LUND UNIVERSITY

Analytical method development for ultra-trace determination of human pharmaceuticals in aqueous samples. Assessing the performance of a sewage treatment plant

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2008

List of Publications

This doctoral thesis includes the following papers that are referred to in the text by their Roman numerals. The papers are appended at the end of the dissertation.

I. Hollow-fibre supported liquid membrane extraction for determination of fluoxetine and norfluoxetine concentration at ultra trace level in sewage samples.
Zorita, S., Mårtensson, L., Mathiasson, L.
Journal of Separation Science, 2007, 30, 2513-2521.

II. A novel hollow-fibre microporous membrane liquid–liquid extraction for determination of free 4-isobutylacetophenone concentration at ultra trace level in environmental aqueous samples.
Zorita, S., Barri, T., Mathiasson, L.
Journal of Chromatography A, 2007, 1157, 30-37.

III. Steroid hormone determination in water using an environmentally friendly membrane based extraction technique.
Zorita, S., Hallgren, P., Mathiasson, L.
Journal of Chromatography A, 2008, 1192, 1-8.

IV. Selective determination of acidic pharmaceuticals in wastewater using molecularly imprinted solid-phase extraction.
Zorita, S., Boyd, B., Jönsson, S., Yilmaz, E., Svensson, C., Mathiasson, L., Bergström, S.
Analytica Chimica Acta, 2008 (accepted).

V. Comparison of solid-phase sorbents for the determination of fluoroquinolone antibiotics in wastewater.
Zorita, S., Larsson, L., Mathiasson, L.
Journal of Separation Sciences, 2008 (accepted).

VI. Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden.
Zorita, S., Mårtensson, L., Mathiasson, L.
Manuscript 2008.

Appendix (not included in defence)

A. **Development of solid-phase extraction method for the determination of polychlorinated biphenyls in water.**
Westbom, R.; Thörneby, L.; Zorita, S.; Mathiasson, L. and Björklund, E.
Journal of Chromatography A, **2004**, 1033, 1-8.

B. Development of a combined solid-phase extraction-supercritical fluid extraction procedure for the determination of polychlorinated biphenyls in wastewater.
Zorita, S.; Westbom, R.; Thörneby, L.; Björklund, E. and Mathiasson, L.
Analytical Sciences, **2006**, 22, 1455-1459.

C. **Determination of free and particle bound pollutants at ultra trace concentrations in water.**
Zorita, S. and Mathiasson, L.
International Journal of Environmental Analytical Chemistry, **2005**, 85, 531-541

The following paper is not included due to the nature of the material and to the extent of my contribution:

- **Optimization and application of homogeneous liquid–liquid extraction in preconcentration of copper (II) in a ternary solvent system**
  Farajzadeh, M.A.; Bahram, M.; Zorita, S. and Mehr, B.G
  Journal of Hazardous Materials, **2008** (in press).

Contribution by the author to the different papers

Paper I. The author was responsible for the experimental design, performed or supervised all the experiments, and wrote the major part of the manuscript.

Paper II. The author was responsible for the experimental design, performed all the experiments, and wrote the major part of the manuscript.

Paper III. The author was responsible for the experimental design, performed all the experiments, and wrote the major part of the manuscript.

Paper IV. The author was heavily involved when the experimental strategy was outlined, performed or supervised all the experiments, and wrote the major part of the manuscript.

Paper V. The author was responsible for the experimental design, performed all the experiments, and wrote the major part of the manuscript.

Paper V. The author was heavily involved in the sampling design, performed all the experiments, and wrote the manuscript.

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ABBREVIATIONS, ACRONYMS AND SYMBOLS

4-IBAP	4-Isobutylacetophenone
APCI	Atmospheric Pressure Chemical Ionisation
BNR	Biological Nutrient Removal
BOD	Biological Oxygen Demand
BSTFA	*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide
C_{18}	Octadecyl Silica
CAS	Conventional Activated Sludge
CZE	Capillary Zone Electrophoresis
DAD	Diode Array Detector
DCM	Dichloromethane
DDD	Defined Daily Dose
DHE	Di-n-Hexyl Ether
DNA	Deoxyribonucleic Acid
E	Extraction Efficiency
E_e	Enrichment Factor
EPA	Environmental Protection Agency
ERA	Environmental Risk Assessment
ESI	Electrospray Ionisation
FS	Flat Sheet
FT-ICR	Fourier-Transform Ion Cyclotron Resonance
GC	Gas Chromatography
HF	Hollow Fibre
HLB	Hydrophilic-Lipophilic Balance
HRT	Hydraulic Retention Time
K_{ow}	Octanol-Water Partition Coefficient
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LIT	Linear Ion Trap
LLE	Liquid-Liquid Extraction
LPME	Liquid-Phase Microextraction
LOEC	Lowest Observed Effect Concentration
MBR	Membrane Bioreactor
MCX	Mixed-Mode Strong Cation-Exchange
MDL	Method Detection Limit
MIP	Molecularly Imprinted Polymer
MISPE	Molecularly Imprinted Solid-Phase Extraction
MMLLE	Microporous Membrane Liquid-Liquid Extraction
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MSTFA	*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide
MTBE	Methyl tert-Butyl Ether

MTBSTFA	*N*-tert-Butyldimethylsilyl-*N*-Methyltrifluoroacetamide
NSAID	Non-Steroidal Anti-Inflammatory Drug
Na_2-EDTA	Ethylenediaminetetraacetic Acid Disodium Salt
OECD	Organisation for Economic Co-operation and Development
PCB	Polychlorinated Biphenyl
PEC	Predicted Environmental Concentration
pK_a	Acid Dissociation Constant
PPCPs	Pharmaceuticals and Personal Care Products
POCIS	Polar Organic Chemical Integrative Sampler
POP	Persistent Organic Pollutants
QqQ	Triple quadrupole
QTRAP	Quadrupole Linear Ion Trap
RO	Reverse Osmosis
RSD	Relative Standard Deviation
SBSE	Stir Bar Sorptive Extraction
SIM	Single Ion Monitoring
SLM	Supported Liquid Membrane
SPE	Solid-Phase Extraction
SPME	Solid-Phase Micro Extraction
SRT	Solid Retention Time
STP	Sewage Treatment Plant
UV	Ultraviolet
US	United States
TOC	Total Organic Carbon
TOPO	Tri-Octyl Phosphine Oxide
TOF	Time of Flight
TSS	Total Suspended Solid

TABLE OF CONTENTS

1. INTRODUCTION

Due to the expected scarcity of clean water resources, and as a result of climate change [1,2], there is an increasing need to monitor water quality across Europe and worldwide. The focus of aquatic environmental research has recently extended beyond classic environmental micropollutants [3] (previously studied and presented in Appendix A-C), such as PCBs [4], pesticides [5,6], and dioxins [7,8], to some polar organic compounds like pharmaceuticals and personal care products (PPCPs) and hydrophilic endocrine-disrupting chemicals [9]. Persistent organic pollutants (POPs) and some pesticides like DDT are considered among the most dangerous contaminants because they do not break down easily, are highly toxic and biomagnify in the food chain [10]. However, scientists are now directing their attention towards pharmaceuticals and polar endocrine-disrupting contaminants such as steroid hormones due to their effects in the aquatic environment [11]. Despite the many efforts that have been made in recent years to study the occurrence of pharmaceuticals and steroid hormones in the environment, the knowledge about the real implications and risks of drugs in the environment is still uncertain. An integrated approach [12] to the problem is needed to be able to regulate the presence of pharmaceuticals (and other pollutants) in the environment.

Although PPCPs are nowadays considered "emerging" pollutants, natural and synthetic steroid hormones were already seen as water contaminants as early as the late sixties [13,14]. At that time scientists already knew and investigated the possibility of removing these compounds from wastewater *via* biodegradation. On the other hand, pharmaceuticals were not seen as environmental contaminants until one decade later [15]. Despite these preliminary investigations about pharmaceuticals in the environment and their biodegradation, it was not until the late nineties that the issue of pharmaceuticals and natural and synthetic steroid hormones in the environment was given significant attention [16-18]. Probably this increasing interest was parallel to the advances in the analytical techniques, which

made it possible to detect these compounds at relevant environmental concentrations (ppt level). Moreover, since the early 1980's, increasing evidence showed that fish intersexuality was closely related to the concentration of sewage effluent entering rivers [19-21]. Despite the low concentration of natural and synthetic estrogens—the female sex steroid hormones—in sewage effluents, these concentrations are high enough to induce negative effects on some aquatic organisms [22,23]. Furthermore, studies have concluded that estrogens are the principal and most potent pollutants of domestic sewage causing endocrine disruption effects in the aquatic environment [23]. However, there are no regulations dealing with the actual levels of pharmaceuticals in various environmental compartments, *e.g.* minimum removal rates in sewage treatment plants (STPs) or maximum drug concentration in surface water. In the European Union there are only a few guidelines advising further environmental risk assessment (ERA) when predicted environmental concentrations (PECs) of pharmaceuticals are equal to or higher than 10 ng/L [24]. Even if the effects of pharmaceuticals on living organisms are often unknown, it is a fact that pharmaceuticals are designed to be biologically active, and some of them are not readily biodegraded [16]. In some cases pharmaceuticals and steroid hormones have also been proven to cause adverse effects in organisms [11]. One example, though not related to the aquatic environment, is the decline of vulture population due to the consumption of livestock treated with diclofenac causing a 95% decrease of oriental white-backed vulture in Pakistan [25].

To address the problem of unwanted occurrence of pharmaceuticals in the environment, a more integrated approach [3,12] is needed to evaluate the real risks of pharmaceuticals and to regulate them. This requires a good and extended knowledge about sources, occurrence, fate, toxicity *etc.* Apart from regulating the waste disposal from the pharmaceutical industry, a deeper understanding of the fate and removal of human drugs and their degradation products in sewage

treatment plants is essential. This would help reduce or minimise the introduction of pharmaceuticals into the environment and thereby protect our water bodies.

1.1. Objectives

This thesis is mainly focused on the development of various sample preparation methodologies preceding the final separation and detection of target analytes on chromatographic systems coupled to DAD or MS. The target analytes were chosen based on consumption (NSAIDs), endocrine disruption potency (steroid hormones) or toxicity (4-IBAP), bio-accumulation properties (fluoxetine and its metabolite norfluoxetine), persistence (clofibric acid) and bacterial resistance (fluoroquinolone antibiotics). The objective of the thesis is to develop novel quantitative, environmentally friendly, cheap, simple and selective analytical strategies to determine seven pharmaceuticals, two metabolites, one degradation product and three steroid hormones from wastewater.

Papers I-III are focused on developing cheap, simple and environmentally friendly membrane-based sample preparation methods for basic drugs, steroid hormones and a degradation product of ibuprofen (4-IBAP).

Paper IV deals with a new MIP sorbent to extract NSAIDs from wastewater, which provides very selective extracts leading to no matrix effects during the analysis with LC-ESI-MS/MS.

Paper V compares different commercially available SPE sorbents in terms of their ability to extract fluoroquinolone antibiotics and to minimise matrix effects during LC-ESI-MS/MS run.

Finally, specific attention is devoted in Paper VI to the evaluation of the occurrence and removal rates of the target analytes in a tertiary STP in the south of Sweden. The analytical methods developed in the work presented in Papers I-V were applied as reported in Paper VI in order to study the occurrence and removal rates of pharmaceuticals and steroid hormones.

2. PHARMACEUTICALS IN THE ENVIRONMENT

2.1. Consumption

The sale of pharmaceuticals is increasing yearly with population as seen in Figure 1, which is consistent with a growing pharmaceutical consumption. These values correspond to an estimated worldwide consumption of active compounds of *ca.* 100000 tons or more *per annum* [26]. However, drug use around the world is not homogeneous. While North America and Europe capture the market with 47% and 30% consumption respectively, the populations of developing countries in Africa, Asia and Latin America only consume 13% of the total market [27].

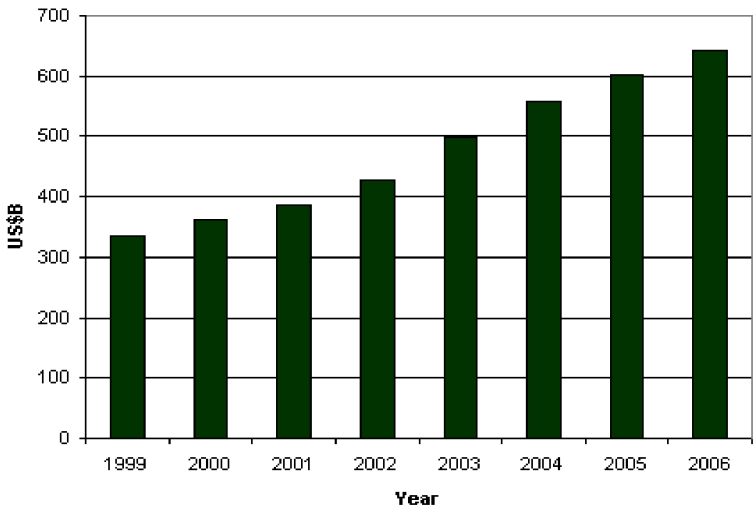

Figure 1. World market for pharmaceuticals. USD billion. Source: IMS World Review 2006.

Lipid regulators, oncology drugs, respiratory agents and acid pump inhibitors are among the highly consumed therapeutic classes [27]. The distribution of use over different therapeutic classes varies from country to country as shown in Table 1.

For example, while cholesterol reducers are the most consumed pharmaceuticals in Australia, diuretics are the most important class in Sweden and Denmark.

Table 1. Consumption of pharmaceuticals in the Defined Daily Dose system (DDD) for 1000 inhabitants in selected countries (n.a. – not available) (OECD Health Data, 2004).

	Australia	Belgium	Denmark	Finland	Sweden
Antidepressants	56.2	45.6	46.5	43.7	59.5
Drugs used in diabetes	39.5	35.3	26.0	52.5	40.9
Diuretics	46.9	42.0	106.0	61.5	84.1
Cholesterol reducers	118.3	74.6	29.5	54.1	55.9
Cardiac glycosides	6.2	6.2	7.0	7.9	55.9
Beta-blockers	23.9	62.3	24.2	63.9	49.4
Anxiolytics	13.9	n.a.	22.1	31.5	16.3
Antibacterials	20.8	24.5	14.7	22.1	16.9
Analgesics	26.3	10.2	89.7	29.7	75.6

2.2. Excretion

After the uptake of pharmaceuticals in the body they are bio-transformed into phase I metabolites by hydrolysis, oxidation and alkylation and into phase II metabolites formed by conjugation with *e.g.* sulphate or glucuronic acid [16]. The aim of metabolism is to change the physicochemical properties of pharmaceuticals to increase their solubility, and hence, to promote their excretion *via* urine or faeces. However, often the metabolism is incomplete resulting in the excretion of not only the conjugates and metabolites, but also the parent compounds [28]. Excretion rates of uncharged compounds can vary from 0 to 100% depending on the type of pharmaceutical (Table 2). The excreted pharmaceuticals, metabolites and conjugates finally end up in the sewage system. Despite the fact that their

excretion rate may be low they are likely to be detected in sewage and surface water if their consumption is high. For instance, this is the case for ibuprofen.

Table 2. Human excretion rates (%) of unchanged, conjugated pharmaceuticals and metabolites. Therapeutic class: [a] Cholesterol reducers, [b] NSAIDs, [c] β-blockers, [d] Antidepressants, [e] Anticonvulsants.

Compound	Excreted unchanged %	Conjugation excretion %	Ref
Benzafibrates[a]	50	22	[17]
Clofibric acid [a]	6	> 90	[17]
Diclofenac[b]	15	< 1	[17]
Ibuprofen[b]	1-8	14	[17]
Atenolol[c]	90	-	[29]
Metaprolol[c]	3-10	-	[17]
Fluoxetine[d]	12	7.5	[30]
Norfluoxetine[d] (metabolite)	7	8	[30]
Carbamazepine[e]	1-3	-	[17]

2.3. Sources

Sewage and sewage treatment plants are believed to be the main source of human pharmaceutical contamination [31], in the sense that STP's compromise the point of release into receiving waters. Pharmaceuticals, used in hospital and household, enter the sewage and sewage treatment plants mainly through urine and faeces [32]. A smaller contribution to the presence of pharmaceuticals in the environment is due to the disposal of outdated medicines down household drains [33] and in the pharmaceutical industry waste [26,34]. Since sewage treatment plants often do not completely remove pharmaceuticals [17,35-38] and steroid hormones [18], these

compounds finally enter surface water and ground water, as discussed in Section 4 and studied in Paper VI. Figure 2 presents a schematic picture showing the sources and pathways of human pharmaceuticals into various water bodies.

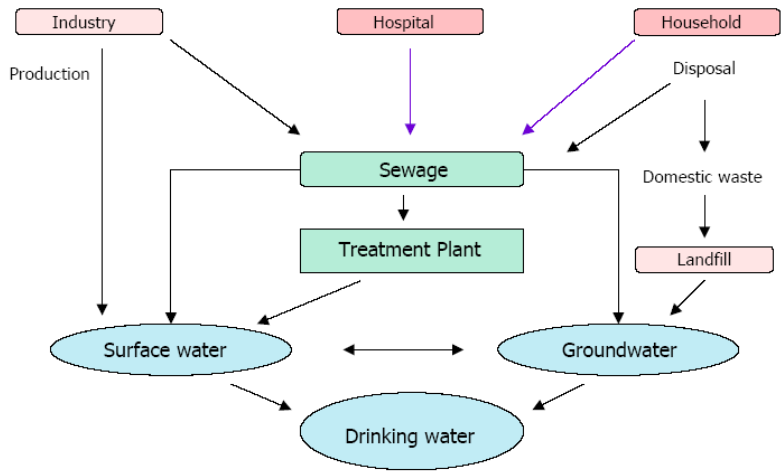

Figure 2. Sources and pathways of human pharmaceuticals and steroid hormones into wastewater and the aquatic environment. Modified from [26].

2.4. Occurrence

The most investigated therapeutic classes in wastewater from 1997 to 2006 (113 studies) have been steroid hormones, NSAIDs and antibiotics with a frequency of 30, 20 and 8.5%, respectively, as described in a review by Miège *et al.* [39]. In many of these works the presence of different pharmaceuticals was confirmed in wastewater effluents leading to contamination of receiving waters. Thus pharmaceuticals and steroid hormones have been detected in different water bodies [11,17,26,31,37,40-57]. Pharmaceuticals have not only been found in wastewater but also in river [46,58-60], ground [61,62], sea [63,64] and drinking water [46,65-68]. In accordance with the data presented in the review by Miége, the most frequently detected therapeutic drug classes in surface waters in the US were

specific steroid hormones and non-prescription drugs [53]. Most likely this is due to their high consumption and/or poor removal rates in STPs. Among individual compounds, carbamazepine is probably the most detected and ubiquitous drug [31,39,69]. More occurrence data (STP influent, effluent and surface concentrations) has been reviewed in a recently published book by D.S. Aga [70]. A few recent studies of occurrence of pharmaceuticals in various important water bodies are given below as an example of their presence in the aquatic environment.

NSAIDs have been detected in **river water** in Finland, Italy and Germany at different concentrations. In a Finish river 32 km downstream from an STP, naproxen and ibuprofen were quantified at 20 and 40 ng/L, respectively [58]. Logically, due to dilution, the further from the sewage treatment plant the lower the pharmaceutical concentration found. Other studies in Germany, Italy and Finland showed lower concentrations for different NSAIDs (0.6-13 ng/L) in rivers [46,59,60]. Other relevant pharmaceutical compounds have been quantified in the Po River, Italy, with median concentrations of 33.0, 17.2 and 23.1 ng/L for ofloxacin, atenolol and carbamazepine respectively [59]. Alternatively, different pharmaceuticals were detected in **groundwater** usually at concentrations between 10 and 100 ng/L [61]. In this study, the most often detected pharmaceuticals were amidotrizoic acid, carbamazepine, sulfamethoxazole and diclofenac with 21, 13, 11 and 4 positive responses in 105 samples. Astonishingly, the maximum detected concentration of amidotrizoic acid and carbamazepine were close to 1 µg/L. Furthermore, pharmaceuticals have even been detected in **sea water** from the North Sea at much lower concentrations with median values of 3.8 and 0.56 ng/L for ibuprofen and clofibric acid, respectively [63,64]. In addition, NSAIDs, antibiotics and X-ray contrast media have been detected in **drinking water** [46,65-67]. The concentration of different pharmaceuticals in drinking water basically depends on: a) the extent of contamination of the surface water; and b) the efficiency of the drinking water treatments. Antibiotics were present at the lowest

concentrations ranging from 1.2 to 4.9 ng/L [67]. Contrast media drugs were found at concentrations between 11 and 60 ng/L [65]. Ibuprofen and ketoprofen were detected one out of three times in Finish drinking water at concentrations of 8.5 and 8.0 ng/L, respectively [46]. However, much higher concentrations were found in a US study, where ibuprofen was detected two out of fifteen times with a mean concentration of 930 ng/L [66].

Pharmaceuticals are today considered "pseudo-persistent" contaminants since they are continuously entering the aquatic environment *via* sewage systems [17], as confirmed by work presented in Paper VI, and can be found in a number of water bodies as shown above. Thus, pharmaceuticals are becoming ubiquitous aqueous pollutants and as a result they are detected in remote aquatic environments [63,64].

2.5. Ecotoxicity

It is known that at least some pharmaceuticals can have toxic effects on non-target species [26,71-81]. However, most available data consists of lethality test (acute) with aquatic algae, daphnids and fish. Therefore, it is difficult to predict long-term effects, resulting in large uncertainties.

In 2006 Fent *et al.* [11] reviewed the aquatic ecotoxicology of human pharmaceuticals. In that review the scattered data regarding the chronic toxicity was highlighted. This means that little is known about the long-term effects of pharmaceuticals in aquatic organisms and that risk assessment is difficult or impossible to perform. On the other hand, most of the lowest observed effect concentrations (LOECs) are substantially above the environmental concentrations that have been observed (ng/L to low μg/L). Therefore, acute toxicity data suggests that the environmental concentrations of most pharmaceuticals are not likely to pose an acute risk to aquatic organisms, except in the case of a spill. In surface water, concentrations are lower than in treated wastewater effluents and so

are the environmental risks. Nonetheless, there are some exceptions where LOECs are close to the observed levels in wastewater effluents, including salicylic acid, diclofenac, propranolol, clofibric acid, carbamazepine, and fluoxetine.

For example, for diclofenac, the LOEC for fish toxicity was 1 μg/L, which is in the range of wastewater concentrations. The LOECs of propranolol (*ca.* 50 μg/L) and fluoxetine (20 μg/L) for zooplankton and benthic organisms were near the maximum measured concentrations in wastewater effluents [11]. Furthermore one should not forget that 17α-ethinylestradiol, which is probably the most potent estrogenic compound, can induce negative effects at extremely low concentrations (low and sub-ng/L) [22,23]. Effects include alteration of sex ratios and sexual characteristics and decreased egg fertilization in fish. Another example of the effects of pharmaceuticals has been investigated by Oetken *et al.* [82]. In this study carbamazepine did not cause any acute toxic effect on three aquatic invertebrate species up to 4 mg/L. However, it produced reproductive effects in an aquatic insect in chronic sediment exposure experiments. So far, most data indicate that some pharmaceuticals may pose an ecological risk, but not a human health risk [83]. For example, a human health risk assessment for 26 active pharmaceutical ingredients and their metabolites (representing 14 different drug classes) indicate that there would be no appreciable human health risk from the presence of these 26 compounds at trace concentrations in surface water or drinking water [84]. Nevertheless, the lack of data on environmental exposure of pharmaceuticals to humans makes it difficult to draw firm conclusions and further studies are needed.

3. DETERMINATION OF PHARMACEUTICALS IN WATER

3.1. Water sampling

In analytical chemistry, sampling is an important part of the experimental determination of chemicals and it can represent the main contribution to the error of the whole analytical process [85,86]. Despite this knowledge, even today samples are not always taken in a representative way in the environmental analysis. For instance, time-integrating sampling techniques should be used in flow systems over a sufficient period of time to avoid over- or underestimation when using *e.g.* grab sampling as shown in Paper I and in [87]. However, only 63% of the total data evaluated on occurrence from 1997 to 2006 used flow-proportional sampling [39] while the rest used grab sampling. This results in uncertain environmental data. In work presented in Papers II-V grab sampling was however conducted because the aim was to evaluate the method performance and not the environmental concentrations.

Time-integrated sampling can be performed by using: a) pumps over a period of time (usually 24h) collecting the water in a recipient; or b) passive sampling by means of placing a device that contains a sorbent in the water [86]. Pump-based time-integrated sampling can be coupled to automated supported liquid membrane (SLM) extraction. In this case pollutants are continuously separated from the matrix, which decreases the risk for undesirable reactions during the sampling period [88,89]. Integrated passive samplers extract analytes from the water over longer periods of time (from days to weeks) [90-96]. Nowadays, there is one commercially available device, which is designed to sample water-soluble organic chemicals from aqueous samples commonly called POCIS (polar organic chemical integrative sampler) [97]. Passive sampling has the ability to reduce the number of sampling periods and correspondingly the number of analyses needed to evaluate environmental concentrations over long periods of time (months) [90]. This will

result in a cost reduction. A further interesting characteristic of POCIS is the elimination of power requirements, making it easy to use in remote areas. Despite these advantages of passive sampling over pump-based time-integrated sampling, there are a few drawbacks to overcome. First, the calculation of accurate concentrations requires calibration studies which may be time consuming, and appropriate reference calibrations compounds are needed [90,93]. Even this does not guarantee the successful application of POCIS [91]. Differences in temperature, flow, salinity and water matrix can influence the analyte uptake kinetics [91]. Therefore, the robustness and reliability of this technique needs to be improved. Additionally, membrane bio-fouling in *e.g.* raw sewage can minimise the uptake kinetics making the laboratory calibration unusable [92,93]. Another problem that may occur over a long sampling period is the degradation of labile pharmaceuticals [92,93]. Extensive laboratory investigations are required to elucidate under which environmental conditions bio- or photo-degradation is relevant.

Probably, as a result of all these limitations, active (by pump) 24-h integrated sampling is widely used to study the fate and occurrence of pharmaceuticals in the environment [29,98-101]. As reported in Paper VI, active integrated sampling was applied to evaluate daily influent and effluent loads of pharmaceuticals in an STP. Nonetheless, extensive development of passive sampling in the last few years for aqueous samples makes it a very attractive technique for the future and has the capacity to supplant active integrated sampling.

3.2. Sample preparation

The analysis of micropollutants, such as pharmaceuticals in wastewater, is often a challenge because of the complex matrix and the low concentrations (often in the low ng/L level). The analysis can require several steps such as filtration, pH adjustment, extraction and clean-up to achieve high enrichment and clean extracts prior to introducing the worked up sample to the final analytical instrument.

Today sample preparation is moving towards environmental friendliness, low cost, miniaturisation, automation and simplicity. Beside these desirable features, sample preparation methods must also be characterised by accuracy and reliability and for pollutants occurring at very low concentrations in complex matrices, they must provide as selective pre-concentration as possible within a reasonable time.

Nowadays, there are several sample preparation techniques used to extract pharmaceuticals, steroid hormones and other polar pollutants from aqueous samples. These include conventional liquid-liquid extraction and solid-phase extraction, employed in the studies presented in Papers IV and V, and different types of membrane-based microextractions that were used for sample preparation of various target analytes as reported in Papers I-III. Pharmaceuticals and steroid hormones can also be extracted by other modern techniques such as solid-phase microextraction (SPME) [102-107] and stir bar sorptive extraction (SBSE) [108-110], but these techniques are not further discussed here since they were not investigated in this thesis.

3.2.1. Liquid-liquid extraction (LLE)

Classically, pollutants from water samples have been extracted by means of LLE. The principle of this technique is based on exploiting the differences in partition coefficients between two analytes in two immiscible solvents, normally an aqueous

phase and an organic phase [87]. Commonly utilised solvents are hexane, isooctane, toluene, chloroform and methylcyclohexane [87,111]. The use of this type of organic solvent is probably the most important drawback of LLE because the solvents themselves pose an environmental problem and result in trouble regarding occupational hygiene [87,112]. In the case of environmental analysis, LLE has traditionally been used for extraction of POPs rather than polar organic pollutants such as pharmaceuticals. However, a continuous LLE method was presented in 2004 for the extraction of pharmaceuticals and hormones from aqueous samples. The method had the advantages of rapidity, simplicity, and there was no need for derivatisation [113]. Nonetheless, the use of dichloromethane as extracting solvent, 40 to 60 mL for each sample, makes the method questionable from an environmental point of view. Other common disadvantages of this simple but labour-intensive technique are the problems regarding emulsions and adsorption to glassware [87,112]. Despite its disadvantages, LLE is still widely used for aqueous sample preparation, mainly because it is prescribed in standardised protocols. Examples of such protocols are those from the US EPA, for environmental samples [114-116] including the analysis of steroids and hormones in a recent protocol [117]. Nevertheless, the latest trend in sample preparation is to substitute LLE by other methods such as the ones presented below. For example, solid–phase extraction (SPE) overcomes the main drawbacks of LLE and most likely this is the reason why SPE is now the preferred extraction technique for pharmaceuticals from the aquatic environment.

3.2.2. Solid-phase extraction (SPE)

The principle of SPE is the adsorption of the analytes to a sorbent when the aqueous phase passes through a cartridge or a disk [118,119]. Conventionally SPE sorbents were made of porous silica particles with a bonded non-polar phase like C_{18}, cyano propyl. Alternatively, the sorbent can comprise an organic polymer,

such as cross-linked divinylbenzene (*e.g.* XAD resin) [118]. However, in most cases, these sorbents do not yield good recoveries for highly polar compounds such as pharmaceuticals. With the introduction of new sorbents such as hydrophilic lipophilic balance resins (HLB), the applicability of SPE for polar compounds has improved considerably. Other sorbents utilised for pharmaceutical extraction in environmental samples are ion-exchange resins [70,120,121], mixed mode sorbents, C_{18} [70,122] and cross-linked polymers (ENV+) [123]. Despite that ion exchange sorbents are becoming more popular for pharmaceutical extractions, HLB is still the preferred sorbent [70] and is the one selected by the US EPA for multi-residue analysis of pharmaceuticals in water [124]. Organic analytes that have been extracted by SPE can be eluted with organic solvents. In most cases the volume of the elution solvent is much smaller than the volume of the original sample and thus a dramatic pre-concentration is achieved. In environmental pharmaceutical analysis the analytes are typically concentrated by a factor of 10^2-10^4 depending on the aqueous matrix.

The extraction procedure of SPE is simple and usually consists of four steps as outlined in Figure 3 [118,119]. To promote better surface contact, conditioning of the sorbent is first done. This is typically carried out by the addition of methanol followed by distilled water with pH adjustment. In some cases the conditioning includes other additives such as Na_2-EDTA [45,124] as reported in Paper V, to avoid complexation with metal ions. Thereafter the sample is loaded onto the cartridge. For a good retention or trapping of the analytes the flow should be controlled. Common flow rates vary between 1 to 10 mL/min. The cartridge size and the amount of sorbent determine the optimal flow rate. Depending on the type of sorbent a washing step is sometimes performed. In some cases distilled water is used to remove remaining salts and ions [45,124]; however this will typically not be sufficient to remove all interferences. Ion exchange sorbents are more selective than the commonly used HLB resins and allow stronger washing

steps even with pure methanol or acetonitrile [121]. In work reprinted in Paper V , mixed-mode cation exchange sorbents were compared to HLB for the determination of fluoroquinolone antibiotics in wastewater. As mentioned, mixed-mode weak cation exchange sorbent allowed pure methanol washing leading to cleaner extracts compared to HLB. However, the latter sorbent showed higher capacity for analyte trapping and better precision. Finally, elution is performed with an organic solvent such as methanol, acetonitrile, methyl tert-butyl ether (MTBE), acetone or with some previously mentioned organic solvents containing acids or bases [70]. The latter is necessary in the case of ion exchange sorbents to break the ionic bonds between the sorbent and the analytes.

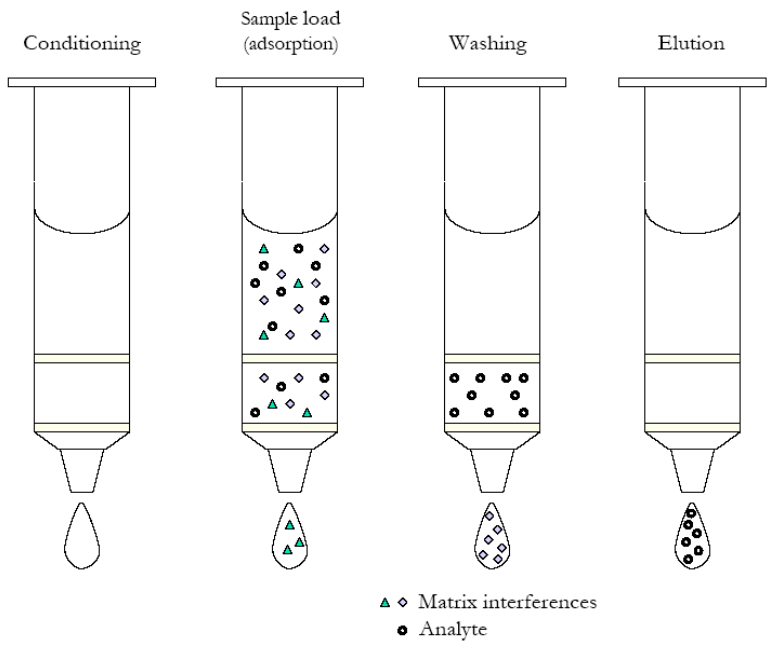

Figure 3. Common SPE steps.

For environmental applications, large volumes (1 L or more) of aqueous matrices can be necessary to achieve relevant method quantification limits. However, due to

analytical improvements these volumes are decreasing. When working with large volumes and/or very complex matrices, it is important to be aware of the potential for the breakthrough of the sorbent [125]. Sample breakthrough depends on the strength of the interaction between the analyte and sorbent, the sample volume, the sample matrix and the mass of sorbent. The presence of natural organic matter can hamper the extraction of analytes from environmental aqueous matrices. The organic matter not only changes the properties of the extracting sorbent (often resulting in smaller breakthrough volume and smaller retention capabilities of the analytes), but it can also affect the final analysis through ion suppression or enhancement [125]. To avoid/diminish ion suppression, highly selective sorbents such as molecularly imprinted polymers (MIPs) can be used. Alternatively, one may employ a different extraction technique with good clean-up capabilities such as supported liquid membrane (SLM) extraction.

Today numerous individual therapeutic class methods are available and have been reviewed in several papers [70,126-129]. Therefore, they will not be covered here. The latest trends in SPE are towards the analysis of multiple compounds with different physicochemical characteristics for screening and monitoring of organic pollutants in different water bodies to identify the source of emission, to study the fate and transport of pollutants and/or to evaluate their occurrence.

Multiresidue methods

Many analytical methods for pharmaceuticals in aqueous matrices are developed for a selected therapeutic family. The benefit of this approach is the high recovery achieved. Moreover, the need to analyse many different type of drugs makes multiresidue methods very attractive. This approach gives a more comprehensive picture of the occurrence and fate of pharmaceuticals in the environment with lower cost and time per analysis. In this section, some of the latest multiresidue

methods for pharmaceutical analysis in the aquatic environment are briefly discussed.

Six investigations have been published since 2007 presenting multiresidue methods for pharmaceuticals and other emerging pollutants [68,130-134]. The number of compounds which were simultaneously determined varies between 10 and 51. The preferred sorbents was mixed-mode strong cation-exchange (MCX) followed by HLB. While HLB was used for methods involving higher structural differences between analytes, MCX was often employed for acidic and neutral pharmaceuticals or for neutral and basic drugs. Zhang *et al.* [130] reported the determination of ten pharmaceuticals with quite different physicochemical properties from nine different therapeutic classes using the HLB sorbent. Recoveries in river water, generally, varied between 71 and 89%, except for tamoxifen and thioridazine with somewhat low recoveries of 55 and 10%, respectively. Kaspryk-Hordern *et al.* [131] developed an analytical method for the determination of 28 basic-neutral pharmaceuticals and illicit drugs. Their sorbent of choice was MCX. In this method, only 18 compounds had relative recoveries over 60%. This means that the analysis of about one third of the target analytes was semi-quantitative and some analytes could not even be extracted (ciprofloxacin and doxycycline). The same authors presented a similar methodology to extract 25 acidic and neutral PPCPs in river water [132]. Generally, relative recoveries with MCX varied between 70 and 138% with a few exceptions like pravastatin, bendroflumethiazide and sulfamethoxazole with relative recoveries below 53%. In a few cases (4-benzophenone, digoxin and pravastatin) HLB sorbent was required to obtain acceptable recoveries. Piram *et al.* [133] developed a method that allowed the quantification of 21 neutral-basic pharmaceuticals from two therapeutic classes, β-blockers and corticosteroids, in sewage water using MCX. The extraction yields for corticosteroids and β-blockers varied between 54-86 and 60-68%, respectively. However, an extraction yield of only 10% was achieved for pindolol. In 2008 Hao

et al. [134] published a multiresidual method for the determination of 51 emerging organic pollutants (many of them being pharmaceuticals) using HLB. With this procedure, absolute recoveries of various drugs ranged between 60 and 115%, except for ciprofloxacin (264%) and acetaminophen (32%). Nonetheless, the use of identical deuterated surrogate standards gave relative recoveries fluctuating between 87 and 108% and better RSD values. The main disadvantage of this quantitation technique is the high price of the isotopically labelled compounds.

These results show that in multiresidue methods, quantitative recoveries are difficult to achieve. For very different compounds, HLB showed slightly better recoveries. As previously mentioned in Paper V, it is also shown that HLB provides a slightly higher trapping capacity over mixed-mode ion exchange sorbents even for the extraction of one pharmaceutical class. The flow rate used to process the sample can be a key parameter in obtaining higher recoveries. Theoretically, low flow rates would result in higher trapping capacity. Zhang, Piram and Kasprzyk-Hordem [130,131,133] used flow rates between 4 and 15 mL/min for 3- and 6- cc cartridges while in work reported in Paper V 1 mL/min was used for 3-cc cartridges.

Although it is natural that multiresidue methods are pursued, such a method is not always the most desirable approach due to the large differences in physicochemical properties of the pharmaceuticals and hormones. For instance, two different extraction methods and four different LC-MS/MS conditions have been considered as optimum in the last US EPA report to determine 74 PPCPs from environmental matrices [124]. Therapeutic-class-based methods often have conditions similar to those of multiresidue methods, but extraction conditions are optimised so that high recoveries are achieved for very similar compounds.

3.2.2.1. Molecularly imprinted solid-phase extraction (MISPE)

MIPs are synthetic polymeric materials that are designed to possess molecular recognition properties [135,136]. Despite their current growing status the first imprinted materials date from the 1940'S-50'S [135,136]. The synthesis of MIPs involves a template, which is the target analyte or a structural analogue, functional monomers, crosslinkers, a porogen and an initiator [135,136]. First, the template molecule interacts with the functional monomers by reversible covalent bonds, metal ion coordination or non-covalent bonds. In this thesis only the non-covalent bond will be discussed (Figure 4). In the second step of the process the polymerisation takes place with the help of a cross-linker in the presence of an initiator. Finally the template is removed, frequently by solvent extraction. The porogen or solvent plays a role in the morphology of the polymer in terms of pore diameter [135,136]. Designing and synthesising a MIP can be challenging due to the many variables involved. Other common drawbacks of this type of sorbent are template bleeding for trace analysis and the presence of non-specific interactions [137].

The use of MIPs as SPE sorbents is relatively recent and has been reviewed recently by Pichon [136]. Sellergren applied MIPs for the first time in 1994 for the extraction of pentamidine in urine [138]. Since then more than 200 compounds have been utilised as templates in non-covalent molecular-imprinting protocols mainly for biological and environmental matrices. Nevertheless there are only a few studies on the application of MISPE for NSAID extraction from river water [139], wastewater (Paper IV) and for diclofenac extraction from wastewater [139,140]. The main advantage of these methods is their high selectivity providing very clean extracts that could be analysed by LC-DAD. The steps in MISPE are the same as in conventional SPE, but efficient washing steps can be applied even by using strong solvents e.g DCM [139], providing good clean-up capabilities. As

reported in Paper IV, cleaner extracts resulted in no appreciable ion suppression/enhancement in the LC-MS/MS when extracting sewage water.

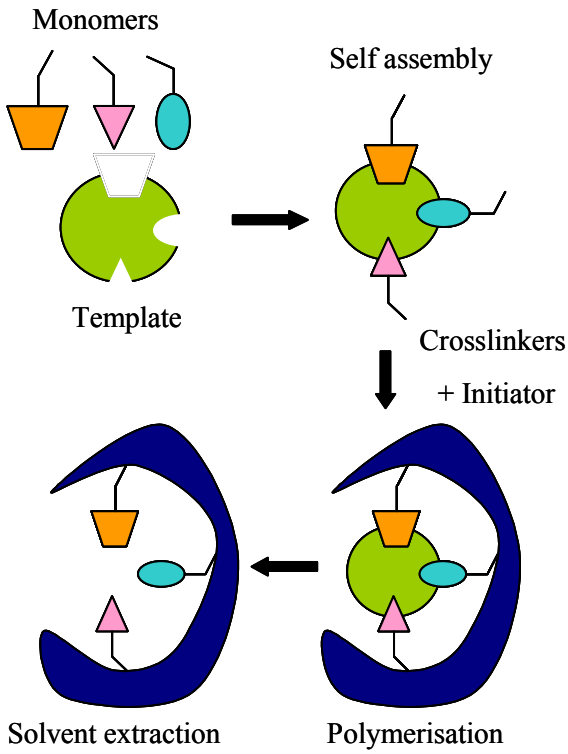

Figure 4. Schematic representation of the non-covalent approach for producing molecular imprinting polymers.

3.2.3. Membrane extractions

In recent years, the extraction of various chemicals with membranes has grown in interest. Membrane extraction has generally been performed with flat sheet (FS) microporous membranes [141-143], but nowadays, the extractions are mainly

performed with hollow fibre (HF) microporous membranes (Papers I-III) [144]. The concept of solvent extraction with microporous HF was already established in 1988 [145], but it was not developed for analytical purposes until approximately until one decade later [146]. Two types of membrane-based extraction techniques have been described since the eighties and nineties (see Table 3): supported liquid membrane (SLM) [141,142], which is a three-phase system, and microporous membrane liquid-liquid extraction (MMLLE) [143], which is a two-phase system. Despite the original use of the terms SLM and MMLLE, these techniques are increasingly referred to as 3-phase and 2-phase liquid-phase microextraction (LPME), respectively [147]. Regardless of the terms used, SLM and MMLLE are considered as subtechniques of LPME.

Table 3. Membrane based extraction techniques and their terminologies

Author	Terminologies	
Rasmusen [144]	3-phase LPME	2-phase LPME
Jönsson [148]	SLM	MMLLE

Membrane-based extractions have the advantages of simplicity, low cost, environmental-friendliness, high enrichment [149,150], and in some cases cleaner extraction compared to than SPE. SLM and MMLLE can be automated [151], however the fibre-based set-ups are difficult to automate. SLM and MMLLE can be used for quantitative extraction shown as in Paper III and [150], partial extraction as shown in Paper I and [152], or for measurement of freely-dissolved compounds as shown in Paper II and [153].

3.2.3.1. Supported liquid membrane (SLM)

SLM, as previously mentioned, is based on a three-phase system with an organic phase immobilised in the pores of a hydrophobic membrane (*e.g.* polypropylene, polytetrafluroethylene) situated between two aqueous phases [148]. In HF-SLM, a water-immiscible organic solvent is immobilised in the pores of a membrane. This can be achieved by dipping the hollow-fibre membrane into the organic solvent. After removing the excess of solvent *e.g.* by immersing the membrane in distilled water or by a short sonication step, the aqueous acceptor phase can be filled with a syringe. Then the fibre is placed in the aqueous sample (donor phase).

The basic principles of HF-SLM are illustrated in Figure 5. An essential feature of a simple SLM set-up without carrier in the membrane liquid is the proper pH adjustment of the donor and acceptor phases. For a depleting extraction, the pH in the donor should be at least two units below the pK_a for acidic analytes or above the pK_a for basic analytes. In this way ionisable target analytes are in uncharged form and will therefore diffuse to the organic phase. Appropriate organic solvents, *e.g.* di-n-hexyl ether (DHE), 1-octanol, undecane, *etc.* should have low solubility in water, low vapour pressure (to avoid losses) and low viscosity (high mass transfer). In work reported in Paper I and [154], the superiority of DHE as an organic solvent was demonstrated, probably due to its low viscosity and low solubility in water. In some cases, carrier-mediated transport (active extractions) can be used to improve the extractability of some compounds [155,156]. As reported in Paper III, higher mass transfer rate was accomplished with the addition of tri-octyl phosphine oxide (TOPO) to the organic phase for the extraction of steroid hormones from water. However, one should also take into account that TOPO will also improve simultaneously the extractability of other non-target analytes, which could result in interferences. From the organic phase, analytes will further diffuse to the acceptor phase, where the pH should be at least 3.3 units above pK_a

(for acidic analytes) or below pK_a (for basic analytes). In this manner analytes will be trapped in the acceptor phase since they will be in an ionised form, preventing them from re-entering the membrane. Charged compounds will not enter the membrane and will therefore not be extracted. The extraction of macromolecules will be very low due to their low diffusion coefficients. Neutral compounds will have a certain tendency to be partitioned in the three phases, but will not be enriched. It has to be mentioned that other trapping mechanisms such as immunological-based trapping with antibodies are possible [157]. SLM is especially applicable for polar-semipolar compounds ($-1 < \log K_{ow} < 3$) [148]. Mass transfer in SLM has previously been described [142,158,159] and will not be further discussed here.

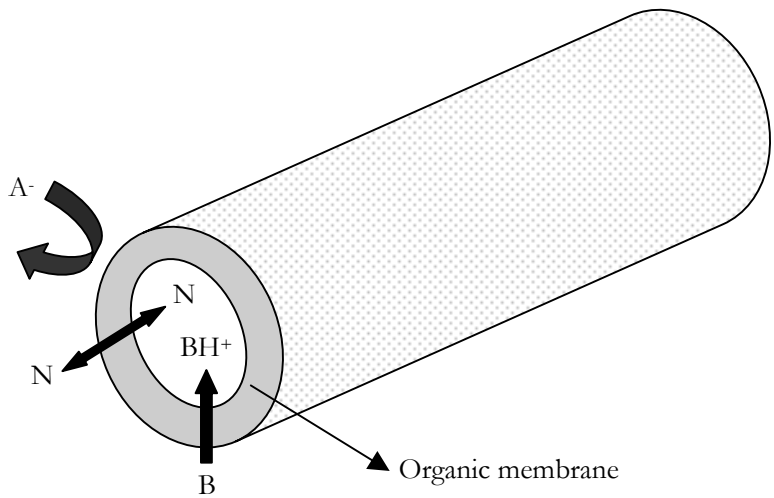

Figure 5. HF-SLM principle for extraction of basic analytes.

The performance of SLM is usually characterised by the enrichment factor (E_e) that generally can be described as the concentration in the acceptor divided by the concentration in the donor, and by the extraction efficiency (E) or recovery, which corresponds to the molar ratio of the analyte between the acceptor and donor. In HF-SLM, high enrichment factors can be obtained even if only low recovery is

achieved, as shown in Paper I. When the volume ratio between the donor and acceptor is high as is often the case in environmental applications [149] the extraction is usually carried out under kinetic conditions and the extraction is stopped before total extraction is achieved. In contrast, when small sample volumes are used (as in bioanalytical applications [160]), quantitative extraction is easy to accomplish. Normally, in HF-SLM set-ups mass-transfer is enhanced by stirring or shaking [153].

Environmental applications

Few analytical papers have been published concerning the extraction of different pharmaceuticals from water using HF-SLM. NSAIDs have been the most investigated pharmaceutical class [160-162], followed by basic antidepressant drugs [42,163], β_2-antagonist drugs [164], estrogens [165] and ivermectin, a veterinary parasiticide [166]. Moreover, the flow system FS-SLM has also been used for industrial purposes such as the removal of diclofenac from aqueous media [167].

High sample volumes (1.1 L) have been used for the extraction of **basic antidepressant drugs** in order to achieve high enrichment factors, *e.g.* 11495 for fluoxetine and 10725 for norfluoxetine in ultrapure water using aqueous formic acid as acceptor phase [42,163]. DHE as organic solvent gave the highest recovery compared to 1-octanol or silicon oil. The acceptor solution consisted of 20 µL of HCl or formic acid at pH = 2, and the sample pH was adjusted to 11.8 with NaOH. Extractions were performed for 120 min supported by magnetic stirring at 800 rpm. Within-day precision showed RSD values between 12 and 21%, whereas between-day precision was within 18 and 37%. The method quantification limits ranged between 57 pg/L for citalopram to 4100 pg/L for desmethylsertraline. However, the method was not validated with more complex water types such as sewage, which means that lower E_e than the values given are probably obtained with complex waters as described in Paper I. In this paper [42] in contrast to Paper

I, the obtained enrichment factors are 2.3 to 2.8 times higher for fluoxetine and norfluoxetine, respectively, in distilled water. Since the fibre used by Vasskog *et al.* [42] is thicker (140-µm versus 50-µm membrane wall used in Paper I) the only possible explanation for those high mass transfer rates leading to high enrichment factors is that magnetic stirring at 800 rpm is a more efficient way to increase the mass transfer than is shaking at 130 rpm.

Yamini *et al.* [164] extracted very polar basic **β_2-antagonist drugs** with active mass transfer using 20% Aliquat 336 (methyltrioctylammonium chloride) as a carrier in the DHE, which was used as organic membrane. The donor phase consisted of 11 mL 0.005M NaOH aqueous solution. In this work it was crucial the use of a salt (1M NaBr) was crucial in activating the transport of analyte though the membrane. For a 60 min extraction under the use of stirring at 500 rpm, the optimised enrichment factors were 53 and 213 for salbutamol and terbutaline, respectively. Despite the use of LC-MS/MS the detection limits were fairly poor, 500 and 2500 ng/L.

Estrogen determination in wastewater using HF-SLM has been carried out by Liu *et al.* [165]. In this case a 4.5-cm fibre impregnated with 1-octanol was placed in 10 mL of an acidic sample containing 20% NaCl (pH =1.5). The acceptor solution composition was 0.5M NaOH. The extraction was performed for 40 min at 1200 rpm stirring speed. Enrichment factors slightly over 300 were obtained, leading to determination limits ranging from 0.25 to 0.5 µg/L, which are extremely poor considering the relevant environmental concentrations. RSD values were below 11% for spiked ultrapure water.

The three published extraction methods for **NSAIDs** and other acidic drugs using HF-SLM are to a great extent quite similar. The type of membrane was in all cases a polypropylene HF (Q3/2 Accuarel, from Membrana) and the same organic

solvent (1-octanol) was used for impregnation. The sample volume varied between 10 [160,162] and 22 mL [161]. The donor phase was adjusted to pH = 2 with HCl or acetic acid. The acceptor volume (5-100 μL) depended on the size of the fibre, which ranged from 2.4 [162] to 53.5 cm [160]. The acceptor phase was a basic solution of 10 mM ammonium carbonate [161], 0.01 or 0.1 M NaOH [160,162]. The extraction times varied between 30 [160] and 60 min [162] with stirring speed ranging between 500 [161] and 990 rpm [162]. Two of these methods achieved recoveries over 79% leading to nearly maximum enrichment, *ca.* 100 fold for Wen *et al.* [160] and 1650-1900 fold for Wu *et al.* [162]. However, despite high enrichment factors obtained by Wu *et al.*, they reported higher MDLs ranging between 30 and 300 ng/L, compared to Wen *et al.* who claimed MDLs between 15-100 ng/L. This is somewhat peculiar since both groups' detection methods were based on LC-UV. Quintana *et al.* [161] also performed the validation in ultrapure water obtaining enrichment factors between 38 (for piroxicam) and 234 (for clofibric acid). The quantification limits using LC-MS/MS improved, fluctuating between 0.5 and 42 ng/L for effluent wastewater. The three methods had a moderate precision in ultrapure water with RSD values ranging from 3.4 to 32%. It has to be stressed that there are some disagreements among the methods. While Quintana *et al.* found *via* an experimental design that NaCl had a negative effect on extraction, Wu concluded that the use of 15% salt was advantageous for the extraction. Furthermore, the LC-UV chromatograms for wastewater in Wu *et al.* and Wen *et al.* methods [160,162] have very different appearance. While Wu *et al.* demonstrated no interferences at all, Wen *et al.* showed a large interfering peak. The latter seems more reasonable due the co-extraction of *e.g.* humic acids present in the matrix. Therefore, the use of LC-MS/MS for this type of matrices is highly recommended. Quintana *et al.* demonstrated that the matrix effect in LC-MS/MS can be minimal with less than 6% ion suppression for clofibric acid, naproxen and diclofenac.

One goal of this thesis was to develop new extraction methods based on membrane techniques for different pharmaceuticals and steroid hormones, which sometimes turned out not to be straightforward. HF-SLM was tested for **acidic pharmaceuticals** (NSAIDs and clofibric acid); however high buffer concentrations (0.5 M acceptor buffer for the extraction of 0.5 L raw sewage) were needed in the acceptor phase in order to avoid pH decrease and associated back-extraction. This high buffer concentration, regardless of the small dilution, led to problems with ion suppression during the LC-ESI-MS/MS run [154]. Furthermore, co-extracting compounds, *e.g.* humic acids, led to higher ion suppression (45-96%), resulting in relatively poor MDLs. As formerly mentioned, the use of TOPO also typically leads to enhancement of the co-extraction of non-target analytes, which can influence quantification in the LC-MS/MS method. As previously described, Quintana *et. al* [161] developed an HF-SLM-based extraction method and the analysis was performed by LC-MS/MS. The authors used 20 µl of 10 mM of acceptor buffer and 22 mL sample volume. After 45 min the enrichment factor started to decrease. This is due to a pH decrease in the acceptor, which leads to back-extraction. This was confirmed in our laboratory by monitoring the pH during extraction. After 45 min of extraction, the pH in the acceptor was 5-5.5 instead of 9. However, no significant matrix effect was observed using this approach, which was also evaluated by our group. Therefore, a successful approach to extract acidic pharmaceuticals by HF-SLM and analyse the extract by LC-MS/MS is to perform the extraction at low acceptor buffer concentrations and stop the extraction before the pH in the acceptor decreases below the pKa values of the analytes. This will lead to small enrichment factors (*ca.* 100-200), but since the QqQ detector offers high sensitivity (discussed in Section 3.3) the MDLs achieved by Quintana *et al.* [161] are satisfactory. LC-UV is not an alternative to LC-MS/MS because of a large amount of co-eluting interferences in the chromatogram, when high buffer concentrations in the acceptor and long extraction times are used. The alternative with low buffer concentration in the

acceptor and short extraction times gives too high detection limits. To overcome these problems, the extraction of the acidic drugs was performed using a novel MISPE procedure (Paper IV), which provides very clean extracts with no ion suppression. Since HF-SLM can achieve very high enrichment factors, another alternative could be to couple HF-SLM with MISPE when extremely low detection limits are needed.

The extraction of **fluoroquinolone antibiotics** with HF-SLM was also investigated. These compounds are zwitteronic and therefore cannot be extracted under regular conditions [168]. The SLM extraction procedure involves use of a carrier in the organic phase and a high concentration of NaCl in the acceptor phase, which is incompatible with LC-MS/MS. This may be overcome with a post-column valve in order to divert the unwanted LC fraction. Another option could be a clean-up step with e.g. MIPs.

3.2.3.2. Microporous membrane liquid-liquid extraction (MMLLE)

An alternative to SLM for moderately or highly hydrophobic (log K_{ow} > 3) compounds is a two-phase system (aqueous/organic) based on the microporous membrane LLE (MMLLE) extraction technique. The extraction is driven by the difference in chemical potential of the analytes in the organic solvent and in the aqueous solution, which is described as a partition coefficient [148]. Usually the membrane, as for SLM, can be a flat sheet (FS) or can have a hollow fibre (HF) format. The latest developments and trends in membrane extraction including MMLLE have been presented by Barri *et al.* [169] in a recent review. This technique has many advantages over conventional LLE. Apart from avoiding emulsion formation, it consumes extremely low amounts (usually on the μL range) of organic solvent and very high enrichment factors can be achieved [150].

For aqueous samples this membrane approach has mainly been applied to POPs, pesticides, phthalate esters and similar highly hydrophobic compounds. Apart from Papers II and III, only one more work [170] is available on the two-phase system membrane extraction for pharmaceuticals, metabolites or degradation products and steroid hormones from water samples. However, MMLLE using FS [143] or HF [171,172] set-ups has previously been used to extract pharmaceuticals from biological fluids.

Müller *et al.* [170] presented an automated hollow-fibre membrane extraction technique coupled to GC-MS for the determination of pharmaceutical and endocrine disrupting compounds in water samples. n-Octanol was used as the acceptor solution inside the HF. Optimum extraction yields were obtained after an enrichment time of 60 min. The influence of pH and saturated NaCl in the donor phase (5 mL) was studied. A saturated NaCl donor solution gave the best performance for carbamazepine, phenazone and ibuprofen while distilled water at pH 2 was found optimal for 17α-ethinylestradiol. Enrichment factors varied between 13 (phenazone) and 415 (ibuprofen) in ultrapure water. Extraction RSD values for pharmaceuticals were below 12%. The limit of detection in ultrapure water ranged from 20 to 40 ng/L in ultrapure water. Paper III deals with extraction of steroid hormones including 17α-ethinylestradiol. Higher E_e, >1500, was obtained in work reported in Paper III as a consequence primarily of a higher sample volume.

3.3. Final analysis

Pharmaceuticals are generally polar compounds with small to moderate octanol-water partition coefficients. Consequently, liquid chromatography is usually preferred as the final analytical technique [173]. Gas chromatography is mainly employed for the analysis of steroid hormones [18,106,108,174-178] and acidic pharmaceuticals after derivatisation [49,60,103,107,170,178,179]. Despite the fact that fluorescence [44,120,121,180] and DAD detectors, as in Paper I and [44,140], have been used in liquid chromatography to detect pharmaceuticals, the latest trend is to perform the analysis with MS or tandem MS. In a few cases, the sample preparation of *e.g.* wastewater samples provides very clean extracts avoiding interferences with fluorescence or DAD detection, as demonstrated in Paper I. These detectors represent a cheap option for analysis compared to MS. However, besides selectivity, MS often provides higher sensitivity, and consequently the majority of the studies performed on the fate and occurrence of pharmaceuticals in wastewater and surface water are performed with mass spectrometry. This section will focus on mass spectrometry based analysis because of its wide usage and greater level of technical development in this area.

3.3.1. Liquid chromatography

Nowadays, the sensitivity achieved by LC coupled to MS has improved to the extent of being as good as GC coupled to mass spectrometry. Consequently, its use for the determination of polar contaminants from environmental samples has grown rapidly [181-188]. LC has the advantage over GC in that derivatisation is not required for *e.g.* acidic compounds and estrogens.

There are some restrictions when coupling LC to MS, such as the requirement that only volatile chemicals be used. However, the main drawback of LC-MS/(MS) is matrix effects, which often result in a signal decrease (ion suppression) or to a

lesser extent a signal increase (ion enhancement) [132]. In both cases, the accuracy of the analysis will suffer. Matrix effects in the widely used ionisation technique electrospray ionisation (ESI) are suspected to be due to the presence of less volatile compounds (from mobile phase additives or co-extracted compounds), which can result in competition for charging or can change the efficiency of droplet formation or evaporation [132,189]. This in turn affects the amount of charged ions in the gas phase [190]. There are different strategies to compensate for or minimise matrix effects, such as the reduction of the liquid flow rate entering the mass spectrometer; the use of smaller amounts of additives, often less than 20 mM, in the mobile phase; as selective sample preparation procedure as possible giving an efficient clean-up; the dilution of the extracts; and the use of deuterated internal standards or the standard addition method to compensate for matrix-dependant signal changes [191].

In most cases, ESI seems to be more sensitive to matrix effects than is atmospheric pressure chemical ionisation (APCI). ESI often presents ion suppression of pharmaceuticals [67,123,131,132,189,192,193]. In one of the few works on the analysis of pharmaceuticals from wastewater using APCI, this technique showed ion enhancement for five of the seven neutral pharmaceuticals considered [194]. However, previously, both ion enhancement and ion suppression have been observed for APCI. The matrix effects observed in APCI are believed to be related to co-precipitation of the analytes with non-volatile materials and gas-phase neutralization processes [194].

Conventionally, the triple quadrupole mass spectrometer (QqQ) has been employed for quantitative trace analysis using single ion monitoring (SIM) or multiple reaction monitoring (MRM) because of its sensitivity. However, other types of detectors are becoming more popular due to technical advances. Recently, linear ion-trap (QTRAP or LIT) mass spectrometers have been commercialised

improving the performance of traditional ion-trap detectors in terms of detection limit [195,196]. The QTRAP provides accurate quantitative results and instrumental detection limits are in the same range (or slightly worse) as in QqQ. Apart from this feature, the capability of the QTRAP to aid in the identification of non-target compounds, metabolites and/or degradation products, makes this detector attractive and promising [195]. Time-of-flight (TOF) mass spectrometry has as well unique capabilities to elucidate/identify unknowns or degradation compounds due to its high mass accuracy measurements and ability to avoid isobaric interferences [181,195,197]. Quadrupole time-of-flight mass spectrometry (Q-TOF-MS), apart from aiding in elucidating unknown compounds, has been proven to have comparable or slightly worse method detection limits than QqQ [197]. Therefore, the great advantages in identifying unknown compounds in complex matrices by Q-TOF-MS and the complementary structural information obtained through fragmentation by the QTRAP (MS^n) point to a future trend of using a variety of MS variants in the study of pharmaceuticals and their degradation products in the environment. The use of other types of high-resolution, high accuracy mass spectrometers (*e.g.* the orbitrap and FT-ICR-MS) is still limited due their price, making them unaffordable in most environmental laboratories [198].

3.3.2. Gas chromatography

Traditionally, the preferred pharmaceutical analysis technique has been GC because of its higher sensitivity over LC and its wide presence in environmental laboratories [199]. As previously mentioned, GC-MS/(MS) has mostly been utilised for moderately polar drugs such as steroid hormones or acidic pharmaceuticals like NSAIDs after derivatisation in both cases. In spite of the availability of alkylating reagents such as diazomethane, which is highly toxic, carcinogenic and explosive, or pentafluorobenzyl bromide, which is corrosive, and

lachrymator, most derivatisation reactions have been performed with silylating agents (MSTFA, MTBSTFA, BSTFA, *etc.*) [126,127,200,201].

The two main advantages of GC-MS over LC-MS are: a) GC-MS/(MS) techniques can benefit from a large electron impact (EI) mass spectrum library which can aid in identifying pharmaceuticals and/or elucidating the presence of metabolites and degradation products in the environment, and b) matrix effects occur to a lesser extent with this technique reducing the problems often observed in LC-ESI-MS/(MS) [201].

The most common MS detectors coupled to GC are quadrupole and ion trap and a combination of these two for tandem MS. However, magnetic sector instrumentation, which provides high mass accuracy, and TOF are as well available. GC-TOF-MS has not been applied for the analysis of pharmaceuticals despite the boom that this detector is experiencing in LC.

The mass spectra obtained in by-full scan mode are usually utilised for identification of compounds while the SIM mode is used for quantification purposes. SIM analysis provides sensitive and selective measurements. However, with complex matrices, the use of GC-MS/MS will further improve selectivity and most often also the signal-to-noise ratio [176,201].

3.3.3. Capillary electrophoresis

Capillary electrophoretic techniques, such as capillary zone electrophoresis (CZE), coupled to UV or MS can as well be employed for the determination of pharmaceuticals and other polar compounds in aqueous environmental samples. However, this option is not very attractive due to its poor detection limit due to *e.g.* the short optical pass-length for UV detectors [87]. On-column preconcentration

techniques can be used to improve sensitivity, but detection limits are still lower than those achieved by means of GC-MS or LC-MS, which remain the technique of choice. The main advantages of capillary electrophoresis-based methods are the high separation efficiency and the short analysis time [126].

4. WATER TREATMENT

4.1. Water quality and treatment

Water is part of our life cycle and a vital resource for the economy. The safeguard of water resources is therefore one of the cornerstones of health and environmental protection. The Romans realised the importance of wastewater isolation to improve hygiene. Therefore, to protect public health and maybe, to a lesser extent, to create a nicer city environment they built what is probably one of the oldest sewers. The Cloaca Maxima, a 600-m-long sewer, was constructed around 600 BC in order to drain local marshes and remove the waste of Rome [202]. However, it was not until the 19th century that the health consequences of the discharge of untreated wastewater into *e.g.* rivers passing urban areas became more obvious leading to the construction of modern sewage systems.

Regardless of the great improvements that have been achieved since the 19th century, problems still arise due to waste and wastewater. For instance, severe eutrophication problems (up to 40%) have been observed in the North Sea, the Baltic Sea and in considerable parts of the Mediterranean Sea, European rivers and lakes [203]. This is a consequence of incomplete removal of contaminants such as nitrogen and phosphorus from sewage treatment plants and/or discharges of wastewaters directly to the aquatic environment [203]. The urban wastewater treatment is, up to date, regulated in Europe by Council Directive 91/271/EEC [204]. In this document the minimum water quality that the effluents of sewage treatment plants should have is stated. However, the directive is not yet satisfactorily fulfilled by all countries. Southern countries like Spain and recent European Union members like Estonia discharged large volumes of their wastewater directly into surface waters at least until 2005 [205]. These countries still have only a few STPs equipped with tertiary and/or advanced treatment (3%

in Spain). On the other hand, in Scandinavian countries like Denmark advanced treatment is present in most STPs (86%) [203,205].

The water quality is measured in terms of total suspended solids (TSS), total organic carbon (TOC), ammonia, phosphate, nitrates and nitrites, biological oxygen demand (BOD), pH, metals and microorganisms *e.g.* faecal coliform bacteria, *etc.* [206]. Nevertheless, few laws regulate the maximum level of organic pollutants such as pesticides and PCBs in water [207]. In addition, there often is no regulation that controls other possible organic pollutants such as PPCPs. While pesticides and PCBs may enter the environment from various sources like agricultural runoff and landfill leachates, human pharmaceuticals mainly enter the environment *via* STPs or from pharmaceutical industrial establishments [26]. Even if the US EPA has a waste effluent limitation guideline for the pharmaceutical industry [208], the document only focuses on substances such as acetone and does not treat the active drug ingredients. The latest effort in chemical regulation from the US EPA is to include pharmaceuticals in the contaminant candidates among other chemicals [32]. However, the main challenge that politicians face is that only scattered data (occurrence, toxicity, risk assessment *etc.*) is available for the large amount of organic chemicals that are discharged to the environment. This makes it difficult to regulate maximum environmental permitted levels.

Since STPs are not specifically designed to eliminate all types of organic compounds, pharmaceuticals among other organic compounds are not always quantitatively removed as proven in Paper VI. Furthermore, the amount of untreated sewage discharged directly to the aquatic environment can reach 40% in countries like Spain and Estonia [205]. This will result in concentrations of different pharmaceuticals in the aquatic environment at a level of ng/L. As mentioned in section 2 this can create environmental problems.

4.2. Description of a sewage treatment plant

Sewage treatment plants can be equipped with primary, secondary and tertiary treatment steps. Primary treatment, often called physical treatment, involves the removal of big objects, floating solids and suspended solids (both fine and coarse) from raw sewage. This step often removes grease as well. Secondary treatment involves biological processes and results in decanted effluents and separated sludge containing microbial mass together with pollutants. The tertiary process removes pollutants not adequately removed by the secondary treatment, particularly nitrogen and phosphorus, often accomplished by some means of chemical treatment, sand filters, or other methods. During the tertiary treatment, microorganisms such as pathogens and viruses should also be removed by disinfection [206,209].

4.2.1. Primary treatment

Primary treatment involves the removal of large object, which is normally performed by automated mechanical treatment. This is usually carried out in two steps: a) coarse contaminant removal, and b) a sedimentation stage [206]. In the first part of the treatment the wastewater is screened (or shredded) to remove large objects. The solids that are normally collected in this step, such as cans, fruits, and tampons, are later disposed at a landfill. Next, sand and grit removal is performed to avoid damaging machinery like pumps. In this process, gravity, velocity and aeration are used to separate grit from organic solids. This step is often performed in a grit chamber, where the flow of the incoming water is controlled to allow sand and small stones to settle, while keeping the majority of the suspended organic material in the water column. Often these basins are aerated to facilitate the suspension of the lighter organic solids. At the end of the mechanical treatment many plants have a sedimentation stage, where the sewage is allowed to pass slowly though large tanks (called primary clarifiers). The purpose of these sedimentation

tanks is to remove suspended particles (often between 40 to 60% of TSS) [209]. This slow flow allows suspended particles to settle down while oils and fats are removed from the surface in this step. In summary, the aim of primary treatment is to eliminate large objects, which can damage the equipment, to produce a more homogeneous liquid, which can be biologically treated, and to produce a sludge that can be treated separately [209]. In some cases the physical treatment can be accompanied by chemical treatment.

4.2.2. Secondary treatment

Secondary treatment is designed to separate and break down the remaining organic matter (*e.g.* sugars, fats, organic short-chain carbon molecules, *etc.*) by the aid of microorganisms. These use the organic waste as their food supply, converting most of the organic matter into stabilised low-energy compounds [209]. The suspended biological content of the sewage is derived from human waste, food waste and detergents. Aerobic biological processes are usually more efficient than anaerobic ones [206] and, therefore, are more extensively used. There are different aerobic biological treatment designs such as activated sludge, membrane bioreactors, fixed or suspended films. However, regardless of the treatment type, the secondary treatment should produce an effluent with no more than 30 mg/L BOD and 30 mg/L TSS [209]. The focus of this section will be on the most extended treatments: conventional activated sludge (CAS) and membrane bioreactors (MBR), which have been studied intensively by the scientific community in the latest years.

Activated sludge

The activated sludge process make use of biological sludge full of microorganisms often combined with bubbling air or oxygen to reduce the organic content from the sewage [206,209]. Under ideal conditions, a nitrification process takes place in

which ammonia is converted to nitrite and nitrate and ultimately to nitrogen gas. This usually takes place in the aeration tank. The microorganisms grow and reproduce by using the organic material as food, and at the same time they are mixed with air, which results in their aggregation. These biological solids or sludges are more readily sedimented in the secondary clarifiers where they are separated from the treated wastewater. Some fraction of the sludge is returned to the head of the aeration system (40 to 60% of the wastewater flow) while the rest goes to waste [209]. This wasted activated sludge is removed from the treatment process to keep the ratio of biomass to food supplied (sewage or wastewater) in balance.

Membrane bioreactors (MBR)

MBR combine activated sludge treatment with a membrane filtration. The membrane component uses low-pressure microfiltration or ultra-filtration membranes and eliminates the need for clarification and tertiary filtration. The membranes are typically immersed in the aeration tank. One of the key benefits of a membrane bioreactor system is that it effectively overcomes the limitations associated with poor settling of sludge in CAS processes and therefore produce solid free treated effluents (TSS<5 mg/l) [100]. Another advantage is that they require less space than traditional activated sludge systems because less hydraulic residence time (HRT) is needed to achieve a given solids retention time (SRT) [210]. Thus increased SRTs—usually exceeding 15 days—ensure complete nitrification even in extremely cold weather. On the other hand, the cost of building and operating an MBR is usually higher than conventional wastewater treatment. Bio-fouling is also a well-known disadvantage which leads to clogging of the membranes, deteriorating MBR performance [100].

4.2.3. Tertiary treatment

Tertiary treatment, often a chemical process, provides a final stage to raise the effluent quality (further nutrient removal) before it is discharged to the receiving environment (sea, river, lake, ground, *etc.*). More than one type of tertiary treatment process may be used at a treatment plant. Flocculation, reverse osmosis, constructed wetlands, chlorination, UV radiation and/or ozonation are examples of such processes. Often there is a sand filtration at the end of the tertiary treatment, which removes much of the residual suspended matter.

Coagulation and flocculation

Chemical treatment is often accomplished by mixing specific chemicals with the wastewater in order to remove TSS, BOD, phosphorous and other substances. In this process coagulation or solid formation and flocculation or particle aggregation are achieved [206,209]. Flocculating agents such as $FeCl_3$ or $Al_2(SO_4)_3$ are chemicals used to promote aggregation by causing colloids and other suspended particles in the liquid phase to agglomerate, forming a floc. Flocculating agents are used in water treatment processes to improve the sedimentation or filterability of small particles. Generally, a sedimentation step follows the flocculation process. In this manner, flocs settle to the bottom of the sedimentation basin, making the separation from the water phase much easier. During this process phosphorus removal is achieved among other chemicals. After the flocculation and sedimentation tanks, remaining solids floating on the water are often removed by some type of filtration [209].

Reverse osmosis (RO)

RO is the process in which water crosses a semi-permeable membrane by applying pressure to concentrated solution while salts and other chemicals are retained by the membrane [211]. RO can remove more than 99% of all dissolved compounds

including colloids from water. The main disadvantage of this treatment technique is the membrane fouling and cost.

Constructed wetlands

The application of secondary effluents to constructed wetlands can provide an effective alternative to the expensive and complicated advanced treatments, but they require large areas [212]. Soil and climate can as well be critical factors achieving good performance [209]. Wetlands include engineered reedbeds and a range of similar methodologies, all of which provide a high degree of aerobic biological improvement.

Disinfection

The purpose of disinfection in the treatment of wastewater is to protect public health by reducing the number of microorganisms in the water to be discharged back into the environment. Common methods of disinfection include chlorination, or ultraviolet light and to a lesser extent ozone.

Chlorination remains the most common form of wastewater disinfection worldwide due to its low cost and long-term history of effectiveness [209]. However, chlorination has some drawbacks such as the generation of chlorinated-organic compounds, which can be harmful to humans and the environment [211]. To avoid this problem a de-chlorination step with *e.g.* sodium sulphite is usually installed after treatment to remove chlorine and toxic compounds such as chloramines [209].

Ultraviolet (UV) light can be used as bacterial disinfectant instead of chlorine, ozone, or other chemicals. UV reduces the number of microorganisms (viruses, pathogens, bacteria, *etc.*) often by damaging their DNA [211]. An advantage over chlorination and ozone is that it carries no residual. On the other hand, the main

disadvantages of UV disinfection are the cost for lamp maintenance and the need for a highly treated effluent (clear and non-turbid) to ensure that the target microorganisms are not shielded from the UV radiation [209,211].

Ozonation is a relatively recent technique. Ozone (O_3) is generated by passing oxygen gas through a high voltage potential resulting in a third oxygen atom becoming attached to form ozone [211]. Ozone is very unstable and reactive and oxidises most organic material it comes in contact with [209], thereby destroying many pathogenic microorganisms and organic compounds. One concern arising with ozonation is the production of by-products. It is not clear what the effects of these by-products on humans and on organisms in the environment might be. The high cost of this technology has been suggested as its main disadvantage. However, a study [213] indicated a reasonable cost of *ca.* 0.05 € per m^3 of wastewater for large-scale installation.

4.3. Removal of pharmaceuticals in STPs

In the last decade, considerable efforts have been made to understand the fate of pharmaceuticals in STPs and their removal during different treatment steps as well as the degree of overall removal. Modelling provides useful tools to pre-evaluate the fate of new drugs entering the environment, to estimate the order of magnitude of pharmaceutical concentrations in the outlet of STPs and to get overall removal rates [214-216]. Unfortunately, few works have focused on models to study fate and transport during primary treatment and activated sludge treatment [215], bio- and abiotic transformation of pharmaceuticals [214] and their removal by specific advanced treatment such as ozone and free chlorine [216]. Thus, for determination of accurate removal rates or wastewater concentrations, experimental data are necessary, particularly since there are many parameters affecting fate and removal rates [210], including: climate/temperature [98,212,217]; physicochemical characteristics of the analyte [211]; hydraulic time; solid retention

time [218,219]; pH [210]; and treatment type [37,219,220]. Some measured removal rates for different pharmaceuticals under different operational conditions or in different STPs are shown in Table 4. This table highlights the incomplete removal of some pharmaceuticals and differences in their removal rates indicating that several factors affect these rates. While some drugs such as ibuprofen and salicylic acid are generally well removed others like carbamazepine and sulfamethoxazole often do not present a satisfactory removal in conventional STPs or using other treatments. In general, from Table 4 one can conclude that NSAIDs are probably the pharmaceuticals that are most effectively removed in STPs, except for diclofenac. One hypothesis for this difference within the same pharmacological group involves the presence of a chlorine atom in the diclofenac molecule. Fluoroquinolone antibiotics also showed some slight differences in removal rates. While ciprofloxacin in most cases was adequately removed, ofloxacin and norfloxacin demonstrated somewhat lower degree of removal. β-blockers, estradiol and clofibric acid generally presented moderate overall removal rates while carbamazepine, estrone and other classes antibiotics such as erythromycin showed low elimination from STPs. Comparable results were obtained in work presented in Paper VI, except for estrone, which showed higher elimination than in the studies presented in Table 4. This table also highlights the fact that the study of degradation products is not widespread. As discussed below, ozonation and RO seem to be necessary for a quantitative removal of some recalcitrant pharmaceuticals and steroid hormones.

Various secondary treatments have been studied, for example CAS [99,100,219,221-224]; extended aeration activated sludge with ferrous chloride [219]; rotating biological contactor [219]; MBRs [99,100,224,225]; and biological nutrient removal (BNR) [224,226]. Batt *et al.* [219] compared several treatments (extended aeration activated sludge with ferrous chloride, rotating biological contactor and pure oxygen activated sludge) to CAS followed in some cases by

sand filtration and chlorination or UV radiation. Activated sludge based treatments showed the best average antibiotic removal. Nevertheless, none of the treatment plants demonstrated superior performance for all four studied antibiotics. This shows the complexity of the topic. In any case, CAS and MBR are the most utilised and studied secondary treatments. Four different works [99,100,210,224] suggested that there is not a real difference between these two treatment processes and that other parameters such as SRT and chemical structure are the key factors. However, removal rates for clofibric acid have been ca 20% higher using MBR treatment than CAS [181]. The main advantage of MBR is its high SRT within a compact reactor volume, but MBR is not free of drawbacks *e.g.* membrane cost and fouling. Neither MBR nor CAS remove quantitatively the majority of the studied pharmaceuticals [181]. However, the combination of MBR and powdered activated carbon (Norit SA UF, 500 mg/L) showed removal rates of quinolone antibiotics in a pilot plant of greater than 94% [225]. Hence, this combination shows potential for future application. On the other hand, BNR has been shown to remove pharmaceuticals 1.5 times more efficiently than CAS [226].

As previously mentioned, the limited removal of some pharmaceuticals and other micropollutants under nominal conditions makes advanced treatment necessary. Lately, ozonation [36,213,225,227,228], sand filtration [36], reverse osmosis [47,49,227], nanofiltration [225], chlorine disinfection [219] and UV [219,229] have been studied as final treatments prior to treated sewage disposal to the environment. While UV has not yielded any significant improvement in chemical removal despite the decrease in biological activity [225,229], chemical degradation via chlorine can contribute a little to the removal of several antibiotics [219]. Photocatalysis (using TiO_2), on the other hand, has been suggested as a promising treatment for removal of pharmaceuticals and other micropollutants. Nonetheless, in some cases toxic, mutagenic and/or carcinogenic intermediates may be created [228]. There is no clear evidence that sand filtration improves drug removal rates

[36]. Ozonation (with or without H_2O_2) and reverse osmosis have been established to be the best option for advanced treatment. Reverse osmosis provides removal rates above 96% even for highly persistent compounds such as carbamazepine [47,227]. Despite the moderate removal in some cases for ozonation, high removal rates (up to 91%) have been achieved for contrast media drugs, which otherwise show low degradation [213]. However, ozonation produces side-products, which have yet not been studied. Therefore it cannot be excluded that toxic degradation products may enter surface water. Ozonation and reverse osmosis, the best suggested treatments for pharmaceutical removal, are considered expensive techniques [230] and therefore constructed wetlands have recently (2008) been suggested as a cheaper possible alternative [212].

Removal rates in an STP are normally achieved by biodegradation [231], particle sorption and to lesser extent by abiotic degradation (hydrolysis and photodegradation) [26]. Ibuprofen for example is eliminated mainly by biodegradation despite its moderate log K_{ow} (4.5) [99,100,222,223]. On the other hand, many antibiotics are not biodegradable [26]. For example, fluoroquinolone antibiotics adsorb to sludge or particles to a great extent [98,219]. They have been detected in sludge at concentrations up to 2.4 mg/kg despite their high hydrophilicity (log K_{ow} below 1) [26,37]. This suggests that apart from log K_{ow}, other physicochemical properties play an important role in the sorption processes. Sorption has also been suggested as the elimination pathway for other pharmaceuticals such as the antidepressant fluoxetine [232]. Having this in mind, if sludge is to be used for land improvement, great effort should be made to ensure delivery of a clean sludge of high quality.

Table 4. Pharmaceutical's removal rates.

Pharmaceutical type	Removal rate %	Treatment	Ref
Ibuprofen	91	Secondary (CAS) STP	[233][c]
	>98	Secondary (CAS/MBR) STP	[100][d]
	62	Secondary (CAS) STP	[221][e]
	38[a]-93[b]	Secondary STPs	[98][j]
	91	Pilot plant (CAS)	[222]
	86	Secondary treatment (CAS)	[223]
	99	Secondary treatment (CAS)	[36]
	>90	Biological treatment	[99][f]
	99	MBR	[47][g,h]
	96	Sand filter	[36]
	36	Ozonation	[36]
	>62	Ozonation	[213]
	>98	Reverse osmosis	[47][g,h]
	95[a]-96[b]	Wetland	[212][c]
Naproxen	95[a]-96[b]	Secondary STPs	[98][e]
	47	Secondary (CAS) STP	[221][c]
	94	Pilot plant (CAS)	[222]
	2	Primary treatment	[221][c]
	59	CAS treatment	[36]
	50-80	Biological treatment	[99] [f]
	36	MBR	[47][g,h]
	-17	Sand filter	[36]
	100	Ozonation	[36]
	>50	Ozonation	[213]

Pharmaceutical type	Removal rate %	Treatment	Ref
Naproxen	36/52	UV photolysis UV/H_2O_2	[229]
	>99	Reverse osmosis	[47][h,g]
	52[a]-92[b]	Wetland	[212]
Ketoprofen	91	Pilot plant (CAS)	[222]
	97[a]-99[b]	Wetland	[212]
Diclofenac	0-70	Secondary (CAS/MBR) STP	[100][d]
	40	Secondary (CAS) STP	[233][c]
	13[b]	Not mentioned	[234][h,i]
	20-40	Biological treatment	[99] [f]
	-150	MBR	[47][g,h]
	59	Pilot plant (CAS)	[222]
	>96	Reverse osmosis	[47][g,h]
	>96	Ozonation	[213]
	73[a]-96[b]	Wetland	[212]
Carbamazepine	-121[a]	Average from secondary and tertiary STP	[29][j]
	13	Secondary (CAS) STP	[233][c]
	0[a]-0[b]	Secondary STPs	[98][e]
	43	CAS treatment	[36]
	-44[a]	CAS treatment	[29]
	0	Biological treatment	[99][f]
	-10	MBR	[47][g,h]
	22	Sand filter	[36]
	-93[b]	Not mentioned	[234][h,i]

Pharmaceutical type	Removal rate %	Treatment	Ref
Carbamazepine	0/13	UV photolysis UV/H_2O_2	[229]
	8	Ozonation	[36]
	>98	Ozonation	[213]
	>98	Reverse osmosis	[47][g,h]
	47[a]-30[b]	Wetland	[212]
Paracetamol	92	Secondary treatment (CAS)	[223]
Salicylic acid	99[b]	Not mentioned	[234][h,i]
Clofibric acid	30[a]-0[b]	Secondary STPs	[98][e]
	49/50	UV photolysis UV/H_2O_2	[229]
	>59	Ozonation	[213]
	32[a]-36[b]	Wetland	[212]
Gemfibrozil	97[b]	Not mentioned	[234][h,i]
Benzafibrate	15[a]-87[b]	Secondary STPs	[98][e]
Furosemide	8[a]-54[b]	Secondary STPs	[98][e]
Acebutolol	47[a]	Average from secondary and tertiary STP	[29][j]
	60[a]	Activated sludge	[29][j]
Atenolol	58[a]	Average from secondary and tertiary STP	[29][j]
	10[a]-55[b]	Secondary STPs	[98][e]
	>86	Secondary (CAS) STP	[219]
	63[a]	Activated sludge	[29][j]
	>86	Ozonation	[213]

Pharmaceutical type	Removal rate %	Treatment	Ref
Metoprolol	17[a]	Average from secondary and tertiary STP	[29][j]
	34[a]	Activated sludge	[29][j]
	>93	Ozonation	[213]
Sotalol	66[a]	Average from secondary and tertiary STP	[29][j]
	54[a]	Activated sludge	[29][j]
Salbutamol	0[a]-0[b]	Secondary STPs	[98][j]
	95	Secondary treatment (CAS)	[223]
Propranolol	>72	Ozonation	[213]
Ciprofloxacin	84[a]	Average from secondary and tertiary STP	[29][j]
	56-75	4 Tertiary STPs	[219]
	60[a]-63[b]	Secondary STPs	[98][j]
	86[a]	CAS treatment	[29][j]
	82[b]	Not mentioned	[234][h,i]
Ofloxacin	82[a]	Average from secondary and tertiary STP	[29][j]
	38	Secondary (CAS) STP	[37][g,h]
	43[a]-57[b]	Secondary STPs	[98][e]
	83[a]	Activated sludge	[29][j]
Norfloxacin	nd[a]	Average from secondary and tertiary STP	[29][j]
	18	Secondary (CAS) STP	[37][g,h]

Pharmaceutical type	Removal rate %	Treatment	Ref
Norfloxacin	-13-5	Primary STPs	[220][g,k]
	20-78	Secondary STPs	[220][g,k]
Sulfamethoxazole	33-74	4 Tertiary STPs	[219]
	0	Secondary (CAS) STP	[37][g,h]
	17[a]-71[b]	Secondary STPs	[98][e]
	0	Biological treatment	[99][f]
	61.5	CAS treatment	[36]
	64	MBR	[47][g,h]
	-81[b]	Not mentioned	[234][h,i]
	27	Sand filter	[36]
	>98	Reverse osmosis	[47][g,h]
	>92	Ozonation	[213]
	87	Ozonation	[36]
Iopamidol	84	Ozonation	[213]
Iopromide	91	Ozonation	[213]
Diatrizote	14	Ozonation	[213]
Iomeprol	90	Ozonation	[213]
Tetracycline	7-8	Primary STPs	[220][g,k]
	-88-78	Secondary STPs	[220][g,k]
	31-83	4 Tertiary STPs	[219]
	11[b]	Not mentioned	[234][h,i]
Clindamycin	-68[b]	Not mentioned	[234][h,i]
Trimethoprim	70-96	4 Tertiary STPs	[219]

Pharmaceutical type	Removal rate %	Treatment	Ref
Roxithromycin	12	Secondary (CAS) STP	[37][g,h]
Erythromycin	4	Secondary (CAS) STP	[37][g,h]
	0[a]-0[b]	Secondary STPs	[98][e]
	10	MBR	[47][g,h]
	39	CAS treatment	[36]
	-12	Sand filter	[36]
	>97.5	Reverse osmosis	[47][g,h]
	89	Ozonation	[36]
Estrone	-81	Secondary (CAS) STP	[221][c]
	0[a]-0[b]	Secondary STPs	[98][e]
	83[b]	Secondary STPs	[18][l]
	-10[a]	Primary STPs	[18][m]
	-41	Primary treatment	[221][c]
	28	CAS treatment	[36]
	34	Sand filter	[36]
	>80	Ozonation	[213]
	66	Ozonation	[36]
Estradiol	68	Secondary (CAS) STP	[221][c]
	100[b]	Secondary STPs	[18][l]
	64[a]	Primary STPs	[18][m]
	43	Primary treatment	[221][c]
	>87	CAS treatment	[36]
Ethinylestradiol	78[b]	Secondary STPs	[18][l]
	-50[a]	Primary STPs	[18][m]

[a] Winter [b] Summer [c] Spain [d] Austria [e] Italy [f] Switzerland [g] China [h] Grab sampling and estimated values if flow in the influent is equal to effluent flow [i] USA [j] Finland [k] Grab sampling [l] Brazil [m] Germany

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The work done and presented in the papers of this thesis compromise one small step towards a better understanding of the occurrence, fate and removal rates of pharmaceuticals in a sewage treatment plant (Paper VI). Furthermore, a number of analytical methods (Papers I-V) were developed with improved selectivity, simplicity, environmentally friendliness and low cost. For instance, in Papers I-III it has been shown that low amounts of organic solvent (< 100 μL) are needed, that the methods are simple and that high enrichment factors (up to 4000) can be obtained by membrane based extraction methods. In Paper I it was highlighted that HF-SLM presented high clean-up capability for the extraction of basic pharmaceuticals from raw sewage, while in Paper II the possibility of extracting only the freely available fraction of neutral compounds by HF-MMLLE was demonstrated. As an alternative to membrane-based methods, MISPE has been presented, which provided highly selective extractions for acidic drugs avoiding common matrix effects in the LC-ESI-MS/MS analysis (Paper IV). The presence of matrix effects during the electrospray ionisation has been studied for HF-SLM, MISPE (Paper IV) and commercially available SPE sorbents (Paper V). In this work the importance of sample preparation towards selective extraction techniques to avoid interferences in the LC-UV runs and to reduce or eliminate the problematic matrix effects when using MS is re-confirmed.

Further deeper studies of the fate of pharmaceuticals during different sewage treatments such as ozonation, reverse osmosis, chlorination, biological steps and so forth are desirable to improve the performance of STPs. Moreover, the study of degradation products and their toxicity is vital to evaluate the real risk of STP effluents. However, one should not forget that pharmaceuticals may be eliminated from water in STPs through sludge adsorption. Consequently, the monitoring of pharmaceuticals during the sludge treatment process will aid in understanding if sludge could present a source of drugs to the environment. Another vital aspect to

consider is the need for relevant studies of chronic toxicity to understand the real risks of low level but persistent pharmaceutical concentration in nature.

Solutions to the problems of pharmaceuticals in the environment should include a proper disposal of pharmaceuticals from industry and households, a sustainable consumption of the drugs, improvement of STPs and, when possible, the installation of local treatments at the source of contamination.

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7. GLOSSARY

Activated sludge or bio-solid
The solids formed when microorganisms are used to treat wastewater using the activated sludge treatment process. These solids comprise organisms, accumulated food materials, and waste products from the aerobic decomposition process.

Aerobic
Conditions under which free, elemental oxygen is present. Also used to describe organisms, biological activity, or treatment processes that require free oxygen.

Anaerobic
Conditions under which no oxygen (free or combined) is available. Also used to describe organisms, biological activity or treatment processes that function in the absence of oxygen.

Anoxic
Conditions under which no free, elemental oxygen is present. The only source of oxygen is combined oxygen, such as that found in nitrate compounds. Also used to describe biological activity of treatment processes that function only in the presence of combined oxygen.

Biochemical oxygen demand (BOD)
The amount of organic matter that can be biologically oxidized under controlled conditions (5 days @ 20 ºC in the dark).

Biodegradation
Organic compounds are broken down into carbon dioxide, water and minerals by the action of microorganisms such as bacteria.

Bio-fouling
The process by which any surface in a marine or freshwater environment acquires a growth of organisms of different types.

Chemical oxygen demand (COD)
The amount of chemically oxidizable materials present in wastewater.

Clarifier
A device designed to permit solids to settle or rise and be separated from the flow. Also known as a settling tank or sedimentation basin.

Dissolved oxygen (DO)
Free or elemental oxygen that is dissolved in water.

Effluent
The flow leaving a tank, channel, or treatment process.

Fecal coliform
a type of bacteria found in the bodily discharges of warm-blooded animals. Used as an indicator organism.

Floc
Solids which join together to form larger particles which will settle more efficiently.

Food-to-microorganism ratio (F:M)
An activated sludge process control calculation based upon the amount of food (BOD or COD) available per pound of mixed liquor volatile suspended solids.

Grit
Heavy inorganic solids such as sand, gravel, egg shells, or metal filings.

Hydraulic retention time (HRT)
The average time a particle or volume element of the culture resides in an STP or step of the STP through which a liquid medium continuously flows.

Influent
The wastewater entering a tank, channel, or treatment process.

Mean cell residence time (MCRT)
The average length of time a mixed liquor suspended solid particle remains in the activated sludge process. May also be known as sludge retention time.

Mixed liquor
the combination of return activated sludge and wastewater in the aeration tank.

Scum
The mixture of floatable solids and water that is removed from the surface of the settling tank.

Settleability
A process control test used to evaluate the settling characteristics of the activated sludge. Readings taken at 30 to 60 min are used to calculate the settled sludge volume and the sludge volume index.

Settled sludge volume (SSV)
The volume in percent occupied by an activated sludge sample after 30 to 60 minutes of settling. Normally written as SSV with a subscript to indicate the time of the reading used for calculation (SSV_{60}) or (SSV_{30}).

Sewage
Wastewater containing human wastes.

Sludge
The mixture of settleable solids and water that is removed from the bottom of the settling tank.

Sludge retention time (SRT)
See mean cell residence time.

Total Suspended Solids (TSS)
Solids in water that can be trapped by a filter. TSS can include a wide variety of material, such as silt, decaying plant and animal matter, industrial wastes, and sewage.

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