Advanced mass spectrometric methods applied to the study of fate and removal of pharmaceuticals in wastewater treatment

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This overview is of analytical methodologies based on gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry, applied in environmental monitoring of pharmaceutical residues and their known degradation products. We also consider the ability of time-of-flight (TOF) and quadrupole-TOF instruments to provide sufficiently accurate-mass measurements and full-scan spectra for unequivocal confirmation of target compounds and investigation of their degradation products, which are either known or unknown.

We focus attention on the fate and the behavior of pharmaceutical residues during conventional and advanced wastewater treatments. Wastewater-treatment plants are designed to remove conventional pollutants (e.g., suspended solids and biodegradable organic compounds), but not low concentrations of synthetic pollutants (e.g., pharmaceutically active compounds).

Membrane bioreactor systems represent a new generation of processes that have proved to outperform conventional activated sludge treatment in terms of sludge production and effluent quality. In the past few years, there has been much attention paid to their capability for removing trace organic contaminants from sewage. This review highlights their improved performance in removing pharmaceutical residues from wastewater compared to conventional treatment.

Keywords: Activated sludge treatment; Environmental analysis; Membrane bioreactor treatment; Pharmaceuticals; Wastewater treatment

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1. Introduction

Production volumes of prescription and non-prescription drugs have been estimated to exceed hundreds of metric tons annually [1]. Unlike pesticides and many industrial chemicals, pharmaceuticals for human usage are discharged into the environment by domestic or industrial sewage systems and surface run-off. Also, their influent loads reported in wastewater-treatment plants (WWTPs) usually reflect local sales figures, which confirm that municipal wastewater is a major disposal path for these compounds. Keeping in mind that, in densely populated areas, significant amounts of raw water used for drinking-water treatment may originate from sewer systems, increasing attention has recently been paid to their efficiency in eliminating trace organic contaminants.

Pharmaceutical residues have been detected in samples of wastewater, surface water, groundwater and even drinking water [2–5]. Although their concentration is too low to pose an acute risk, it is not known whether other receptors in non-target organisms are sensitive to individual residues, or the drugs that share a common mechanism of action exhibit synergetic effects [1]. However, due to the widespread usage of pharmaceutically active compounds (PhACs) in everyday life and because their purpose is to produce specific biological effects on organisms or living tissue, unwanted environmental effects are to be expected. Since they are continually being introduced into the environment, they do not need to be persistent to cause negative effects. Nevertheless, toxic effects can be chronic, rather than acute, considering their low environmental concentrations.

These findings have raised concerns about the ubiquity of PhACs in the environment, and highlight the importance of monitoring them. Because of the need to study their environmental occurrence and fate, numerous analytical methods for determination of pharmaceuticals and their metabolites in aqueous solutions

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have been developed. Solid-phase extraction (SPE) methods combined with gas chromatography and mass spectrometry gas chromatography-mass spectrometry (GC-MS) and GC with tandem MS (GC-MS^2) [5–7] or liquid chromatography-mass spectrometry (LC-MS) and LC with tandem MS (LC-MS^2) [8–11] are the usual methods of choice for identification and quantitation. The pharmaceuticals more frequently included in such multi-residue methods include analgesics and anti-inflammatory drugs, antibiotics, lipid regulators, psychiatric drugs, and β-blockers. These drugs have very high consumption worldwide and are the most ubiquitous in both surface waters and wastewaters.

The prerequisite for proper risk assessment and monitoring of the quality of waste water, and surface and drinking waters is the availability of multi-residue methods that permit measurement at low ng/L level (or even below that). A single method of analysis for these various compound classes has several advantages (e.g., shorter analysis time, reduced field sampling and overall cost reduction).

This overview covers methodologies developed in recent years for the pharmaceutical-residue analysis (PRA) in the aquatic environment by using GC and LC techniques in combination with MS. Furthermore, we discuss elimination of pharmaceutical residues during wastewater treatment, considering conventional activated sludge (CAS) processes frequently applied in WWTPs, as well as advanced treatment processes (e.g., membrane bioreactor (MBR)).

2. Analytical method

2.1. Sample preparation

The procedure for PRA in water samples includes the enrichment step followed by chromatographic determination of target analytes and MS detection. Nowadays, very few analytical approaches are based on classical techniques for sample enrichment (e.g., liquid-liquid extraction (LLE) [6,12]). Newly developed polymeric sorbents with improved wetting characteristics and mass transfer, and with additional possibilities for interaction with functional groups of analytes have allowed efficient preconcentration of analytes and high enrichment factors. One of the major advantages of polymeric cartridges is the possibility of carrying out a multi-residue method working at neutral pH [13], which greatly simplifies the sample-handling procedure. Due to the high retention capabilities of these sorbents, acidic compounds can be extracted from water samples without previous acidification. This is of great importance when performing a multi-residue analysis, because the risk of acidic hydrolysis of other compounds is not enhanced. Furthermore, no clean-up step is needed for the removal of humic and fluvic acid, and also there is a possibility of on-line extraction using large sample volumes [14].

SPE and solid-phase microextraction (SPME) are the two methods most widely used for sample extraction and analyte enrichment. SPME has several advantages over SPE when the analysis is performed by GC-MS, since less sample volume is required, it is solvent-free and easily automated, which allows high enrichment factors in the concentration of organic compounds in aqueous matrices [15,16]. However, there are some drawbacks of SPME (e.g., often sorption capacity of the sorption coatings used is insufficient to accomplish the analytical task [17]).

One of the challenges when optimizing a multi-residue analytical method is simultaneous extraction and pretreatment of analytes that have different polarities. All target compounds can be extracted in one single step, or they can be classified in two or more SPE groups, according to their physico-chemical properties. Also, two SPE materials can be operating in series for simultaneous extraction of all compounds.

Several SPE materials have been employed to enrich pharmaceutical residues in water samples (e.g., RP-C_{18}, Oasis HLB, StrataX, and Lichrolut ENV+). Lin et al. [18] investigated the effectiveness of various SPE sorbents for extracting several pharmaceutical residues (i.e. clofibric acid, carbamazepine, ibuprofen, naproxen, ketoprofen and diclofenac) from water samples. Hydrophobic cartridges RP-C_{18} and PS-DVB performed badly, whereas Oasis HLB had high rates of recoveries. The Oasis HLB sorbent (polystyrene-divinylbenzene-*N*-vinylpyrrolidone terpolymer), which exhibits both hydrophilic and lipophilic retention characteristics, has often been used for simultaneous extraction of neutral and acidic pharmaceutical residues [3,19,20]. This material has excellent wetting properties, thus providing the advantage of no negative ''running dry'' effects on the analyte recovery [21]. Neutral and acidic compounds are retained on a solid phase exhibiting Van der Waals and H-donor-H-acceptor interactions [22].

The choice of elution solvent used to desorb the target compounds from the SPE cartridge depends on the physico-chemical properties of the analytes and the elution strength of the solvent. Ethyl acetate, acetone and methanol, which have different elution strengths and polarities, are the most commonly used [23]. The type and the volume of the elution solvent are important factors that affect the recoveries of target compounds.

2.2. GC-MS methods

For numerous compounds occurring in the environment, the use of GC-MS after appropriate derivatization is a sensitive, cost-effective and suitable technique for routine analysis. It is also less prone to matrix interferences than HPLC coupled to an atmospheric pressure

ionization (API) MS^2 interface. Due to possible problems related to signal suppression and the availability of GC-MS systems in environmental laboratories, GC-MS is still a technique of choice.

When employing a GC-MS technique for the analysis of polar compounds, derivatization is a necessary step after sample clean-up. The purpose of derivatization is to convert polar substances into less polar analogues. Volatility and thermal stability of analyte are increased, thus making it accessible to GC analysis and affording an improved GC separation, detection and quantitation.

Reddersen et al. [24] optimized an analytical method for the determination of 19 pharmaceuticals (e.g., clofibric acid, fenofibrate, diclofenac, gemfibrozil) and seven related polar contaminants in water. It involved SPE extraction using RP-C_{18} material, chemical derivatization, and, finally, detection of analytes by capillary GC-MS in selected ion monitoring (SIM) mode. In this study, two types of derivatization agents were tested: pentafluorobenzyl bromide (PFBBr) and N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA), the second one being found to be less sensitive and reproducible. When using PFBBr, limits of detection (LODs) below 1 ng/L were obtained for most of the target compounds, whereas the recoveries were between 70–110% and standard deviations less than 15%.

By contrast, in a study by Yu et al. [23], who analyzed various acidic and neutral PhACs and endocrine-disrupting compounds (EDCs) in surface and tap waters, PFBBr gave various by-products. Sylation agents MTBSTFA and N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) reacted better with both hydroxyl and carboxyl groups of the target compounds, whereas most acidic drugs had a higher response as TBS derivatives (from MTBSTFA) than as TMS derivatives (from BSTFA).

Another common derivatization agent is diazomethane [21], but its high toxicity, carcinogenicity and explosiveness must be considered.

However, injection-port derivatization with ion-pairing agents has been reported to be a rapid, simple alternative to conventional derivatization methods for aliphatic, aromatic acids and sulfonic acids [18].

Table 1 summarizes some representative GC-MS methods for PRA in aqueous environmental samples.

For compounds that have a basic character (e.g., β-blockers and β-sympathomimetics), efficient derivatization prior to GC analysis is of crucial importance [25]. Also, in some cases of hydrophobic compounds, derivatization may not be necessary, but is used to improve the sensitivity of detection. For example, carbamazepine can be detected without derivatization, but the peak shape is poor, and, during injection, the compound is partially thermo-degraded [25]. When derivatizing carbamazepine with MTBSTFA, the resulting derivative does not decompose inside the hot inlet liner and the analyte peak in the GC-MS chromatogram also has a good peak shape [24].

Derivatization reactions can be affected by several factors (e.g., solvent, time, temperature and reagent dose). Also, dosage of derivatization agent (100–200 μL) was reported as having the most significant influence on the derivatization of several acidic drugs [26].

2.3. LC-MS methods

GC-MS can be successfully applied for only a limited number of non-polar and volatile pharmaceutical compounds, while analysis of polar pharmaceuticals requires time-consuming, often irreproducible derivatization. In many cases, unequivocal identification by GC-MS was not possible, mainly because the compounds are not volatile in the inlet of GC. This can be avoided by applying LC-MS instrumental techniques.

LC-MS and LC-MS^2 have mostly been applied for the environmental analysis of pharmaceuticals, operating in the SIM and selected reaction monitoring (SRM) mode, respectively. The interfaces most widely used are API technologies (e.g., electrospray (ESI) and atmospheric pressure chemical ionization (APCI)). ESI is particularly well suited for the analysis of polar compounds, whereas APCI is very effective in the analysis of medium-polarity and low-polarity substances. The introduction of API interfaces and triple quadrupole (QqQ) analyzers has greatly improved the sensitivity and the selectivity of detection. Nevertheless, API-ionization interfaces are susceptible to matrix interferences, and, in the case of a very complex matrix (e.g., wastewater), even when using SRM detection, both false-negative results (due to ''ion suppression'') and false-positive results (due to insufficient selectivity, ''ion enhancement'') can be obtained [28]. In order to minimize problems of inaccurate quantification, several strategies have been adopted as standard practices [11,29]. The most often applied approach involves suitable calibration (e.g., standard addition or internal standard calibration using structurally similar unlabeled pharmaceuticals or isotopically labeled standards).

Besides avoiding the derivatization step and its high selectivity and sensitivity, an additional benefit of these techniques is that analytes do not have to be fully resolved to be quantified. However, it is always advisable to have a good chromatographic separation in order to reduce matrix effects. To achieve good chromatographic separation, short columns are generally used, the C_{18} column being the one preferred. As mobile phases, acetonitrile, methanol or mixtures of both solvents are generally used, with the mixtures obtaining shorter retention times (RTs) and better resolution of the analytes [30]. In order to obtain sufficient retention and reproducible RTs for acidic drugs (e.g., anti-inflammatories and lipid regulators), use of eluent buffer or acidification of mobile phase is recommended, although it

Table 1. Summary of representative GC-MS methodologies for pharmaceutical-residue analysis (PRA) in aqueous environmental samples

Compounds	Matrix	Sample pretreatment	Extraction method	Elution solvent	Derivatization agent	LOD (ng/L)	Ref.
NSAIDs	Sewage water	pH adjustment (pH 2–2.5)	SPE Oasis HLB	Ethyl acetate	MTBSTFA	20–50 (LOQ)	[26]
Multi-residue method including NSAIDs, lipid regulators and cholesterol-lowering statin drugs, hemorheological agent, phenazone-type metabolite	Sewage, surface water and groundwater	pH adjustment (pH 2)	SPE RP-C_{18}	Methanol	(1) PFBBr (2) MTBSTFA	1–10	[24]
NSAIDs, lipid regulators and cholesterol-lowering statin drugs, antiepileptic	Sewage, tap, water, river water and groundwater	Natural water pH	SPE (1) RP-C_{18} (2) PS-DVB (3) Oasis HLB	Acetone–ethyl acetate	On-line injection with TBA-HSO_4	1–8 (LOQ in tap water)	[18]
Multi-residue method including lipid regulators, anti-epileptic, psychiatric, NSAIDs and a stimulant drug	Sewage water	pH adjustment (pH 3), addition of ascorbic acid and sodium sulfate	On-line continuous LLE with dichloromethane		No derivatization required	20–80	[6]
Multi-residue method including NSAIDs, antiepileptic drugs, lipid regulator and β-blocker	Surface water and tap water	Addition of $Na_2S_2O_3$ (3%)	SPE Oasis HLB	Acetone–ethyl acetate	(1) MTBSTFA (2) BSTFA	0.7–2.4 (tap water) 0.9–7.0 (drinking water)	[23]
Multi-residue method including analgesics/anti-inflammatory drugs, lipid regulator, antiepileptic	Sewage and natural water	pH adjustment (pH 3)	SPE Oasis HLB	Acetone–ethyl acetate	Diazomethane	0.3–8.7 (surface water)	[19]
NSAIDs	Sewage water	pH adjustment (1) pH 2–6 (2) pH 2–2.5	(1) SPME (2) SPE Oasis HLB	(1) No elution (2) Ethyl acetate	On-fiber with MTBSTFA	12–40 (LOQ)	[15]
Multi-residue method including NSAIDs, Lipid regulators and cholesterol-lowering statin drugs	Sewage water	pH adjustment (pH 3)	SPE Oasis MAX	(1) Methanol (2) 2% formic acid in methanol	(1) PFP (2) TBDMS	10–20	[27]

can cause a reduction in signal intensities due to effects of suppression in the MS interface. Quintana and Reemtsma [10] used volatile ion-pairing agent tri-*n*-butylamine (TrBA), which led to very strong retention of the analytes, thus allowing more polar metabolites (e.g., salicylic acid) and adducts from selected drugs to be retained in the column. However, when analyzing drugs with basic character (e.g., β-blockers or β-sympathomimetics), neutral pH is preferable.

Due to their comprehensive approach, multi-residue analytical methods have become preferred tools for determining different categories of therapeutics to low-ng/L levels. They allow determination of a large number of micropollutants in a single analysis, thus reducing time and cost. In an ideal case, a multi-class/multi-residue analytical method for determination of trace organic pollutants in environmental matrices should fulfill several criteria, as follows:

1. sample preparation and preconcentration is achieved in a single step although analytes possess different physicochemical properties;
2. LODs and limits of quantification (LOQs) low enough for each analyte;
3. substance-specific detection; and,
4. easy application to various matrices (e.g., natural water, drinking water, and wastewater).

However, simultaneous analysis of multi-class compounds with quite different physico-chemical

characteristics often imposes compromises between the performance parameters. For example, Andreozzi et al. [19] studied 26 pharmaceuticals. Some macrolide antibiotics, clofibrate and gemfibrozil yielded quite low recoveries (35%, 36% and 46%, respectively), whereas, for other compounds investigated, recoveries were over 75%. Castiglioni et al. [31] reported recoveries in the range 65–131% for all pharmaceuticals analyzed, except for amoxicillin, which had a very low recovery (only 36%). Nevertheless, in all these methods, low recoveries are not considered as drawbacks, since other parameters (e.g., LODs or standard deviations) have very low values. Another potential pitfall of multi-residue determination methods is enhanced signal suppression, particularly when studying complex samples (e.g., wastewater). In multi-residue methodologies, sample pretreatment is usually simple to reduce the analysis time. This simplification of sample clean-up step can result in dirty extracts with high co-extractive substance content. Therefore, even when using LC-MS^2, matrix effects must be taken into account.

Identification and confirmation criteria for the analysis of environmental contaminants are defined in the EU Commission Decision 2002/657/EC [32]. To confirm the presence of a compound in environmental samples when using LC-MS^2, two transitions between precursor and product ions should be monitored, working in multiple-reaction monitoring (MRM) mode, earning four identification points (IPs). Other criteria used are the MRM ratio (calculated as the ratio between the abundances of both transitions) and the LC RT. If there is poor fragmentation of some compounds (e.g., anti-inflammatory drugs ibuprofen and ketoprofen and lipid regulator gemfibrozil), their confirmation can be performed by matching their LC-RTs with those obtained with standards [8]. This shift between the RTs compared should not exceed 2.5% for the confirmation to be considered accurate enough.

Representative chromatograms obtained by a multi-residue analysis with LC-MS^2 in negative ionization (NI) and positive ionization (PI) mode of 29 pharmaceutical residues [8] are illustrated on Fig. 1. MRM transitions are fitted in RT windows, in order to enhance the sensitivity of the method. The dependence of peak intensity on dwell time has to be considered in order to determine the maximum number of MRM transitions possible in a single period. It is generally accepted that the distribution of MRM functions into windows based on analyte RTs permits the use of longer dwell times, thereby increasing the signal-to-noise (S/N) values of less intense peaks while maintaining a short overall scan time. Total analytical run is therefore usually divided into RT windows [8].

2.4. Recent trends

Over the past 20 years, LC-MS techniques have advanced dramatically in their sensitivity, specificity and reliability. Many pharmaceutical compounds that were not possible to detect at concentrations lower than the ng/L level are nowadays part of the routine analysis in environmental laboratories, with excellent instrumental detection limits (IDLs). At the same time, with progress in analytical instrumentation, extraction techniques have become simpler, faster and less expensive, providing the enrichment of analytes of interest from matrices as complicated as wastewater. Besides SPE and SPME methods, molecularly-imprinted polymers (MIPs) and immunosorbent technologies have been developed for more selective isolation of the desired compounds [33,34]. These selective enrichment techniques have become extremely important when treating complex environmental matrices (i.e. wastewater and sewage-sludge samples), whereas, with conventional SPE, bulk material (e.g., proteins and surfactants) gets easily co-extracted with target analytes.

Because of the great complexity of some samples, techniques of high resolving power are needed to provide additional structural information. Recently developed hybrid mass spectrometers can combine methods into one single instrument and collect more information on the sample, while significantly reducing the time needed for analysis. There are several types of hybrid instruments available (e.g., quadrupole-time of flight (Q-TOF), quadrupole-linear ion trap (Q-LIT), linear ion trap-Fourier transform-ion cyclotron resonance (LIT-FT-ICR) and LTQ Orbitrap). Among these, Q-TOF is probably the most frequently employed.

The recent introduction of orthogonal acceleration TOF (oa-TOF) and Q-TOF instruments has provided accurate-mass measurements with an error typically lower than 2 mDa, which is an impressive improvement over the conventional nominal-mass information of quadrupole or ion-trap instruments. TOF-MS instruments combine ease of operation, excellent ion transmission and virtually unlimited mass range. The only significant disadvantage with respect to other mass spectrometers is limited mass resolution. However, the application of TOF instruments in screening and identification of unknown metabolites provides qualitative information that is impossible to obtain by QqQ. Q-TOF has proved an excellent analyzer to study degradation, transformation and metabolism of organic pollutants. Since transformation of organic contaminants in the environment or during wastewater and drinking-water treatment may lead to formation of more toxic products, detection and identification of these substances is of crucial importance. For example, Eichhorn et al. [35] identified two microbial degradation products of trimethoprim. This study revealed the potential of nitrifying activated sludge for breaking down this frequently found pharmaceutical residue, which is not amenable to biodegradation in CAS treatment.

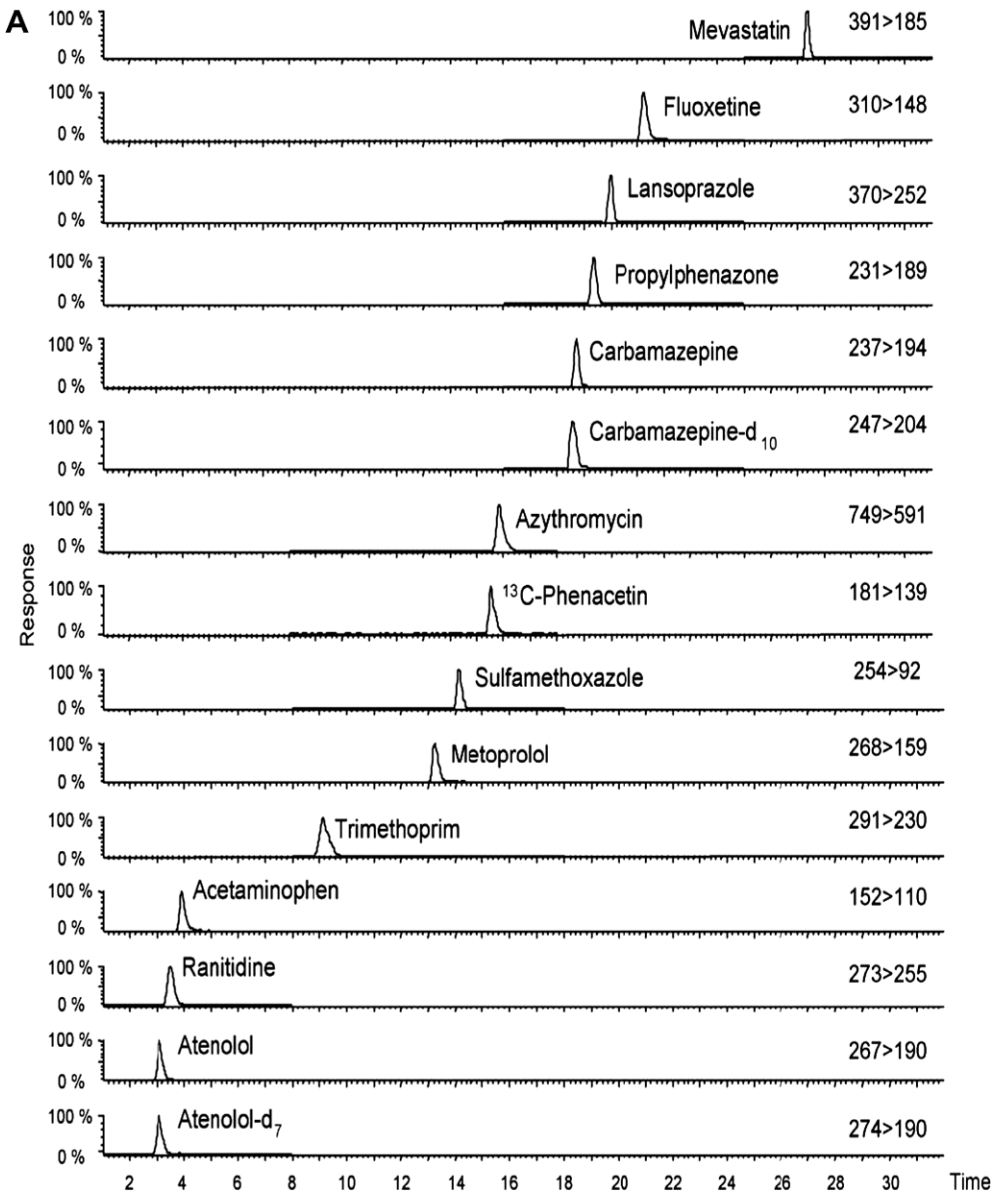

Figure 1. Example of a MRM chromatogram of a standard mixture at 1 ng/L for target compounds analyzed by (A) positive ionization mode and (B) negative ionization mode [8].

Furthermore, Q-TOF has been successfully used for target analysis of environmental samples [9,36,37]. Marchese et al. [36] compared Q-TOF and QqQ for determination of non-steroidal anti-inflammatory drugs (NSAIDs) in water samples. Although the LOQs obtained in the TOF experiment were 3–5 times higher than those obtained with QqQ, LC-Q-TOF provided a sensitive, highly selective quantitative assessment of environmental concentrations of selected NSAIDs. Stolker et al. [9] concluded that satisfactory results were obtained with both QqQ and Q-TOF MS analyzers; however, full MS^2 spectra and accurate-mass measurements provided by Q-TOF had the distinct advantages of enhanced selectivity and greater qualitative information. Petrović et al. [37] developed a method for screening and confirmation of 29 pharmaceutical residues belonging to different therapeutic categories (e.g., analgesics, anti-inflammatory drugs, antibiotics, lipid regulators and cholesterol-lowering statin agents, and β-blockers). They applied a novel approach to chromatographic separation, ultra-performance liquid chromatography (UPLC), which afforded better chromatographic resolution and reduction of run time, compared with conventional HPLC techniques. Unequivocal identification of target PhACs was based on accurate-mass measurements of the molecular ions in TOF mode, and of the product ions obtained in Q-TOF mode, by performing collision-induced dissociation (CID). This method was successfully

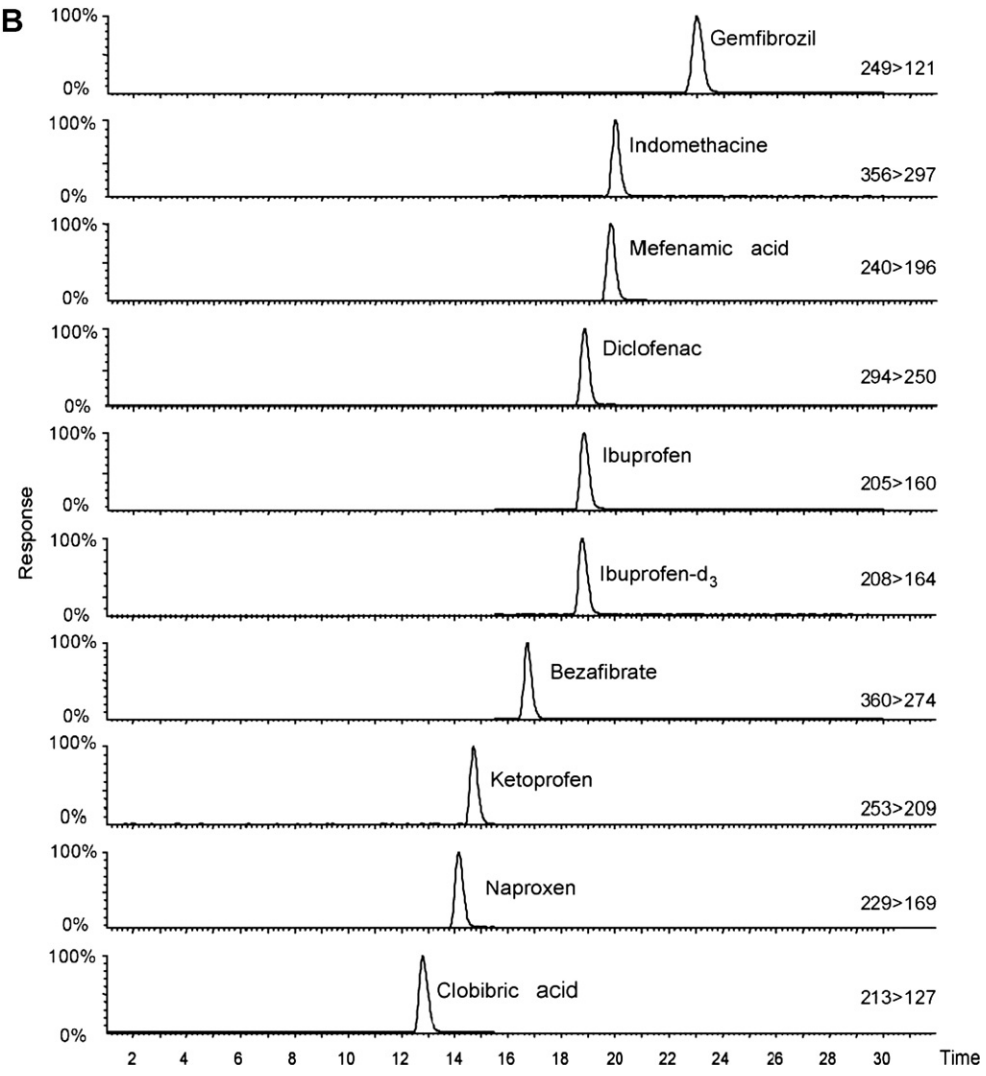

Figure 1 (*continued*)

applied for the analysis of selected pharmaceuticals in wastewater, though sensitivity was slightly lower than that obtained by QqQ.

As mentioned before, LC-MS^2 (QqQ) analysis requires two MRM transitions to confirm the identity of substances. However, with Q-TOF, only one precursor and one product ion are sufficient, affording 4.5 IPs. This is due to its unique feature of generating full-scan product-ion spectra acquired with high mass accuracy. However, lower sensitivity and also lower linear dynamic range are still the main drawbacks to wider acceptance of Q-TOF methods for quantitative purposes. The typical reported linear range spanned over two orders of magnitude, which, in some cases, required an additional adjustment of sample-preparation protocols and re-analysis of samples after appropriate dilution or concentration. However, the trend of pushing the detection limits of analysis towards lower and lower values holds out the promise of Q-TOF instruments with IDLs and linear ranges equal to or superior than those of current QqQs. Another major pitfall of Q-TOF when investigating completely unknown compounds is lack of searchable databases. In future, unambiguous identification of environmentally relevant compounds should be possible by simple matching of sample spectra and library source.

As previously stated, Q-TOF instruments have great advantages in identifying unknown biodegradation and photodegradation products of pharmaceuticals. However, Q-LIT can give complementary structural information through fragmentation of pharmaceuticals in MS^n experiments [38]. For example, Eichhorn et al. [39] performed structural identification of metabolites formed in nitrifying sludge of antibiotic trimethoprim by using ESI combined with an IT mass spectrometer. Other types of high-resolution, accurate-mass instruments (e.g., LTQ Orbitrap and LIT-FT-ICR) still do not have such a widespread use due to their higher costs. Nevertheless,

the Orbitrap technology introduced by Thermo Electron (San Jose, CA, USA) points to a future trend in MS due to the high resolving power of the Orbitrap and the high sensitivity gained by Fourier transform ion detection, followed by a significantly reduced initial cost and maintenance requirements compared to the LIT-FT-ICR instrument [40].

The need for faster, cheaper analysis, more accurate mass measurement and black-box use of instruments is expected to lead to improvements, thus finally enabling application of analytical methods of great traceability in routine environmental laboratories. Some practical problems (e.g., lack of available standards) still limit the possibilities of environmental analysis, and become especially relevant when dealing with metabolites and/or transformation products. Although identification can be performed without them (e.g., with hybrid instruments), standards are still necessary for final confirmation and quantitation.

In conclusion, analytical methods should be integrated with toxicity tracking and toxicity identification evaluation (TIE), in order to provide an integrated approach to the complex problem of environmental contamination. Also, most of the analytical efforts have so far focused on the water phase, leaving a gap in knowledge about what happens with contaminants present in soil, sediment and sewage sludge. All these fields represent new challenges for analytical chemists and biologists.

3. Removal of pharmaceutical residues in wastewater treatment

3.1. CAS treatment

Wastewater treatment generally comprises a primary, secondary and sometimes an advanced tertiary treatment, employing different physico-chemical and biological techniques. During the primary treatment, solids are removed from wastewater entering for further processing, typically in a biological secondary treatment, in order to remove organic matter and/or other nutrients. In some WWTPs, effluent is also disinfected before it is released into the environment (e.g., chlorination, or ultraviolet radiation). In addition, advanced wastewater treatments can be applied to enhance the removal of nitrogen, phosphorus and other pollutants.

While existing treatment technologies produce water that satisfies current legislation on water-quality standards, it has been demonstrated that the removal of many emerging contaminants, including pharmaceuticals, personal-care products and hormones, is incomplete [2,41,42]. In the light of escalating population growth and increased agriculture and industrialization, use of reclaimed wastewater has become the most popular and sustainable option. Considering very high influent concentrations frequently found for some PhACs, their attenuation during wastewater treatment is of crucial importance.

The most important removal pathways of organic compounds during wastewater treatment are biotransformation/biodegradation, abiotic removal by adsorption onto the sludge (excess sludge removal) and stripping by aeration (volatilization). Considering the low values of Henry coefficient (K_H) of most of the PhACs detected in wastewater streams [43], the stripped fraction removed by volatilization can be neglected [44]. It is expected that physico-chemical properties of a contaminant, especially its hydrophobicity and biodegradability, will influence the efficiency of WWTPs. Also, process operating parameters (i.e. hydraulic RT (HRT), solids RT (SRT), temperature) will strongly affect the performance of a system. HRT is the average length of time that a soluble compound remains in the reactor. SRT is the mean residence time of microorganisms, whereas only organisms that can reproduce themselves during this time can be detained and enriched in the system. Higher SRT will therefore allow the enrichment of slowly growing bacteria, so a more diverse microbial biocenosis will be established than with lower SRT. If a micropollutant is biodegradable and if this degradation can be described by Michaelis-Menten kinetics, then the critical SRT can be determined (below which no biological removal of a substance occurs) [45]. If a compound is present at only trace levels of concentration (ng/L or lower μg/L range), it will probably be transformed by co-metabolism (i.e. mixed substrate growth), whereas the specific SRT of such trace organics will depend on the maximum specific growth rate (μ_{max}) on the primary substrate. The nature of microbial population can have a significant impact on the biodegradation of some persistent PhACs (e.g., it was found that a longer SRT that provides growth of nitrifying bacteria is favorable for degradation of antibiotic trimethoprim [46]).

Clara et al. [45] reported a correlation between the observed removals of ibuprofen and bezafibrate, and the operating SRT. NSAID ibuprofen is considered to be readily biodegradable [47], and removal rates (RRs) traditionally reported in literature were in the range 80–100% (see Fig. 2). However, stronger fluctuations were observed for bezafibrate [45].

In another study by Clara et al. [48], it was found that the removal of lipid regulator bezafibrate was found to be strongly influenced by temperature. Moreover, variable removal of bezafibrate in WWTPs can be explained by cleaving of its conjugates during sewage treatment [49]. Unexplained variations of concentration over time were also observed for sulfonamide antibiotics, probably because of unknown conjugation and deconjugation processes that may occur during contact with activated sludge. For example, a significant amount of sulfamethoxazole enters the WWTP in metabolized form as

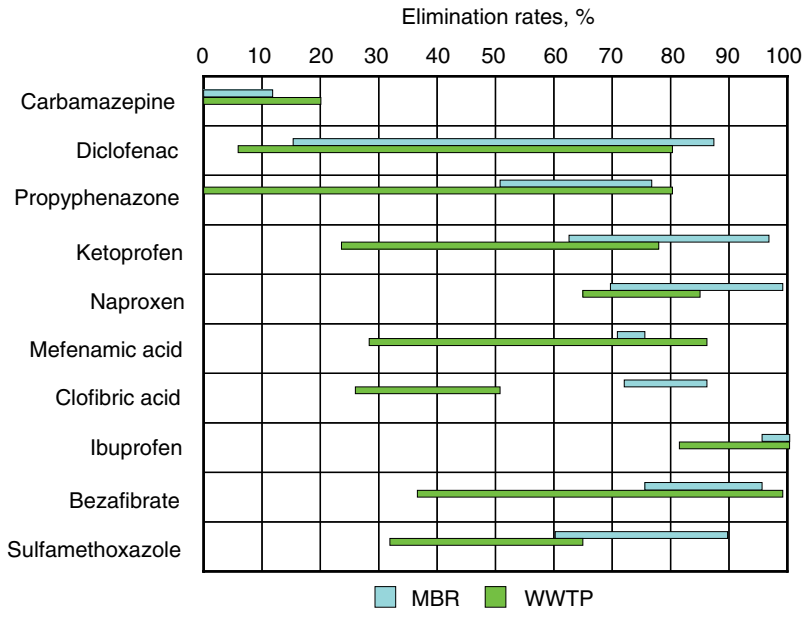

Figure 2. Elimination rates of pharmaceutical residues in conventional activated sludge treatment and membrane bioreactor treatment [2,41,45,48,49,51,52,54,56,59,61].

N_4-acetyl-sulfamethoxazole, that can be converted back to the original compound [50].

Nakada et al. [51] investigated the removal of several PhACs belonging to different therapeutic categories (e.g., analgesics and anti-inflammatory drugs, antiseptics, and an anti-epileptic drug) during activated sludge treatment in a municipal WWTP in Tokyo, Japan. They noted very high removals (>90%) of ibuprofen, thymol and aspirin, whereas amide-type pharmaceuticals (e.g., carbamazepine and crotamiton), ketoprofen and naproxen turned out to be more persistent (<50% removal).

However, Lindqvist et al. [49] reported an average removal of ketoprofen in a WWTP of 78 ± 18%.

Contradictory results have been reported in the literature for the behavior of NSAID diclofenac during conventional wastewater treatment. No influence of the increased SRT on its biodegradation was found [45]. In some WWTPs, attenuation of 50–70% of diclofenac was reported [2,41,45,52,53]. By contrast, many studies showed conventional treatment had extremely low efficiency (only 10–30% removal) [49,54–56].

For anti-epileptic drug carbamazepine, poor elimination in WWTPs was reported by many authors [41,42,45,48,54,57]. Also, carbamazepine does not adsorb onto sludge [58]. Pharmacokinetic data indicate that only 1–2% of carbamazepine is excreted unmetabolized. However, glucuronide conjugates of carbamazepine can presumably be cleaved in the sewage, and thus increase its environmental concentrations [2]. This is confirmed by its high ubiquity in the environment at concentrations levels of several hundred ng/L in different surface waters [55]. Due to its recalcitrant nature, it can be used as an anthropogenic marker for contamination of the aquatic environment.

Besides the operational conditions of the WWTP and the properties of a trace-organic pollutant, its removal might also be affected by various environmental conditions (e.g., temperature and light intensity). In real conditions of a full-scale WWTP, other factors (e.g., rainfall) can affect performance. Ternes et al. [2] found a decrease in the elimination rates of several PhACs (i.e. bezafibrate, diclofenac, naproxen and clofibric acid) after rainfall. It could be explained by reduced microbial activity and/or altered sorption and flocculation conditions.

Castiglioni et al. [52] evaluated seasonal variability in RRs of PhACs. They collected samples from six municipal WWTPs in dry-weather conditions during summer and winter seasons. The pharmaceuticals investigated were divided into three groups:

1. with higher RRs in summer than in winter (i.e. amoxicilin, atenolol, bezafibrate, enalpril, furosemide, ibuprofen, ranitidine and sulfamethoxazole);
2. with similar RRs in summer and in winter (i.e. ciprofloxacin, hydrochlorothiazide, ofloxacin); and, finally,
3. compounds that were not removed at all (i.e. carbamazepine, clarithromycin, erythromycin, lincomycin, salbutamol and spyramycin).

For example, ibuprofen was reported to be removed in winter time with a median RR of 38%, compared to 93% reported for the summer season.

3.2. MBR treatment

In a CAS-treatment process, after passing the aeration basin, treated sewage is separated from the sludge in a secondary clarifier. The ability of sludge to sediment is of elementary interest, since biomass concentration in the mixed liquor is limited by the capacity of the secondary clarifier. In MBR treatment, this parameter is of minor importance since separation is achieved via membrane filtration.

In recent years, MBR technology that combines a biological activated sludge process and membrane filtration has became a very popular and accepted alternative for the treatment of many types of wastewaters. MBR treatment managed to overcome many limitations of conventional treatment with activated sludge (e.g., poor sludge settling, microbiological contamination of the effluent, wash-out of slow-growing species). It is able to cope easily with wide fluctuations in wastewater flow rate, turbidity and/or organic matter content, while affording a good-quality effluent (e.g., in terms of chemical oxygen demand (COD), biological oxygen demand (BOD), and total nitrogen removal). Also, MBR effluent is ''solids-free'', due to complete removal of suspended solids (SS) by membrane separation.

The membrane activated sludge process is also expected to enhance removal of trace organics to a greater extent than the conventional treatment. There are many reasons for this assumption (e.g., higher sludge age, higher biomass concentration, complete retention of solids and microorganisms). Due to the avoidance of biomass wash-out problems commonly encountered in a CAS process, stable conditions for growth of specialized microorganisms are provided and they are able to remove biodegradable components slowly. Higher sludge age, which is achieved by long SRT, allows an adaptation of microorganisms to less biodegradable compounds. Together with sludge retention, it allows a biomass to acclimatize to wastewater without being restricted to fast-growing and floc-forming microorganisms. Thus, synthesis of specialized enzymes for biodegradation of micropollutants is induced.

Several studies [41,47,59–61] have confirmed that MBR treatment has an advantage over CAS treatment when it comes to reduction of polar trace organic pollutants and pharmaceutical residues (Fig. 2).

Radjenović et al. [41] found improved removal of lipid regulators and cholesterol-lowering statin drugs (gemfibrozil, bezafibrate, clofibric acid and pravastatin), β-blockers (atenolol and metoprolol), antibiotics (ofloxacin and erythromycin), anti-ulcer agent (ranitidine) and some analgesics and anti-inflammatory drugs as well (propyphenazone, mefenamic acid and diclofenac).

Kimura et al. [61] reported greater attenuation of influent concentrations of ketoprofen, mefenamic acid and naproxen, which was explained by better adaptation of MBR microbial consortia to these compounds.

Lesjean et al. [59] also correlated improved removal in MBR treatment to the enrichment of specialized microorganisms. Besides a shift in RRs when increasing SRT, biodegradation of trace organics was also enhanced with rises in reactor temperature and influent concentrations. Maximum elimination was achieved when operating with a pre-denitrification tank before MBR treatment.

MBR technology is generally considered suitable for the treatment of wastewater containing refractory compounds. However, some compounds (e.g., anti-epileptic drug carbamazepine) pass through both WWTP and MBR without any reduction. Effluent concentrations of carbamazepine in the range of influent ones were measured in many studies [41,51,52,54,56]. Moreover, in many cases, the significantly higher concentrations recorded in the effluent could be explained by the presence in the input of conjugate compounds of carbamazepine that are retransformed into the original compounds during treatment.

NSAID diclofenac and a lipid-regulator clofibric acid were also found to be slightly recalcitrant pharmaceutical residues in some studies on MBR and CAS performance [56,61]. Kimura et al. [61] related the persistence of diclofenac and clofibric acid in both MBR and CAS processes to the presence of chlorine in their structures, which makes them hardly degradable. However, Clara et al. [62] reported a higher removal of diclofenac when increasing SRT in MBR treatment. González et al. [63] also suggested that faster diminution of diclofenac was because of better acclimatizaton of microorganisms to the influent water of the MBR.

Radjenović et al. [41] noted a significant improvement in the removal of these compounds when using an MBR unit. In MBR treatment, eliminations of diclofenac and clofibric acid were 87% and 72%, compared to 50% and 28% in CAS treatment, respectively.

Besides possible changes in microbial consortia during adaptation to wastewater contaminants, another explanation for better performance of MBR treatment could be its enhanced elimination by sorption. Since sorption processes are relevant for diclofenac [58], and bearing in mind that MBR sludge has higher organic matter content which means higher sorption potential [64], the amount of the adsorbed compound can be expected to be greater than in CAS treatment. As well as for diclofenac, relevant sorption coefficients were also found for the macrolide antibiotics azithromycin and clarithromycin [60,65]. For other PhACs (e.g., acetaminophen, naproxen, indomethacin, ibuprofen, fenoprofen, diclofenac, roxithromycin, bezafibrate, clofibric acid, fenofibric acid, gemfibrozil and sulfamethoxazole), sorption effects can be neglected [66]. In these cases, biological transformation can be estimated by direct

comparison of the soluble concentration in the influent and effluent.

Theoretically, increase in SRT should have a positive influence on the biodegradation of organic micropollutants. In a study by Göbel et al. [60], a clear increase in attenuation percentages was observed at sludge ages of 60–80 days for antibiotics trimethoprim, azithromycin, erithromycin and clarithromycin, whereas a greater reduction of roxythromycin had already occurred by an SRT of 33 days. However, Joss et al. [67] found no improvement in degradation of roxythromycin and some other PhACs with increased SRT. They noted a comparable performance of CAS treatment and pilot-scale MBR treatment run in parallel regarding removal of several selected PhACs (i.e. ibuprofen, naproxen, diclofenac, carbamazepine and roxythromycin). Higher elimination rates in MBR treatment were found only for iopromide and sulfamethoxazole. Moreover, in another study conducted by Joss et al. [66], kinetic biodegradation constants (Kbiol, $kg_{SS}^{-1}d^{-1}$) were estimated for a wide range of pharmaceuticals, afforded from batch biodegradation experiments with CAS and MBR sludge. Based on the average results for kinetics, they established three different classes of compounds according to their susceptibility to biological degradation:

1. compounds with $k_{biol} < 0.1/kg_{SS}/d$, which had no removal (e.g., carbamazepine, diclofenac, diazepam);
2. partially removed compounds, $0.1 < k_{biol} < 10/kg_{SS}/d$ (e.g., roxythromycin, fenoprofen, acetylsalicylic acid, naproxen, bezafibrate, clofibric acid, fenofibric acid, gemfibrozil, piracetam, and some iodinated contrast agents);
3. compounds removed with more than 90% efficiency, $k_{biol} > 10/kg_{SS}/d$ (e.g., ibuprofen and acetaminophen).

However, there are many data in the literature that contradict these results. Good elimination in CAS treatment has been reported for both indomethacine and diclofenac [2]. Removal of bezafibrate was over 80% in some investigations [2,45]. Discrepancies in results could be due to different concentrations of pharmaceuticals and/or different sludge origins (e.g., sludge age or wastewater composition). When performing batch experiments, their outcome will also depend on the way sludge is handled prior to the experiment. Although the biodegradation of some pharmaceuticals in batch reactors has been described [47,68], it is unclear how this information relates to biotransformation processes under real conditions in WWTPs.

The current understanding of biotransformation of pharmaceutical residues in both WWTPs and MBRs and of their biodegradation pathways and mechanisms is still incomplete. Since products of their biological degradation by activated sludge are often more polar, and hence more persistent metabolites, it is of interest to identify and to monitor these unknown compounds. However, MBR treatment is expected to remove polar trace organics more efficiently than CAS treatment. Quintana et al. [47] investigated microbial degradation of several pharmaceuticals and the performance of MBR treatment in their elimination from wastewater. They found formation of potentially stable metabolites during ketoprofen and bezafibrate transformation, which may deserve further attention when analyzing removal of pharmaceutical residues in wastewater treatment technologies. In laboratory degradation experiments, ketoprofen yielded two metabolites formed along the biphenyls, biphenyl ethers and related compounds pathway. Bezafibrate was hydrolytically cleaved along the amide bond, yielding one well-degradable metabolite (4-chlorobenzoic acid) and another metabolite that was not mineralized. Ibuprofen, bezafibrate and naproxen were degraded only with the addition of external carbon source (co-metabolism), whereas diclofenac was not transformed. In both WWTP and MBR treatment, ibuprofen has been found to be completely degraded to hydroxyl-ibuprofen and carboxy-ibuprofen, with removals above 95% [47,48,54,56]. By contrast, Stumpf et al. [69] found that hydroxyl-ibuprofen was quite stable during conventional treatment in WWTP, while carboxy-ibuprofen, the main metabolite in humans, disappeared. Quintana et al. [47] found that the only two metabolites in wastewater were hydroxyl-ibuprofen and 4-chlorobenzoic acid, which they detected in the MBR influent, whereas they were not present in the effluent.

Radjenović et al. [41] found concentrations for most of the PhACs investigated in MBR effluent significantly lower than in the effluent of a CAS treatment (see Fig. 3). On the one hand, compounds that are very well removed in the CAS treatment (e.g., ibuprofen and naproxen) are also successfully eliminated in MBR treatment. The same goes for poorly degradable compounds (e.g., carbamazepine, with less than 10% eliminated). On the other hand, the study showed that removal of pharmaceutical residues that are not so readily degraded in CAS treatment (e.g., atenolol, metoprolol, mefenamic acid, gemfibrozil, etc.) can be enhanced by applying MBR technology. They are removed from wastewater during MBR treatment by sorption, degradation or a combination of both. Better removal of readily biodegradable micropollutants in MBR treatment could be due to the smaller flock size of sludge, which enhances mass transfer by diffusion and therefore increases elimination. Considering the composition of sludge originating from an MBR (specialized microorganisms, large portion of active biomass in suspended solids), improved removal is to be expected. In general, no relationship has been found so far between the structures of micropollutants and their removal during wastewater treatments.

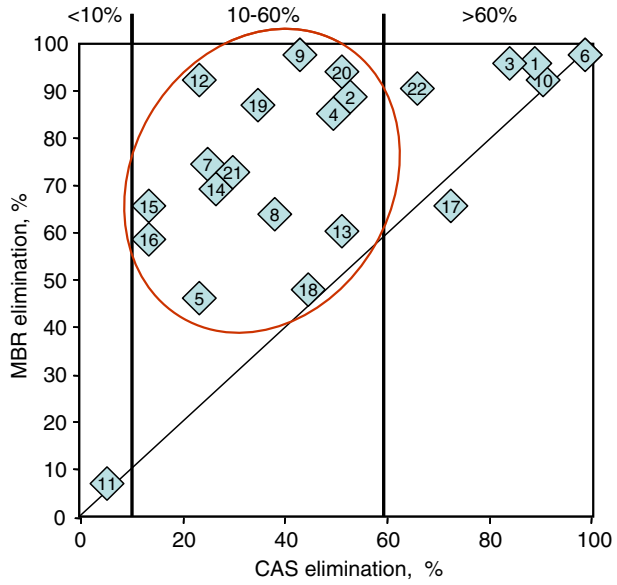

Figure 3. Enhanced removal of pharmaceutical residues in membrane bioreactor treatment compared to conventional activated sludge treatment: 1-naproxen, 2-ketoprofen, 3-ibuprofen, 4-diclofenac, 5-indomethacin, 6-acetaminophen, 7-mefenamic acid, 8-propyphenazone, 9-ranitidine, 10-paroxetine, 11-carbamazepine, 12-ofloxacin, 13-sulfamethoxazole, 14-erythromycin, 15-atenolol, 16-metoprolol, 17-hydrochlorothiazide, 18-glibenclamide, 19-gemfibrozil, 20-bezafibrate, 21-clofibric acid, 22-pravastatin [41].

4. Conclusions

Urbanization and constant population growth are likely to keep increasing the quantity of wastewater discharged to WWTPs. Also, considering the fast development of pharmaceutical industry and the general aging of population, it can be assumed that greater quantities and a more diverse array of PhACs will be consumed, with development of new compounds that have unknown fates and effects on the environment. At the same time, the demand for clean water will also increase.

Nowadays, a wide range of pharmaceutical residues can be determined down to the low ng/L level. GC-MS and LC-MS are the prevailing techniques, although only LC-MS allows analysis of extremely polar micropollutants, usually due to the incomplete derivatization of functional groups of such compounds. GC-MS is limited to low-polarity and low-molecular weight compounds, and thermally-labile compounds cannot be analyzed at all. However, GC still offers some advantages over LC (e.g., higher separation efficiency and lower cost). Also, matrix effects are expected to be lower with GC. Nevertheless, the aforementioned drawbacks, as well as extensive sample preparation and derivatization, can be avoided by employing LC-MS^2 methods, which provide reliable quantitative data. Swift growth in the use of LC-MS^2 for analysis of drugs in environmental matrices has been driven by the need for high-quality data on their occurrence in the environment at very low concentration levels.

TOF and Q-TOF instruments are expected to be widely applied for the analysis of trace organic contaminants, due to their capabilities in achieving exact mass measurements and acquiring indispensable qualitative information through full-scan spectra.

Novel UPLC systems reduce total analytical run time while increasing the resolution and S/N ratio (i.e. sensitivity) of the method.

Both conventional and innovative analytical techniques are widely applied to study the occurrence of pharmaceutical residues in wastewater, which are also commonly found in surface water and groundwater. The processing of wastewater in municipal WWTPs cannot prevent the entry of drugs into surface water, because of the high stability of some drugs and their metabolites subjected to biological degradation. The advanced technology of MBR treatment has proved to be more efficient in eliminating various classes of organic compounds, including pharmaceutical residues. Although the efficiency of MBR treatment as a barrier for micropollutants (e.g., PhACs) is still not clear, it seems a promising means for their removal.

In most studies, LC-MS technologies are the main ones applied for tracking down parent compounds. At the same time, there is a great lack of information on biodegradation pathways of pharmaceutical residues. Biodegradation products formed during activated sludge processes are expected to be more polar and thus more persistent than original compounds. TOF and Q-TOF instruments can provide valuable information for their identification and determination of their environmental fate.

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