28 h) Cod TP 316(2)	302.13834 (-1.2) $C_{17}H_{20}NO_4$	284.12796 (-0.6)	$C_{17}H_{18}NO_3$	$[MH-H_2O]^+$
			227.07010 (-0.8)	$C_{14}H_{11}O_3$	$[MH-H_2O-C_3H_7N]^+$
			209.05956 (-0.7)	$C_{14}H_9O_2$	$[MH-H_2O-C_3H_7N-H_2O]^+$
			185.05957 (-0.7)	$C_{12}H_9O_2$	$[MH-H_2O-C_3H_7N-C_2H_2O]^+$
 **MO TP 316** (proposed)	5.2 min $2.3x10^5$ (t=2 d) Cod TP 330	316.11850 (1.7) $C_{17}H_{18}NO_5$	298.10703 (-1.2)	$C_{17}H_{16}NO_4$	$[MH-H_2O]^+$
			270.11243 (-0.2)	$C_{16}H_{16}NO_3$	$[MH-HCOOH]^+$
			241.04953 (-0.1)	$C_{14}H_9O_4$	$[MH-H_2O-C_3H_7N]^+$
			228.10184 (-0.3)	$C_{14}H_{14}NO_2$	$[MH-HCOOH-C_2H_2O]^+$
			213.05466 (0.2)	$C_{13}H_9O_3$	$[MH-H_2O-C_3H_7N-CO]^+$
			197.05969 (-0.1)	$C_{13}H_9O_2$	$[MH-H_2O-C_3H_7N-CO_2]^+$
			185.05971 (0.1)	$C_{12}H_9O_2$	$[MH-H_2O-C_3H_7N-CO-CO]^+$
 **MO TP 318** (proposed)	5.2 min $2.7x10^5$ (t=2 d) Cod TP 332(2)	318.13358 (-0.1) $C_{17}H_{20}NO_5$	300.12289 (-0.5)	$C_{17}H_{18}NO_4$	$[MH-H_2O]^+$
			282.11240 (-0.3)	$C_{17}H_{16}NO_3$	$[MH-H_2O-H_2O]^+$
			254.11758 (0.1)	$C_{16}H_{16}NO_2$	$[MH-H_2O-H_2O-CO]^+$
			225.05463 (0.1)	$C_{14}H_9O_3$	$[MH-H_2O-H_2O-C_3H_7N]^+$
			197.05972 (0.1)	$C_{13}H_9O_2$	$[MH-H_2O-H_2O-C_3H_7N-CO]^+$
 **MO TP 334** (proposed)	8.3 min $3.0x10^4$ (t=48 d) Cod TP 348	334.12820 (-0.9) $C_{17}H_{20}NO_6$	316.11761 (-1.1)	$C_{17}H_{18}NO_5$	$[MH-H_2O]^+$
			298.10709 (-1.0)	$C_{17}H_{16}NO_4$	$[MH-H_2O-H_2O]^+$
			288.12289 (-0.5)	$C_{16}H_{18}NO_4$	$[MH-HCOOH]^+$
			270.11230 (-0.6)	$C_{16}H_{16}NO_3$	$[MH-H_2O-HCOOH]^+$
			148.07562 (-0.5)	$C_9H_{10}NO$	$[MH-H_2O-C_8H_8O_4]^+$
 **2-Nitromorphine**	17.2 min $1.7x10^6$ (t=5 h)	331.12888 (0.1) $C_{17}H_{19}N_2O_5$	285.13565 (-0.3)	$C_{17}H_{19}NO_3$	$[MH-NO_2]^+$
			274.07068 (-0.3)	$C_{14}H_{12}NO_5$	$[MH-C_3H_7N]^+$
			230.04448 (-0.5)	$C_{12}H_8NO_4$	$[MH-C_3H_7N-C_2H_4O]^+$

continues

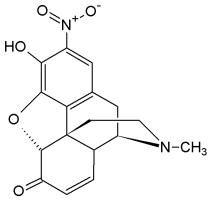
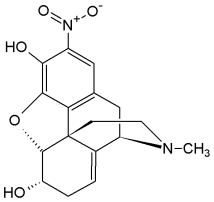
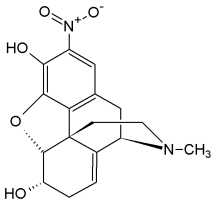
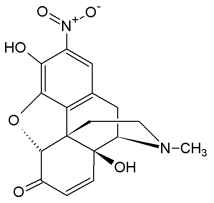
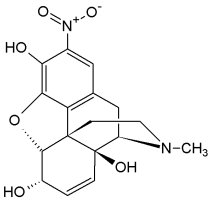
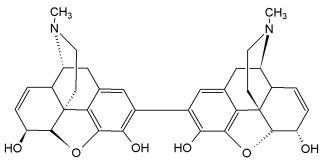
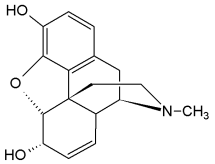
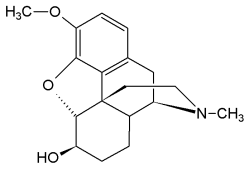
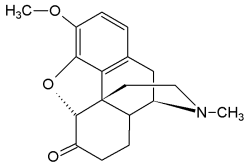
 **2-Nitromorphinone**	20.1 min 1.9×10^5 (t=28 h)	329.11325 (0.2) $C_{17}H_{17}N_2O_5$	283.12027 (-0.1) 272.05542 (0.3) 270.03970 (0.1) 242.04480 (0.1) 230.04485 (0.3)	$C_{17}H_{17}NO_3$ $C_{14}H_{10}O_5N$ $C_{14}H_8O_5N$ $C_{13}H_8O_4N$ $C_{12}H_8O_4N$	$[MH\text{-}NO_2]^+$ $[MH\text{-}C_3H_7N]^+$ $[MH\text{-}C_3H_9N]^+$ $[MH\text{-}C_3H_9N\text{-}CO]^+$ $[MH\text{-}C_3H_7N\text{-}C_2H_2O]^+$
 **MO TP 331(1)** **2-Nitroneomorphine** (proposed)	12.7 min 3.0×10^4 (t=2 d)	331.12897 (0.3) $C_{17}H_{19}N_2O_5$	313.11786 (-1.4) 274.07068 (-1.2) 256.06021 (-0.9) 230.04463 (-0.7)	$C_{17}H_{17}N_2O_4$ $C_{14}H_{12}NO_5$ $C_{14}H_{10}NO_4$ $C_{12}H_8NO_4$	$[MH\text{-}H_2O]^+$ $[MH\text{-}C_3H_7N]^+$ $[MH\text{-}H_2O\text{-}C_3H_7N]^+$ $[MH\text{-}C_3H_7N\text{-}C_2H_4O]^+$
 **MO TP 331(2)** **2-Nitroisoneomorphine** (proposed)	19.2 min 9.0×10^4 (t=2 d)	331.12949 (1.9) $C_{17}H_{19}N_2O_5$	similar to MO TP 331(1)		
 **14-Hydroxy-2-nitromorphinone**	19.5 min 2.6×10^5 (t=2 d)	345.10861 (1.4) $C_{17}H_{17}N_2O_6$	327.09747 (-0.2) 285.08685 (-0.5) 268.08417 (-0.3) 239.09404 (-0.2)	$C_{17}H_{15}N_2O_5$ $C_{15}H_{13}N_2O_4$ $C_{15}H_{12}N_2O_3$ $C_{15}H_{13}NO_2$	$[MH\text{-}H_2O]^+$ $[MH\text{-}H_2O\text{-}C_2H_2O]^+$ $[MH\text{-}H_2O\text{-}C_2H_2O\text{-}OH]^{+\bullet}$ $[MH\text{-}H_2O\text{-}C_2H_2O\text{-}NO_2]^+$
 **14-Hydroxy-2-nitromorphine**	15.1 min 3.6×10^4 (t=4 d)	347.12384 (0.2) $C_{17}H_{19}N_2O_6$	no MS^2 available		
 **Pseudomorphine** 12.3 min 3.5×10^5 (t=5 h) confirmed with authentic standard		569.26428 (-0.6) $C_{34}H_{37}N_2O_6$	551.25378 (-0.5) 512.20648 (-0.6) 494.19589 (-0.6) 466.20096 (-0.7)	$C_{34}H_{35}N_2O_5$ $C_{31}H_{30}NO_6$ $C_{31}H_{28}NO_5$ $C_{30}H_{28}NO_4$	$[MH\text{-}H_2O]^+$ $[MH\text{-}C_3H_7N]^+$ $[MH\text{-}H_2O\text{-}C_3H_7N]^+$ $[MH\text{-}H_2O\text{-}C_3H_7N\text{-}CO]^+$

Table SI 5-6. List of identified TPs from dihydrocodeine with chemical structures, retention times, maximum intensities, names of corresponding codeine TPs and precursor $[M+H]^+$ and product ions determined in batch samples using a LTQ-Orbitrap-MS screening with data dependent acquisition.

Compound structure	Formation details	Precursor ion $[M+H]^+$	Characteristic product ions	Elemental composition	Fragmentation
	Retention time Max. intensity Corr. codeine TP	Measured MW (Error in ppm) Elemental comp.	Measured MW (Error in ppm)		
 **Dihydrocodeine**	18.4 min $4.0x10^7$ (t=0 h) parent compound	302.17496 (-0.4) $C_{18}H_{24}NO_3$	245.11725 (0.1) 243.10156 (-0.1) 227.10667 (0.1) 201.09099 (-0.1) 199.07539 (0.2)	$C_{15}H_{17}O_3$ $C_{15}H_{15}O_3$ $C_{15}H_{15}O_2$ $C_{13}H_{13}O_2$ $C_{13}H_{11}O_2$	$[MH-C_3H_7N]^+$ $[MH-C_3H_9N]^+$ $[MH-C_3H_7N-H_2O]^+$ $[MH-C_3H_7N-C_2H_4O]^+$ $[MH-C_3H_9N-C_2H_4O]^+$
 **Isodihydrocodeine**	20.6 min $3.5x10^6$ (t=6 d) Cod TP 300(2)	similar to dihydrocodeine			
 **Hydrocodon**	21.1 min $1.1x10^7$ (t=4 d) Cod TP 298	300.15930 (-0.4) $C_{18}H_{22}NO_3$	241.08594 (0.1) 213.09108 (0.3) 199.07533 (-0.1)	$C_{15}H_{13}NO_3$ $C_{14}H_{13}O_2$ $C_{13}H_{11}O_2$	$[MH-C_3H_9N]^+$ $[MH-C_3H_9N-CO]^+$ $[MH-C_3H_9N-C_2H_2O]^+$

Curriculum Vitae

Name	Arne Wick
Date of birth, birthplace	31.01.1979, Delmenhorst, Germany
Citizenship	German
Adress	Alexanderstrasse 44 56075 Koblenz Germany

09/1985-07/1989	Primary school, Iprump, Delmenhorst
09/1989-07/1991	Orientierungsstufe, Am Steinacker, Ganderkesee
08/1991-06/1998	Gymnasium, Am Steinacker, Ganderkesee, **Abitur** (German A-level)
08/1998-12/1998	Voluntary work in the chemical and biological laboratory of the brewery Beck & Co (Dr. Gromus), Bremen, Germany
01/1999-08/1999	Voluntary work in the National Park Harz, Torfhaus (Dr. Heydenreich), Germany
10/1999-05/2005	Diploma in Environmental Science at the University of Oldenburg, Germany **Diploma thesis** at the Institute of Chemistry and Biology of the Marine Environment (ICBM), University of Oldenburg, working group of Jun.-Prof. Dr. Harder, Laboratory for Environmental Biochemistry Title of the thesis: "*Analysis of 3-bromo-4-hydroxycinnamic acid methyl ester in sediments of the wadden sea and in the epithelium of the benthic polychaete Heteromastus filiformis"*
08/2005-03/2006	Public employee, city of Delmenhorst, youth work
05/2006-10/2006	Research associate at the University of Oldenburg, working group of Jun.-Prof. Dr. Harder, Laboratory for Environmental Biochemistry
since 01/2007	**PhD program** at the Federal Institute of Hydrology, Koblenz, Germany, working group of PD Dr. T.A. Ternes Funded by the EU-project NEPTUNE (Project No.036845)