Enantiomeric profiling of chiral drugs in wastewater and receiving waters

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ABSTRACT

The aim of this paper is to discuss the enantiomer-specific fate of chiral drugs during wastewater treatment and in receiving waters. Several chiral drugs were studied: amphetamine-like drugs of abuse (amphetamine, methamphetamine, MDMA, MDA), ephedrines (ephedrine and pseudoephedrine), antidepressant venlafaxine and beta-blocker atenolol. A monitoring programme was undertaken in 7 WWTPs (utilising mainly activated sludge and trickling filters technologies) and at 6 sampling points in receiving waters over the period of 9 months. The results revealed enantiomer-specific fate of all studied drugs during both wastewater treatment and in the aqueous environment. The extent of stereoselectivity depended on several parameters including: type of chiral drug (high stereoselectivity was recorded for atenolol and MDMA), treatment technology used (activated sludge showed higher stereoselectivity than trickling filters) and season (higher stereoselectivity was observed in the aqueous environment over the spring/summer time).

KEYWORDS: chiral, illicit, drugs of abuse, pharmaceuticals, wastewater, environment, river, enantiomeric profiling, enantiomeric fractions

1. Introduction

Chiral drugs of abuse including illicit amphetamine-like compounds and abused prescription medications such as antidepressants are regarded as new environmental contaminants. Amphetamines are frequently found in rivers at levels reaching 50 ng/L. Concentrations of amphetamine-like compounds in wastewater were found to vary between a few ng/L and <0.5 μg/L in different wastewater treatment plants and different countries and are a reflection of local drug abuse trends *(1,2)*. Due to the limited extent of research undertaken in this field, there is minimal understanding of the environmental fate and ecotoxicity for these compounds. The phenomenon of chirality of drugs of abuse has also been overlooked by environmental researchers and has to be considered as it is a major parameter determining the potency and possible toxicity of chiral drugs *(2,3)*. This aspect of environmental studies concerning pharmacologically active compounds has been recently reviewed by Kasprzyk-Hordern *(2)*.

A chiral molecule usually has at least one chiral centre (e.g. asymmetric carbon) as a result of which it shows optical activity. It exists in the form of two enantiomers, being the non-superimposable mirror images of each other. Enantiomers of the same drug have similar physico-chemical properties but may differ in their biological properties. Distribution, metabolism and excretion usually favour one

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enantiomer over the other. This results from the fact that enantiomers stereoselectively react in biological systems, for example with enzymes. Additionally, due to different biological activity, chiral drugs can differ in toxicity. Therefore, the enantiomeric composition of chiral drugs can change significantly after its administration, followed by metabolism in and excretion from the body. It can be subsequently altered during wastewater treatment and as a result of biodegradation processes in the environment. These processes can lead to stereoselective enrichment or depletion of enantiomeric composition of chiral drugs. Therefore the very same drug might have different activity and toxicity and this will depend on its origin and exposure to factors governing its fate in the environment *(2, 3)*. Furthermore, the environmental fate and toxicity of chiral drugs are currently assessed without taking into consideration their enantiomeric form. This might lead to a significant under or overestimation of toxicity of chiral drugs and to incorrect environmental risk assessment as chiral drugs will very likely be present in the environment in their non-racemic forms. Fluoxetine is a great example. It is the most toxic human pharmaceutical reported so far. Its toxicity is currently assessed for the racemate. However, recent research indicates that toxic effects of fluoxetine are enantiomer dependent: *S*-fluoxetine is 9.4 times more toxic than *R*-fluoxetine in *Pimephales promelas (11)*. This enantiomer dependent toxicity of fluoxetine is of vital importance if fluoxetine is not released to the environment in a racemic form. According to preliminary studies undertaken by MacLeod et al. *(8)*, raw sewage was found to be enriched with *R*(-)-fluoxetine, but after treatment the enantiomeric ratio of fluoxetine's enantiomers changed and led to an enrichment of fluoxetine with *S*(-)-enantiomer, which is more potent and toxic to certain organisms. Additionally, as fluoxetine was also found in tap water *(2)*, actual human exposure to this compound might be higher than expected due, primarily, to the presence of a more potent enantiomer. Therefore to understand and predict the mechanisms governing the fate of chiral drugs, their possible toxicity and impact on the environment, their enantiomeric profiling in environmental matrices is essential. Unfortunately no reports exist on the enantiomeric analysis of chiral drugs of abuse in the environment and only a few reports are available on the analysis of chiral pharmaceuticals (beta-blockers, NSAIDs and antidepressants) in environmental matrices *(4-10)* and on ecotoxicity studies *(11-12)*.

This paper is, to the authors' knowledge, the first study aiming at enantiomeric profiling of chiral drugs of abuse during wastewater treatment and in receiving waters. It also discusses the enantiomer-specific fate of beta-blocker atenolol in the aqueous environment and during wastewater treatment.

2. Experimental

2.1. Chemicals and materials

Reference standards: *R*/*S*(±)-amphetamine (*R*/*S*(±)-AMPH), *R*/*S*(±)-methamphetamine (*R*/*S*(±)-METH), *1R,2S*(-)-ephedrine (*1R,2S*(-)-EPH), *1S,2R*(+)-ephedrine (*1S,2R*(+)-EPH), *1S,2S*(+)-pseudoephedrine (1S,2S(+)-PSEUDOEPH), *1R,2R*(-)-pseudoephedrine (*1R,2R*(-)-PSEUDOEPH), *R*/*S*(±)-MDA (3,4-methylenedioxyamphetamine), *R*/*S*(±)-MDMA (4-methylenedioxymethamphetamine), *R*/*S*(±)-venlafaxine and *R*/*S*(±)-atenolol (Tab. S1) were purchased from LGC Standards (Teddington, UK) and Sigma-Aldrich (Gillingham, UK). Enantiomerically pure standards of amphetamine and methamphetamine were obtained from LGC Standards. Internal standards (IS): *R*/*S*(±)-amphetamine-d11, *R*/*S*(±)-methamphetamine-d14, *R*/*S*(±)-MDMA-d5, *R*/*S*(±)-MDA-d5 were purchased from LGC standards (Teddington, UK). All internal standards were added to the samples before extraction and were also used for the quantification of the analytes.

2.2. Sampling

Wastewater samples (both influent and effluent) were collected from 7 WWTPs utilising either activated sludge or trickling filter bed technologies and 6 locations alongside a river receiving treated wastewater from all studied WWTPs (Tab. S2, Fig. S1). Grab samples were collected over the period of

9 months (December 2009 – August 2010) during 6 sampling campaigns in order to understand the impact seasons might have on enantiomer-specific fate of drugs resulting from different microbial activity in different seasons. Grab sampling was chosen as opposed to widely used composite sampling due to the possibility of enantiomer-specific degradation of drugs occurring during 24h composite sampling time. Significant degradation of several drugs of abuse was observed in another study conducted by the authors (*33*) during 24h experiments imitating composite sampling, which undermines the validity of this sampling technique.

Samples (2 per each sampling point during every sampling campaign) were collected in 2.5 L silanized glass amber bottles with teflon faced phenolic caps and filtered through GF/D 2.7 μm followed by GF/F 0.7 μm glass fibre filters (Whatman, UK). The sample collection times within plants were not adjusted for plant hydraulic residence times.

2.3. Sample preparation and analysis

Two methodologies were used to identify and quantify chiral drugs in environmental matrices. SPE-chiral-LC-MS/MS was used only for the verification of enantiomeric fractions of chiral drugs. Quantification of drugs was undertaken with non-chiral SPE-UPLC-MS/MS method. Both methods are outlined below and described in detail elsewhere *(3, 13)*.

2.3.1. The verification of enantiomeric fractions of chiral drugs with SPE-Chiral LC-MS/MS

Chiral drugs were extracted from filtered wastewater (100 mL) and surface water (500 mL) using SPE Gilson ASPEC XL4 (Anachem, UK) and Oasis HLB adsorbents (Waters, UK). All samples were filtered, adjusted to pH 7.5 with NaOH and then spiked with 100 ng of each internal standard. Analytes were eluted from HLB cartridge with 4 mL of MeOH. The extracts were evaporated to dryness with TurboVap evaporator (Caliper, UK, 40^{o}C, N_2, 5-15 psi) and finally reconstituted in 0.5 mL of mobile phase.

Waters ACQUITY UPLC^{TM} system (Waters, Manchester, UK) equipped with Chiral-CBH column, 100x2mm, 5μm (Chromtech, Congleton, UK) and Chiral-CBH 10x2.0mm guard column (Chromtech, Congleton, UK) were used for the analysis of enantiomers of chiral drugs. The separation of chiral drugs was undertaken at 25^{o}C, under isocratic conditions and with the usage of mobile phase (pH, 5.0; flow rate, 0.075 mL/min) composed of 90% H_2O, 10% 2-propanol and 1 mM ammonium acetate. An injection volume of 20 μL was used.

A TQD (triple quadrupole) mass spectrometer (Waters, Manchester, UK), equipped with an electrospray ionisation source was used for the quantification of drugs of abuse. The analyses were performed in positive mode. MassLynx 4.1 (Waters, UK) software was used to collect and analyse the obtained data. Mass spectrometry analyses were performed in the multiple reaction monitoring mode(Tab. S3). All method validation parameters and sample mass chromatograms are presented in Tab. S4, Fig. S2 and S3.

The relative concentration of enantiomers of chiral drugs was expressed as the enantiomeric fraction (*EF*) and was calculated with the following equation:

$$EF = \frac{E1}{E1 + E2}$$

where *E1* and *E2* are peak areas for the first (*E1*) and the last (*E2*) enantiomer of a chiral drug eluting from the CBH column (Fig. S2). *EF* equals 1 or 0 in the case of single enantiomer form and 0.5 in the case of racemate. In the case of amphetamine, methamphetamine, MDMA and MDA, *E1* and *E2*-enantiomers were identified as *R*(-) and *S*(+)-enantiomers respectively. In the case of ephedrine, *E1* and *E2* denoted *1R,2S*(-)-ephedrine and *1S,2R*(+)-ephedrine respectively. In the case of atenolol, *E1* and *E2*

denoted *R*(+) and *S*(-)-atenolol respectively. Enantiomers of amphetamine, methamphetamine and ephedrine were confirmed in this research through the usage of enantiomerically pure standards. Identification of elution order of MDMA, MDA and atenolol was based on data published by others *(14, 15, 16). EFs* were calculated for all detected drugs with S/N ratios >10.

Ephedrine/pseudoephedrine has two chiral centres and as a result two pairs of enantiomers. The ephedrine enantiomers have a diastereomeric relationship with pseudoephedrine enantiomers. Therefore, for *1R,2S*(-)-ephedrine and *1S,2S*(+)-pseudoephedrine diastereomeric fractions (*DF*) were also calculated:

$$DF = \frac{1R,2S(-)\text{-}ephedrine}{1R,2S(-)\text{-}ephedrine + 1S,2S(+)\text{-}pseudoephedrine}$$

2.3.2. Quantification of chiral drugs with SPE-UPLC-MS/MS

Solid phase extraction of samples was carried out as described above. Oasis MCX cartridge was used for the extraction of all analytes. 500 mL of acidified river water and 100 mL of wastewater were spiked with 50 ng of each surrogate/internal standard and then passed through the MCX cartridge. Cartridges were washed with 0.6%HCOOH/MeOH (2 mL, pH 2) followed by elution with 7%NH_4OH/MeOH (3 mL) into silanised vials. Extracts were evaporated to dryness (40 °C, N_2, 2-10 psi) and reconstituted with 0.3%CH_3COOH/5%MeOH/H_2O (0.5 mL).

Analyses were carried out with the usage of Waters ACQUITY UPLC™ system (Waters, UK) and ACQUITY UPLC BEH C18, 150x1mm, 1.7µm column (Waters, UK). The UPLC method employed mobile phase A (pH 2.9): 79.7%H_2O, 20%MeOH, 0.3%CH_3COOH and mobile phase B (pH 3.30): 99.7%MeOH, 0.3%CH_3COOH at a flow rate of 0.04 mL min^{-1} and a temperature of 30 °C. The gradient programme was as follows: 0min-100%A, 17min-41.3%A, 17.2min-0%A, 20.2min-0%A, 20.3min-100%A, 34.0min-100%A. An injection volume of 20 µL was used.

A triple quadrupole mass spectrometer (TQD, Waters, UK) was used as described above. All method validation parameters were determined and are presented in Tabs. S3 and S5.

3. Results and discussion

3.1. MDMA and MDA

Both MDMA and MDA, which are ring-substituted amphetamine analogues, are characterised by one asymmetric carbon centre and exist in the form of two enantiomers. The pharmacological actions of both the MDMA and MDA enantiomers differ both quantitatively and qualitatively. *S*(+)-enantiomers are thought to be more amphetamine-like stimulants and *R*(-)-enantiomers are more hallucinogenic *(17)*. MDMA is a selective serotonin (5-HT) neurotoxin. The *S*(+)-isomer of MDMA has been reported to be a more potent neurotoxin than the *R*(-)-isomer. Unlike MDMA, however, both isomers of MDA cause long-term serotonin neurotoxicity *(18)*. In fact, much of the neurotoxicity originally attributed to MDMA may actually be a result of its more potent neurotoxic metabolite, MDA *(19)*.

MDMA does not currently have medical applications and its clandestine manufacture (e.g. reductive amination or the Leuckart method) leads to the production of racemic MDMA *(20)*. It is however known to undergo stereoselective metabolism in humans and animals with preferential metabolism of *S*(+)-MDMA, which leads to enrichment of MDMA with *R*(-)-enantiomer and formation of *S*(+)-MDA. Moore et al. *(18)* observed that both primary routes of excretion in human (bile and urine) had greater concentrations of *R*(-)-MDMA than the *S*(+) isomer (*EF* of 0.57, autopsy findings). These fluids also contained twice the concentration of *S*(+)-MDA than the *R*(-)-isomer (*EF*=0.37, autopsy findings) *(18)*.

MDMA was indeed found in this study, in raw wastewater samples, to be enriched with *R*(-)-MDMA. Its concentrations varied from <LOQ to 455 ng/L in raw wastewater and from <LOQ to 115 ng/L in treated wastewater. This high variability in concentrations of MDMA might be due to both different patterns of usage as well as the influence of dry/wet weather conditions. Mean *EFs* values for raw and treated wastewater collected during 5 sampling campaigns and at 7 different WWTPs were found to be 0.68 and 0.78 respectively (Fig. 1, Fig. S4 and Tab. S6). This indicates further enrichment of MDMA with *R*(-)-enantiomer as a result of wastewater treatment, probably due to enantioselective microbial activity. Over the course of the sampling campaign the change of *EF*s of MDMA as a result of wastewater treatment varied from -11 to 73.6%. The highest stereoselectivity of the transformation of MDMA was observed in WWTPs 5, 6 and 7, which is where activated sludge processes are implemented. For example, in the August sampling campaign *EFs* values increased from 0.63±0.02 0.72±0.07 and 0.64±0.01 in raw wastewater to 0.84±0.01, 0.91±0.00 and 0.78±0.01 in treated wastewater in WWTPs 5, 6 and 7 respectively. During the same sampling campaign, *EFs* of MDMA increased only slightly from 0.64±0.00 and 0.68±0.02 in raw wastewater to 0.69±0.00 in treated wastewater in WWTPs 2 and 3 respectively. This is an important observation indicating that different consortia of microorganisms, which are utilized in both activated sludge (WWTPs 4-7) and trickling filter bed treatment (WWTPs 1-3), can show different stereoselectivity.

MDMA was also quantified in receiving waters at low ppt levels not exceeding 20 ng/L (2.3 g/day) (Fig. 1, Tab. S6). Change in *EFs* (enrichment of MDMA with *R*(-) enantiomer) was observed with the course of the river, which might be due to microbial processes occurring or as a result of a discharge of non-racemic MDMA with treated wastewater effluent. This process was found to be more prominent in April and August, when microbial activity is higher than during winter times, thus suggesting stereoselective microbial processes occurring. For example, during the August sampling campaign *EF* of MDMA denoted only 0.56±0.01 in sampling point located before WWTP 1 (see Fig. S1 for sampling points locations) and increased to 0.80±0.01 in sampling point located after WWTP 6 over 50 km downstream from sampling point 1. It has to be however noted that the change of *EF* of MDMA throughout the course of the river can also be affected by a discharge of non-racemic MDMA to the river from WWTPs 1-6.

MDA is a demethylated metabolite of MDMA and is also available on the illicit market. It is synthesized (similarly to MDMA) in racemic form. It is also known that (similarly to MDMA) *S*(+)-MDA is preferentially metabolized leading to enrichment of excreted MDA with *R*(-)-enantiomer *(21)*. However, if the presence of MDA in urine is expected due to MDMA abuse and not direct MDA use, an enrichment of MDA with *S*(+)-enantiomer should be anticipated in urine and subsequently in wastewater. Almost twice as much S(+)-MDA is excreted in urine as compared to *R*(-)-MDA *(19)*. This was also the case in the discussed study. MDA was only quantified in the August sampling campaign (at concentrations not exceeding 50 ng/L and 20 ng/L in the case of raw and treated wastewater respectively, see Fig. 2) and it was found in raw wastewater to be enriched with *S*(+)-enantiomer (*EF* = 0.26±0.04 and 0.30±0.03 in the case of WWTP 2 and 3), which suggests that its presence might be associated with MDMA abuse and not intentional MDA use. Interestingly, an enrichment of MDA with *R*(-)-enantiomer (ranging from 23 to 45%) was observed during wastewater treatment (*EF* = 0.38±0.03 and 0.40±0.01 in the case of WWTP 2 and 3 effluents respectively), which suggests stereoselective processes occurring (Fig. 2, Tab. S7). MDA was also quantified in receiving waters at concentrations not exceeding 5 ng/L (0.1g/day) (Fig. 2, Tab. S7). EFs recorded in surface water were higher than those observed in WWTP 2 and 3 but in line with EFs recorded in WWTP1 and ranged from 0.56±0.02 to 0.58±0.08. This again might indicate that preferential removal or transformation of S(+)-enantiomer in water takes place leading to enrichment of MDA with *R*(-)-enantiomer.

3.2. Amphetamine and methamphetamine

Amphetamine, similarly to MDMA, contains one asymmetric carbon and as a result can exist in the form of two enantiomers, which significantly differ in potency: *S*(+)-amphetamine has twice as high stimulant activity than *R*(-)-amphetamine *(3)*. The most common route of clandestine synthesis of amphetamine is the Leuckart method which yields a racemic amphetamine *(20)*. Furthermore, both *S*(+)- and *S*(+)-/*R*(-)-amphetamine are prescription medications and have medical applications. Amphetamine is used in narcolepsy, attention deficit disorder in children and short term weight loss *(22)*. According to NHS statistics *(23)* in England, only *S*(+)-amphetamine is prescribed (dexamfetamine and lisdexamphetamine: 25 and 0.01 kg/2010). These are relatively low levels when compared to illegal use (estimated usage of amphetamine and methamphetamine: 4.0 tonnes in 2003/2004; calculated with reference to seizures, purity and survey-based estimates of usage) *(32)*. Amphetamine can also be excreted in different enantiomeric forms as a result of metabolism of methamphetamine and certain prescription drugs (e.g. selegiline, prescription in England: 13 kg/2010 *(23)*, which leads to the metabolic formation of *R*(-)-amphetamine and *R*(-)-methamphetamine). Similarly to MDMA, *S*(+)-amphetamine metabolises faster than *R*(-)-enantiomer if administered in racemic form *(20, 22, 24)*. This wide usage of different forms of amphetamine and its stereoselective metabolism make an understanding of enantiomer-specific fate of amphetamine in the environment difficult, although not impossible.

The results of this study indicated that amphetamine in wastewater was enriched with *R*(-)-enantiomer. Recorded *EFs* were ranging from 0.52±0.01 to 0.84±0.01 with average value of 0.64. This was to be expected in raw wastewater because, as mentioned above, amphetamine is usually abused in racemic form with preferential metabolism of *S*(+)-amphetamine leading to an enrichment of excreted amphetamine with *R*(-)-enantiomer. It has to be however emphasized here that (although unlikely due to low usage) possible contribution to enantiomeric composition of amphetamine from legally prescribed S(+)-amphetamine and selegiline should be taken into account. Although based on only a few samples, a faster removal of *S*(+)-amphetamine, probably due to stereoselective microbial activity, was also observed during wastewater treatment (Fig. 3, Tab. S9). This led to further enrichment of amphetamine with *R*(-)-enantiomer in treated wastewater (*EFs* > 0.70). As an example, during April sampling campaign, *EF* of amphetamine in WWTP 3 and 4 denoted 0.65±0.00 and 0.59±0.04 respectively in raw wastewater and increased to 0.71±0.03 and 0.78±0.01 as a result of WWTP treatment (Tab. S9). Amphetamine was also quantified in receiving waters at single ng/L (<1.7 g/day) levels and was found to be enriched with *R*(-)-enantiomer at all times (*EFs* > 0.80; see Fig. 3 and Tab. S9).

Psychostimulant effects of methamphetamine, similarly to amphetamine, are enantioselective, and *S*(+)-enantiomer is much more active than *R*(-)-enantiomer. As opposed to amphetamine, *S*(+)-enantiomer of methamphetamine is the most commonly abused drug and produced in clandestine laboratories *(20)*. Methamphetamine is also used for valid medical treatment as *S*(+)-methamphetamine (to treat attention deficit disorder in children, narcolepsy and exogenous obesity) *(22, 24)*. In the USA also *R*(-)-methamphetamine is used as decongestant in the Vicks Inhaler (an over-the-counter medication). Methamphetamine can be also formed as a result of metabolism of certain prescription medications (e.g. selegiline leading to the formation of *R*(-)-methamphetamine). Currently, there is no medical use of methamphetamine in England. Therefore, if present in wastewater it is thought to result from its abuse or metabolism of other drugs. In this study, methamphetamine was only quantified in the August sampling campaign and was found to be racemic in WWTP 1 influent (EF, 0.53±0.05) but enriched with S(+)-enantiomer in the influent of WWTPs 2 and 4 (EF, 0.22±0.00 and 0.28±0.04 respectively) (Fig. 2, Tab. S8). In all WWTPs, treatment of wastewater resulted in stereoselective removal of methamphetamine leading, similarly to amphetamine, to an enrichment of methamphetamine with *R*(-)-enantiomer (EF, 0.87±0.19, 1.00±0.00 and 0.70±0.06 in the case of WWTP 1, 2 and 4 respectively).

3.3. Ephedrine and pseudoephedrine

Ephedrine has two chiral carbons and can therefore exist in the form of four stereoisomers: *1R,2S*(-)-ephedrine, *1S,2R*(+)-ephedrine, *1S,2S*(+)-pseudoephedrine and *1R,2R*-(-)-pseudoephedrine. However, only two stereoisomers: *1R,2S*(-)-ephedrine and *1S,2S*(+)-pseudoephedrine can be found in natural sources such as ephedra *(25)*. *1R,2S*(-)-Ephedrine finds wide applications as a bronchodilator to treat bronchospasm associated with asthma, bronchitis and emphysema. It is also abused for its stimulant properties. *1S,2S*(+)-Pseudoephedrine is used as a decongestant *(20)*.

Ephedrine has been detected before in environmental matrices *(26, 27)*, but with the usage of non-enantioselective methodology, which did not allow for enantiomeric and diastereomeric ephedrine profiling and as a result did not allow for an accurate assessment of possible effects ephedrine might have on the environment. Enantiomeric/diastereomeric profiling is of vital importance as different stereoisomers of ephedrine differ significantly in potency (*1R,2S*(-)-ephedrine has much higher stimulant properties than *1S,2S*(+)-pseudoephedrine) and possibly also toxicity to certain organisms.

In this study a verification of the enantiomer-specific fate of ephedrine isomers was undertaken (Fig. 4, Fig. S6, Tab. S10). Out of the two enantiomers of ephedrine (*1S,2R*(+)- and *1R,2S*(-)-) only natural *1R,2S*(-)-enantiomer was frequently detected. Synthetic *1S,2R*(+)-Ephedrine was detected at low levels in only certain WWTPs throughout the sampling campaign. It was detected on several occasions in WWTP 2 but only in treated wastewater (EF, 0.55±0.00, 0.22±0.00, 0.89±0.00, 0.91±0.00 during January, February, March and August sampling campaigns respectively), which might suggest stereoselective processes occurring during treatment (e.g. chiral inversion although there is currently no experimental evidence to support this claim) leading to enrichment of ephedrine with *1S,2R*(+)-enantiomer. The possibility of chiral inversion occurring during treatment is of critical importance in understanding the fate of ephedrines in the environment and has to be studied further. A similar situation was observed in WWTP 7 (only in the August sampling campaign: EF, 0.72±0.03 in treated wastewater). In WWTP 3, 4 and 6 during the August campaign *1S,2R*(+)-ephedrine was detected in both influent and effluent wastewater (Fig. S3). A low enrichment of ephedrine with *1S,2R*(+)-enantiomer was observed in all three WWTPs (decrease of *EF* from on average 0.90 to on average 0.82), which indicates stereoselectivity of processes occurring during treatment. For example, *EF* of ephedrine in WWTP 3, 4 and 7 denoted 0.95±0.07, 0.96±0.01 and 0.81±0.01 respectively in raw wastewater and decreased to 0.90±0.02, 0.83±0.05 and 0.76±0.02 as a result of WWTP treatment (Tab. S9). As a result of enrichment of ephedrine with *1S,2R*(+)-enantiomer during wastewater treatment, *1S,2R*(+)-ephedrine was also detected in receiving waters (Fig. 4, Tab. S10). *EFs* for ephedrine varied from 1.00 (December-March) to 0.80±0.00, and were the lowest in the April and August campaign, which might suggest enantioselective processes occurring during spring/summer due to potentially higher activity of microorganisms.

The verification of cumulative concentrations of ephedrines in raw wastewater indicated that higher levels of these compounds were observed during winter time (reaching 180 g/day in February in all studied WWTPs) than during summer time (< 80 g/day in August). Interestingly, the analysis of diastereomeric fractions of *1R,2S*(-)-ephedrine and *1S,2S*(+)-pseudoephedrine in raw wastewater revealed that over the winter months ephedrines were enriched with *1S,2S*(+)-pseudoephedrine (Fig. 4, Fig. S6, Tab. S10). This is possibly due to higher usage of over-the-counter medications (containing *1S,2S*(+)-pseudoephedrine) for the treatment of mild symptoms of cold. During the spring and summer months a reverse situation was observed as ephedrine was found to be enriched with a much more potent stimulant, *1R,2S*(-)-ephedrine. For example, *DFs* values increased from 0.09±0.00, 0.02±0.00, 0.12±0.01, 0.25±0.01, 0.19±0.01 and 0.07±0.02 in the January sampling campaign to 0.44±0.01, 0.56±0.01, 0.51±0.04, 0.47±0.00, 0.44±0.00 and 0.66±0.00 in the August sampling campaign WWTPs 1, 2, 4, 5, 6 and 7 respectively. This is a very important finding indicating that non-enantioselective

measurement of ephedrines cannot be a reliable indicator of actual potency of ephedrines in the environment. Higher cumulative concentrations of ephedrines, which are enriched with less potent *1S,2S*(+)-pseudoephedrine (as was during winter time in this study) might be of lower environmental significance than lower concentrations of ephedrines enriched with much more potent *1R,2S*(-)-ephedrine (in summer in this study). Furthermore, wastewater treatment resulted in almost all cases in further enrichment of ephedrines in aqueous phase with more potent *1R,2S*(-)-ephedrine, with an average increase of *DFs* from 0.25 in raw wastewater to 0.35 in treated wastewater (see Tab. S10). For example, in the August sampling campaign, *DF* of ephedrine in WWTP 1, 5 and 6 denoted 0.44±0.01, 0.47±0.00 and 0.44±0.00 respectively in raw wastewater and increased to 0.82±0.01, 0.66±0.02 and 0.68±0.02 as a result of WWTP treatment. Interestingly, the monitoring of receiving waters revealed that ephedrine was enriched with *1R,2S*(-)-ephedrine at the beginning of the course of the river and its *DFs* decreased over the course of the river indicating an increase of *1S,2S*(+)-pseudoephedrine (e.g. during the August sampling campaign *DF* of ephedrine denoted 0.93±0.03 in sampling point located before WWTP 1 and decreased to 0.33±0.03 in sampling point located after WWTP 6 over 50 km downstream from sampling point 1); a reverse situation to the one observed during wastewater treatment (Fig. 4 and Tab. S10). This might indicate that different microbial communities are responsible for transformation of ephedrines during wastewater treatment and in the environment.

3.4. Venlafaxine

Venlafaxine is an inhibitor of reuptake of both serotonin and noradrenaline and is one of the most frequently prescribed antidepressant drugs worldwide (England: 9 tonnes/2010 *(23)*). It is marketed as a racemate. Both of the enantiomers exhibit pharmacological activity, but interact with different signal molecules in the central nervous system. The *R*(-)-enantiomer is a potent inhibitor of both serotonin and noradrenaline reuptake, while the *S*(+)-enantiomer is more selective in inhibiting serotonin reuptake *(2, 28)*. Oral clearance of venlafaxine is higher for the *R*(-)-enantiomer *(29)*.

In this study venlafaxine was found to be omnipresent in both wastewater and in receiving waters (Fig. 5, Fig. S7 and Tab. S11). Its concentrations in raw wastewater varied from tenths to a few hundred ng/L. WWTP treatment did not effectively remove this compound as treated wastewater contained its significant concentrations, sometimes higher than in raw wastewater. This is probably due to cleavage of free venlafaxine from its conjugated form due to microbial processes occurring during wastewater treatment (although there is currently no experimental evidence to support this claim). As a result it was found in receiving waters at concentrations of tenths ng/L (<35 g/day). *EFs* of venlafaxine were on average 0.48 in raw sewage and were observed to slightly increase in almost all cases to on average 0.52 in treated wastewater. For example, in the August sampling campaign, *EF* of venlafaxine in WWTP 1, 3 and 7 denoted 0.46±0.02, 0.39±0.03 and 0.47±0.00 respectively in raw wastewater and increased to 0.51±0.01, 0.52±0.00 and 0.50±0.01 as a result of WWTP treatment. This indicates some weak stereoselective processes occurring during wastewater treatment, although not as significant as in the case of other chiral drugs studied in this work (the difference in EFs, although very small, is statistically significant, see Tab. S11). Interestingly, in receiving waters, more significant changes of *EFs* of venlafaxine (both with the flow of the river and time of year) were observed. The lowest *EF* of 0.40±0.02 indicating enrichment of venlafaxine with *E2* enantiomer was observed in the December sampling campaign (Fig. 5, Tab. S11). The highest *EF* of 0.65±0.01 indicating enrichment of venlafaxine with *E1* enantiomer was recorded in the August sampling campaign, which is when microbial activity is the highest. Surprisingly *EFs* of venlafaxine in receiving waters did not always correspond with *EFs* of venlafaxine in discharged wastewater effluent. It is also worth noting that higher variability in EFs of venlafaxine (usually enrichment with *E1*-enantiomer) was observed in sampling points located further from a WWTP discharge point (see Fig. 5, Tab. S11, sampling points: 'before WWTP 1, 3 and 6') than those just after a discharge of treated wastewater. This suggests that some

stereoselective processes regarding venlafaxine take place in the river and are more significant than those observed during wastewater treatment. Despite the fact that in the discussed research distinction between levorotary and dextrorotary enantiomers of venlafaxine was not possible, one can observe that analysis of this compound at enantiomeric level provides yet another dimension to the understanding of its fate in the environment.

3.5. Atenolol

Atenolol belongs to the group of beta-blockers. It's prescription in England exceeds 30 tonnes annually *(2)*. Pharmacological action of beta-blockers in humans is highly stereoselective. *S*(-)-Enantiomers usually reveal much higher cardiac beta-blocking potency than *R*(+)-enantiomers in most beta-blockers, with an activity ratio being in the region of *S* : *R* from 33 to 530. On the other hand *R*(+)-enantiomers have higher activity in blocking β_2 receptors in ciliary processes *(2)*. Modest stereoselectivity in renal clearance of atenolol in humans in favour of *S*(-)-enantiomer results in an enrichment of excreted atenolol with this enantiomer *(30)*. Indeed atenolol was found to be enriched with *S*(-)-enantiomer in both raw and treated wastewater (average *EF*=0.40 for raw wastewater and 0.46 in treated wastewater) (Fig. 6, Fig. S8, Tab. S12). These results correspond with those already published by Nikolai et al. *(7)* and MacLeod et al. *(31)*. However, it is important to emphasize that during wastewater treatment enrichment of atenolol with *R*(+)-enantiomer took place in several cases. This outcome is contrary to the outcome reported by Nikolai et al. *(7)* and MacLeod et al. *(8)*, where an enrichment of atenolol with *S*(-)-enantiomer was observed. The highest stereoselectivity of the removal of atenolol was observed in the case of WWTP 4 (an increase of EF from 0.35±0.02 in raw wastewater to 0.49±0.02 (0.48±0.04) in treated wastewater in the March (April) sampling campaign), WWTP 6 (an increase of EF from 0.33±0.04 (0.31±0.05) in raw wastewater to 0.49±0.01 (0.45±0.01) in treated wastewater in the March (April) sampling campaign) and WWTP 7 (an increase of EF from 0.33±0.03 (0.32±0.02) in raw wastewater to 0.61±0.00 (0.52±0.02) in treated wastewater respectively in the March (April) sampling campaign) where activated sludge technology is being utilized. No or low stereoselectivity was observed in WWTPs 1, 2 and 3 where trickling filters are used. For example during the August sampling campaign *EFs* of atenolol did not change as a result of wastewater treatment in WWTP 1, 2 and 3 and denoted 0.44±0.01, 0.44±0.01 and 0.43±0.01 respectively. This is an important observation indicating the importance of a type of technology in use. In the case of activated sludge treatment aerobic microbial processes take place. While in the case of trickling filter bed technology anaerobic microbial processes also occur and these might not be stereoselective in nature. To test this hypothesis and verify which consortia of microorganisms might be responsible for enantiomer-specific fate of drugs, further research will need to be undertaken.

In receiving waters, the lowest EF was recorded for atenolol in August (0.39±0.00) and the highest in March (0.56±0.09). There was also a strong tendency during the spring/summer months (April-August) for an enrichment of atenolol with the flow of the river with *S*(-)-enantiomer. It has to be remembered here that a reverse pattern was observed in this study during wastewater treatment processes. This suggests that different consortia of microorganisms and/or environmental conditions are involved in stereoselective degradation of atenolol in receiving waters and during wastewater treatment.

To summarise, the phenomenon of the enantiomer-specific fate of chiral drugs such as amphetamines, atenolol or venlafaxine is of vital importance and needs to be taken into account in environmental risk assessment. This is because:

- these drugs are distributed as racemates,
- their enantiomers reveal different pharmacological activity (and possibly different ecotoxicity),
- their metabolism in humans is stereoselective and as a result they are discharged into wastewater in non-racemic mixtures,

- their fate during wastewater treatment shows stereoselectivity, which depends on the type of technology utilised (e.g. activated sludge shows higher stereoselectivity than trickling filters in the case of atenolol)
- their fate in the aqueous environment is also stereoselective and is season and location dependant (in certain cases observed stereoselectivity is different to the one recorded during wastewater treatment)

After taking the above discussion into consideration, one can hypothesize that the environmental risk assessment of chiral pharmacologically active compounds, which currently does not take into account their enantiomer-specific fate, might significantly under- (or over-) estimate their actual impact on the surrounding environment.

ACKNOWLEDGMENTS

The support of the Natural Environment Research Council (NE/I000534/1) is greatly appreciated. The authors would like to thank Yorkshire Water and Dr Ilyas Dawood for assistance. David Baker would like to acknowledge the University of Huddersfield Research Fund for funding his PhD studies. Special thanks to Jo Spence and Hycinth Osobe for help with sample collection and preparation.

SUPPORTING INFORMATION

Supporting information material includes: structures, molecular weights and *CAS* numbers of selected chiral drugs; general information on the studied WWTPs and receiving waters; optimised MRM conditions for the analysis of chiral drugs by LC-MS/MS; validation parameters for SPE-Chiral LC-MS/MS and SPE-UPLC-MS/MS methods; aLC-MS/MS chromatograms of chiral drugs in wastewater; and detailed tables and figures on environmental occurrence and enantiomeric fractions of studied drugs in wastewater and receiving waters. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

[1] Illicit drugs in the environment, Eds. Castiglioni, S.; Zuccato, E.; Fanelli, R. Wiley 2011.

[2] Kasprzyk-Hordern, B. Pharmacologically active compounds in the environment and their chirality. Chemical Society Reviews, 2010, 39, 4466-4503.

[3] Kasprzyk-Hordern, B.; Kondakal, K.; Baker, D.R. Enantiomeric profiling of drugs of abuse in wastewater by chiral liquid chromatography coupled with tandem mass spectrometry. Journal of Chromatography. A, 2010, 1217, 4575-4586.

[4] Fono, L.J.; Sedlak, D.L. Use of chiral pharmaceutical propranolol to identify sewage discharges into surface waters. Environmental Science and Technology, 2005, 39, 9244-9252.

[5] Fono, L.J.; Kolodziej, E.P.; Sedlak, D.L. Attenuation of wastewater-derived contaminants in the effluent-dominated river. Environmental Science and Technology, 2006, 40, 7257-7262.

[6] Buser, H.-R.; Poiger, T.; Müller, D. Occurrence and environnemental behavior of the chiral pharmaceutical drug ibuprofen in surface waters and wastewaters. Environmental Science and Technology, 1999, 33, 2529-2535.

[7] Nikolai, L.N.; McClure, L.; MacLeod, S.L.; Wong, C.S. Stereoisomer quantification of the β-blocker drugs atenolol, metoprolol, and propranolol in wastewaters by chiral high-performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A, 2006, 1131, 103-109.

[8] MacLeod, S.L.; Sudhir, P.; Wong, C.S. Stereoisomer analysis of wastewater-derived β-blockers, selective serotonin re-uptake inhibitors, and salbutamol by high-performance liquid chromatography-tandem mass spectrometry. Journal of Chromatography A, 2007, 1170, 23-33.

[9] Matamoros, V.; Hijosa, M.; Bayona, J.M. Assessment of the pharmaceutical active compounds removal in wastewater treatment systems at enantiomeric level. Ibupforen and naproxen. Chemosphere 2009, 75, 200-205.

[10] Winkler, M.; Lawrence, J.R.; Neu, T.R. Selective degradation of ibuprofen and clofibric acid in two model river biofilm systems. Water Research 2001, 35, 3197-3205.

[11] Stanley, J.K.; Ramirez, A.J.; Chambliss, C.K.; Brooks, B.W. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. Chemosphere 2007, 69, 9-16.

[12] Stanley, J.K.; Ramirez, A.J.; Mottaleb, M.; Chambliss, C.K.; Brooks, B.W. Enantiospecific toxicity of the β-blocker propranolol to Daphnia Magna and Pimephales Promelas. Environmental Toxicology and Chemistry 2006, 25, 1780-1786.

[13] Baker, D.R.; Kasprzyk-Hordern, B. Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography-positive electrospray ionization tandem mass spectrometry. Journal of Chromatography A, 2011, 1218, 1620-1631.

[14] Buechler, J.; Schwab, M.; Mikus, G.; Fischer, B.; Hermle, L.; Marx, C., Grön, G; Spitzer, M.; Kovar, K.-A. E nantioselective quantitation of the ecstasy compound (*R*)- and (*S*)-*N*-ethyl-3,4-methylenedioxyamphetamine and its major metabolites in human plasma and urine. Journal of Chromatography B, 2003, 793, 207–222.

[15] Fornstedt, T.; Hesselgren, A.-M.; Johansson, M. Chiral Assay of Atenolol Present in Microdialysis and Plasma Samples of Rats Using Chiral CBH as Stationary Phase. Chirality, 1997, 329-333.

[16] Isaksson, R.; Pettersson, C.; Pettersson, G.; Jönsson, S.; Stålberg, J.; Hermansson, J.; Marle, I. Cellulases as chiral selectors. Trends in Analytical Chemistry 1994, 13, 431-439.

[17] Fantegrossi, W.E. In vivo pharmacology of MDMA and its enantiomers

in Rhesus Monkeys. Experimental and Clinical Psychopharmacology, 2008, 16, 1–12.

[18] Moore, K.A; Mozayani, A.; Fierroc, M.F.; Poklis, A. Distribution of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4 methylenedioxyamphetamine (MDA) stereoisomers in a fatal poisoning. Forensic Science International, 1996, 83, 111-119.

[19] Levine, B. Principles of Forensic Toxicology, AACC Press, 2003.

[20] King, L.A. Forensic chemistry of substance misuse. RSC Publishing, 2009.

[21] Meyer, M.R.; Peters, F.T.; Maurer, H.H. Investigations on the human hepatic cytochrome P450 isozymes involved in the metabolism of 3,4-methylenedioxy-amphetamine (MDA) and benzodioxolyl-butanamine (BDB) enantiomers. Toxicology Letters, 2009, 190, 54–60.

[22] Cody, J.T. Precursors medications as a source of methamphetamine and/or amphetamine positive drug testing results. JOEM, 2002, 44, 435-450.

[23] Prescription Cost Analysis, England, National Health Service, 2010 (http://www.ic.nhs.uk/pubs/prescostanalysis2010; accessed: January 2011)

[24] Cody, J.T.; Schwarzhoft, R. Interpretation of methamphetamine and amphetamine enantiomer data. Journal of Analytical Toxicology, 1993, 17, 321-326.

[25] McCooeye, M.; Ding, L.; Gardner, G.J.; Fraser, C.A.; Lam, J.; Sturgeon, R.E.; Mester, Z. Separation and quantitation of the stereoisomers of ephedra alkaloids in natural health products using flow injection-electrospray ionization-high filed asymmetric waveform ion mobility spectrometry-mass spectrometry. Analytical Chemistry, 2003, 75, 2538-2542.

[26] Chiaia, A.C.; Banta-Green, C.; Field, J. Eliminating solid phase extraction with Large-Volume Injection LC/MS/MS: analysis of illicit and legal drugs and human urine indicators in US wastewaters. Environmental Science and Technology, 2008, 42, 8841-8848.

[27] Postigo, Ch.; Lopez de Alda, M.J.; Barceló, D. Fully automated determination in the low nanogram per liter level of different classes of drugs of abuse in sewage water by On-line Solid-Phase Extraction-Liquid Solid-Phase Extraction-Liquid Chromatography-Electrospray-Tandem Mass Spectrometry. Analytical Chemistry, 2008, 80, 3123-3134.

[28] Kingbäck, M.; Josefsson, M.; Karlsson, L.; Ahlner, J.; Bengtsson, F.; Kugelberg, F.C.; Carlsson, B. Stereoselective determination of venlafaxine and its three demethylated metabolites in human plasma and whole blood by liquid chromatography with electrospray tandem mass spectrometric detection and solid phase extraction. Journal of Pharmaceutical and Biomedical Analysis, 2010, 53, 583–590.

[29] Eap, Ch.B.; Lessard, E.; Baumann, P.; Brawand-Amey, M.; Yessine, M.-A. O'Hara, G.; Turgeon, J. Role of CYP2D6 in the stereoselective disposition of venlafaxine in humans. Pharmacogenetics, 2003, 13, 39-47.

[30] Mehvar, R.; Brooks, D.R. Stereospecific pharmacokinetics and pharmacodynamics of beta-adrenergic blockers in humans. Journal of Pharmacy and Pharmaceutical Sciences, 2001, 4, 185-200.

[31] MacLeod, S.L.; Wong, Ch.S. Loadings, trends, comparisons, and fate of achiral and chiral pharmaceuticals in wastewaters from urban tertiary and rural aerated lagoon treatments. Water Research, 2010, 44, 533-544.

[32] Singleton, N.; Murray, R.; Tinsley, L.; (Eds.) (2006). Measuring Different Aspects of Problem Drug Use: Methodological Developments. Home Office Online Report 16/06. www.homeoffice.gov.uk/rds/pdfs06/rdsolr1606.pdf.

[33] Baker, D.R.; Kasprzyk-Hordern, B. Critical evaluation of methodology commonly used in sample collection, storage and preparation in the analysis of pharmaceuticals and illicit drugs in surface water and wastewater by SPE-LC/MS. Journal of Chromatography A, 2011, DOI: 10.1016/j.chroma.2011.09.012.

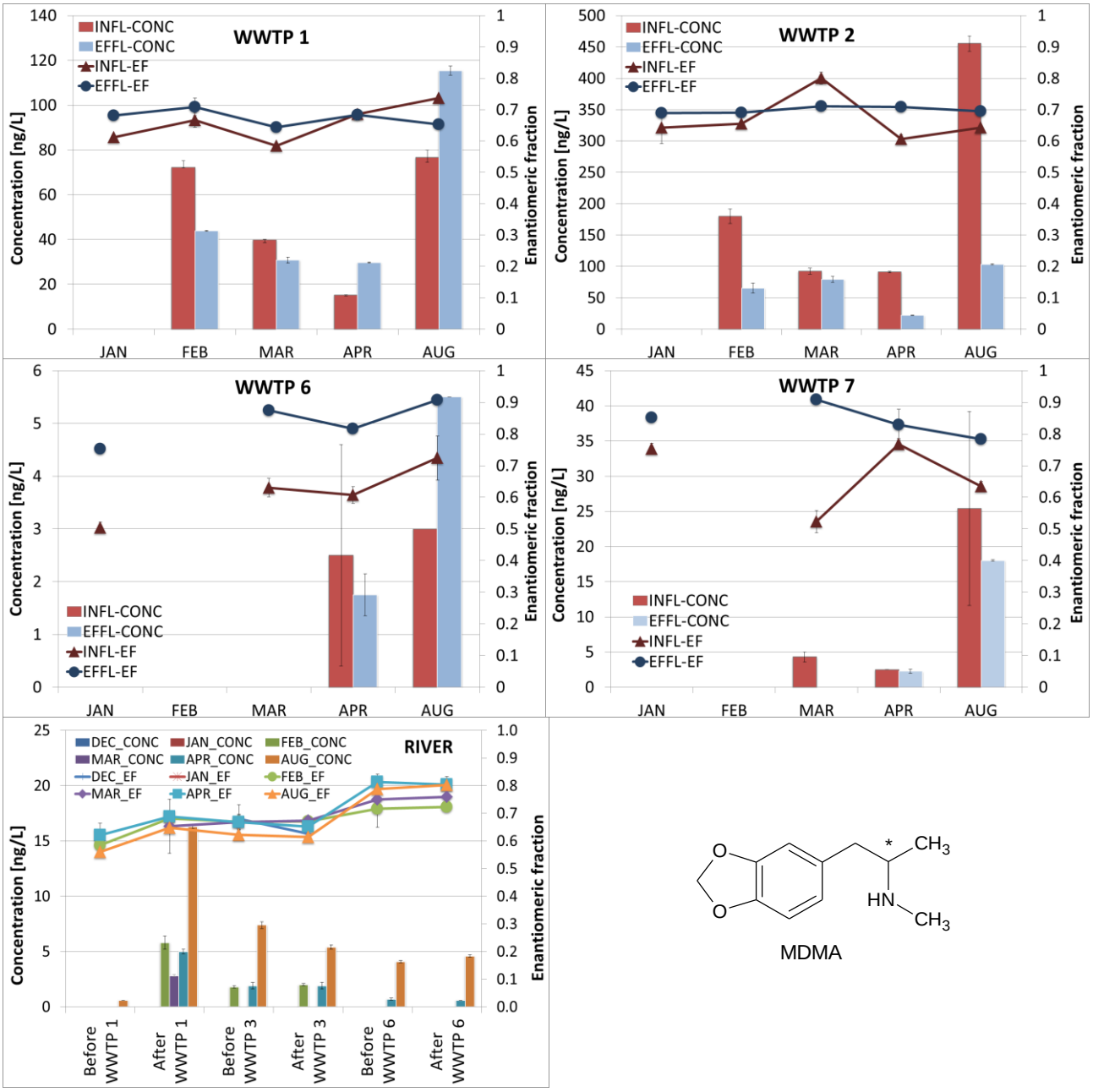


Figure 1. Concentrations and enantiomeric fractions of MDMA in WWTPs and receiving waters (note: different methods were used for quantification of drugs and enantiomeric profiling; see Tabs. S4 and S5 for validation parameters).

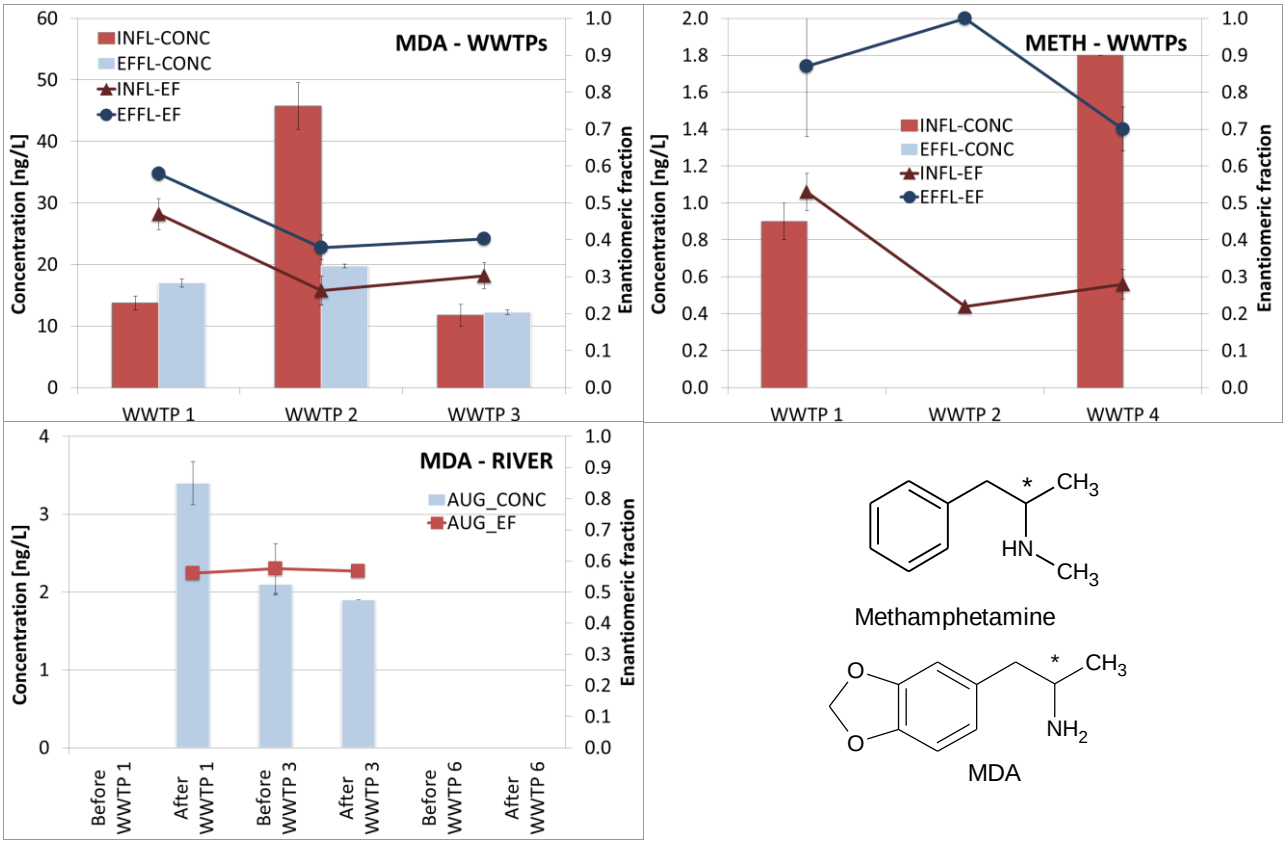
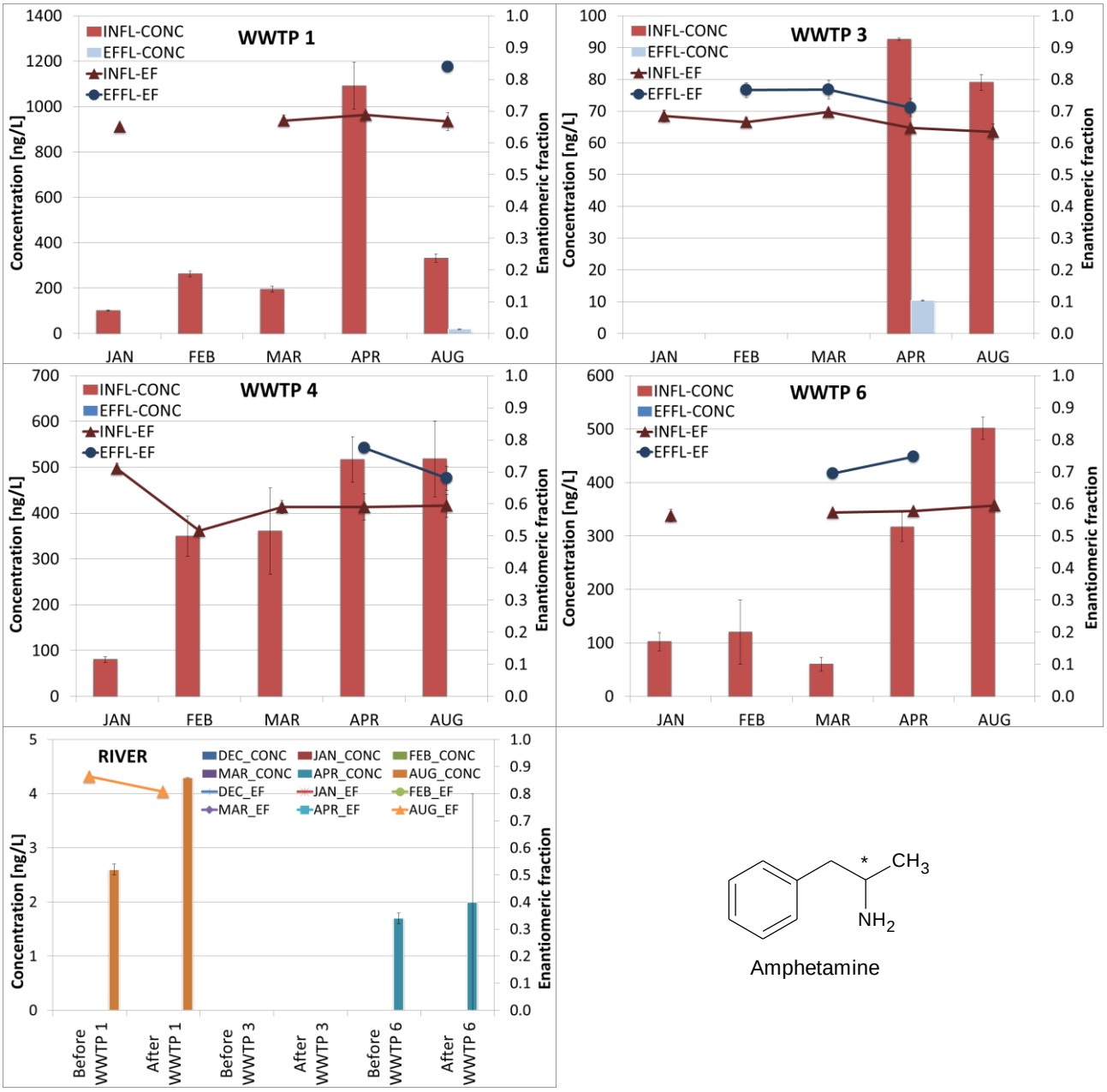


Figure 2. Concentrations and enantiomeric fractions of MDA and methamphetamine in WWTPs and receiving waters (note: different methods were used for quantification of drugs and enantiomeric profiling; see Tabs. S4 and S5 for validation parameters).



Amphetamine

Figure 3. Concentrations and enantiomeric fractions of amphetamine in WWTPs and receiving waters (note: different methods were used for quantification of drugs and enantiomeric profiling - see Tabs. S4 and S5 for validation parameters. Due to high variability of MS ion ratios in the case of amphetamine quantitation results should be considered on semi-quantitative basis).

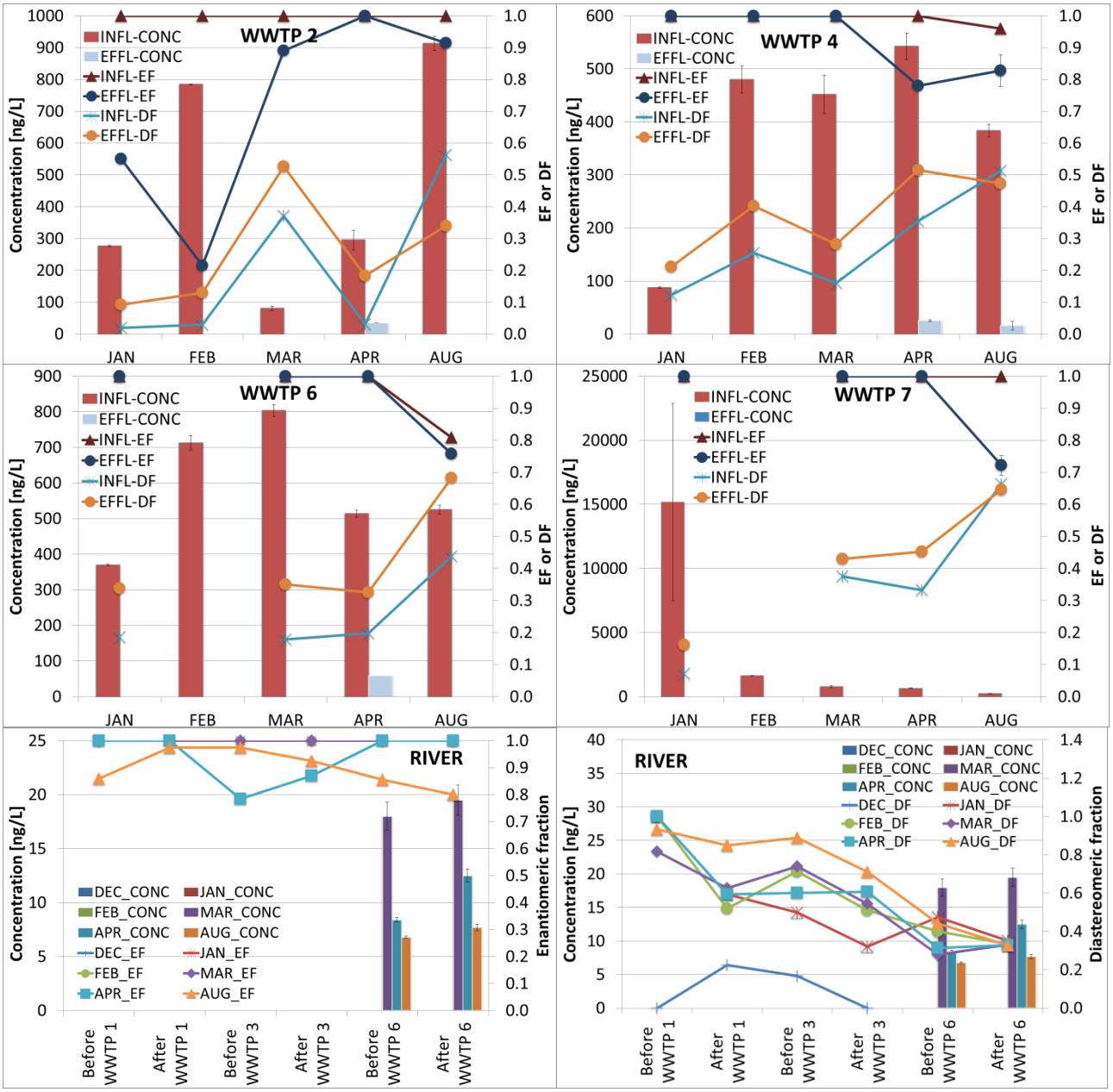


Figure 4. Concentrations and enantiomeric/diastereomeric fractions of ephedrine in WWTPs and receiving waters (note: different methods were used for quantification of drugs and enantiomeric profiling; see Tabs. S4 and S5 for validation parameters)

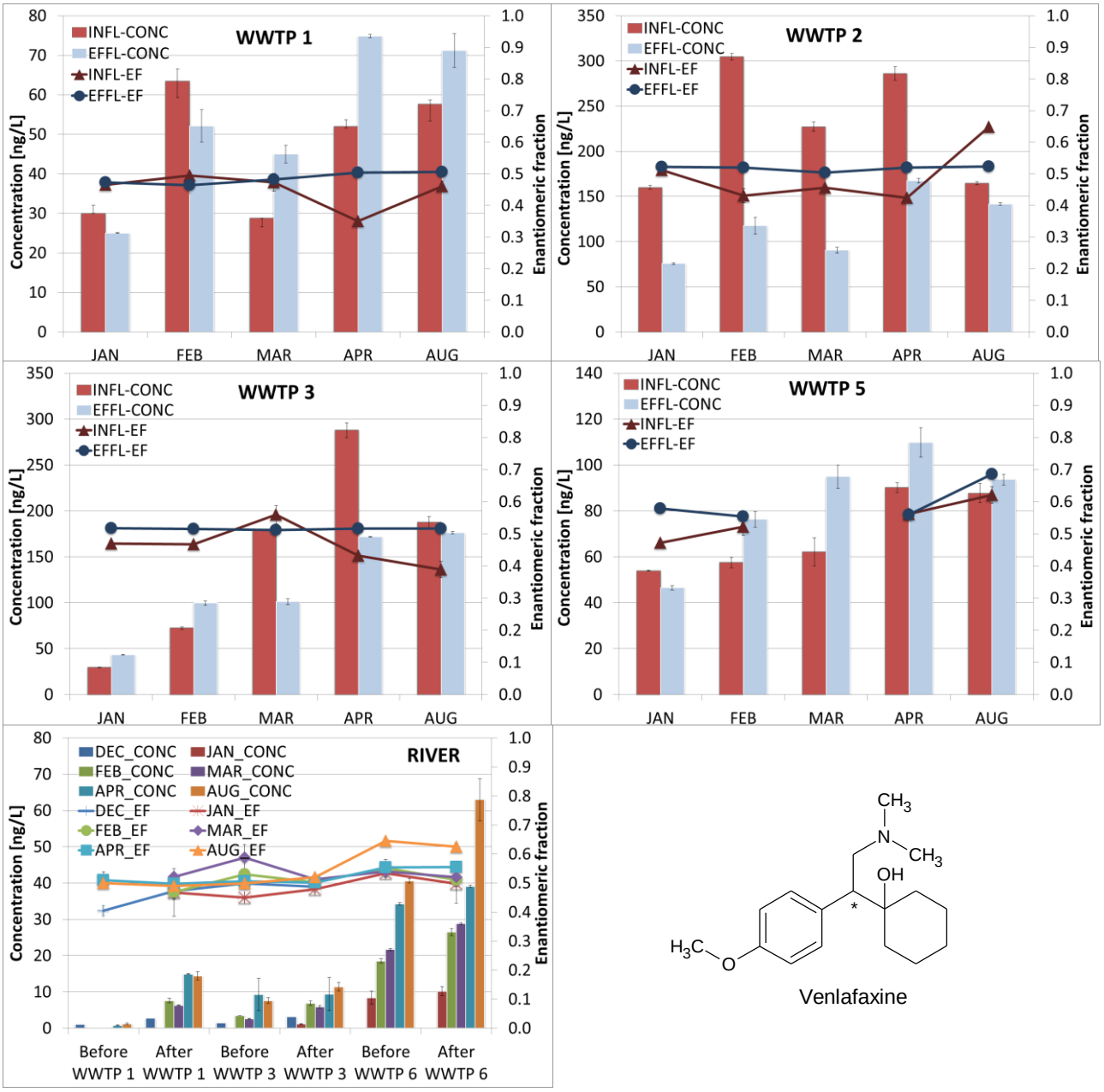


Figure 5. Concentrations and enantiomeric fractions of venlafaxine in WWTPs and receiving waters (note: different methods were used for quantification of drugs and enantiomeric profiling; see Tabs. S4 and S5 for validation parameters).

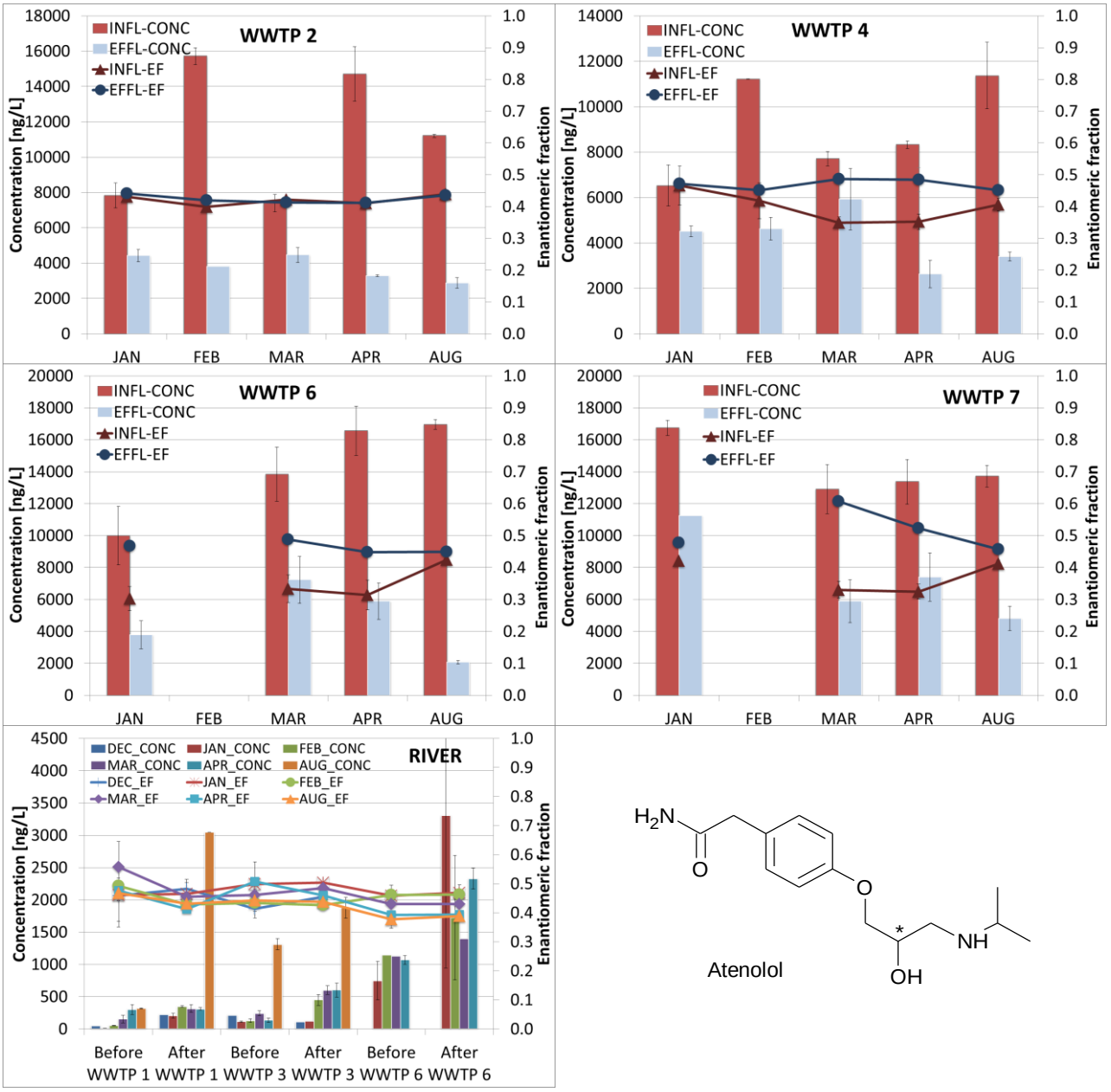


Figure 6. Concentrations and enantiomeric fractions of atenolol in WWTPs and receiving waters (note: chiral-LC-MS/MS method was used for both quantification of drugs and enantiomeric profiling; see Tab. S4 for validation parameters).

Supporting Information

Enantiomeric profiling of chiral drugs in wastewater and receiving waters

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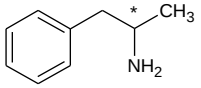
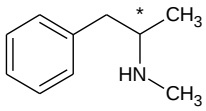
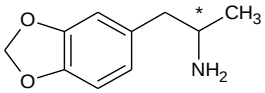
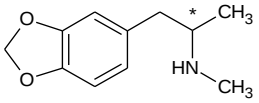
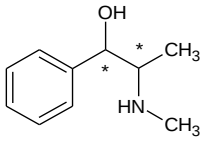
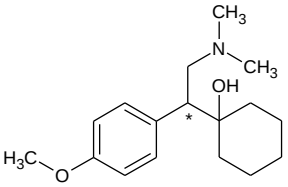
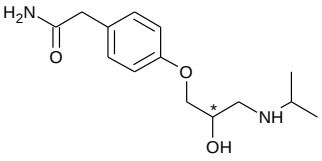
Table S1. Chemical formulas, molecular weights and *CAS* numbers of selected chiral drugs.

Name	CAS	MW	Structure
R/S-(±)-Amphetamine $C_9H_{13}N$	R(-): 300-62-9 S(+): 51-64-9 R/S-(±): 300-62-9	135.2	
R/S-(±)-Methamphetamine $C_{10}H_{15}N$	R(-): 33817-09-3 S(+): 537-46-2	149.2	
R/S-(±)-MDA $C_{10}H_{13}NO_2$	4764-17-4	179.2	
R/S-(±)-MDMA $C_{11}H_{15}NO_2$	42542-10-9	193.2	
1R,2S-(-)-Ephedrine HCl $C_{10}H_{15}NO \cdot HCl$	50-98-6	201.7	
1S,2R-(+)-Ephedrine HCl $C_{10}H_{15}NO \cdot HCl$	24221-86-1	201.7	
1S,2S-(+)-Pseudoephedrine HCl $C_{10}H_{15}NO \cdot HCl$	345-78-8	201.7	
1R,2R-(-)-Pseudoephedrine $C_{10}H_{15}NO$	90-82-4	165.2	
R/S-(±)-Venlafaxine HCl $C_{17}H_{27}NO_2 \cdot HCl$	99300-78-4	313.9	
R/S-(±)-Atenolol $C_{14}H_{22}N_2O_3$	29122-68-7	266.3	

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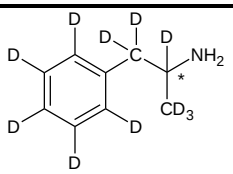
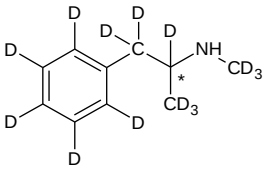
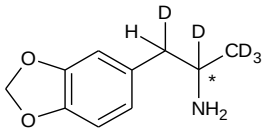
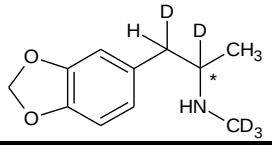
R/S-(±)-Amphetamine-d11 $C_9H_2D_{11}N$	NA	146.3	
R/S-(±)-Methamphetamine-d14 $C_{10}HD_{14}N$	NA	163.1	
R/S-(±)-MDA-d5 $C_{10}H_8D_5NO_2$	136765-42-9	184.2	
R/S-(±)-MDMA-d5 $C_{11}H_{10}D_5NO_2$	136765-43-0	198.2	

*- *chiral centre*

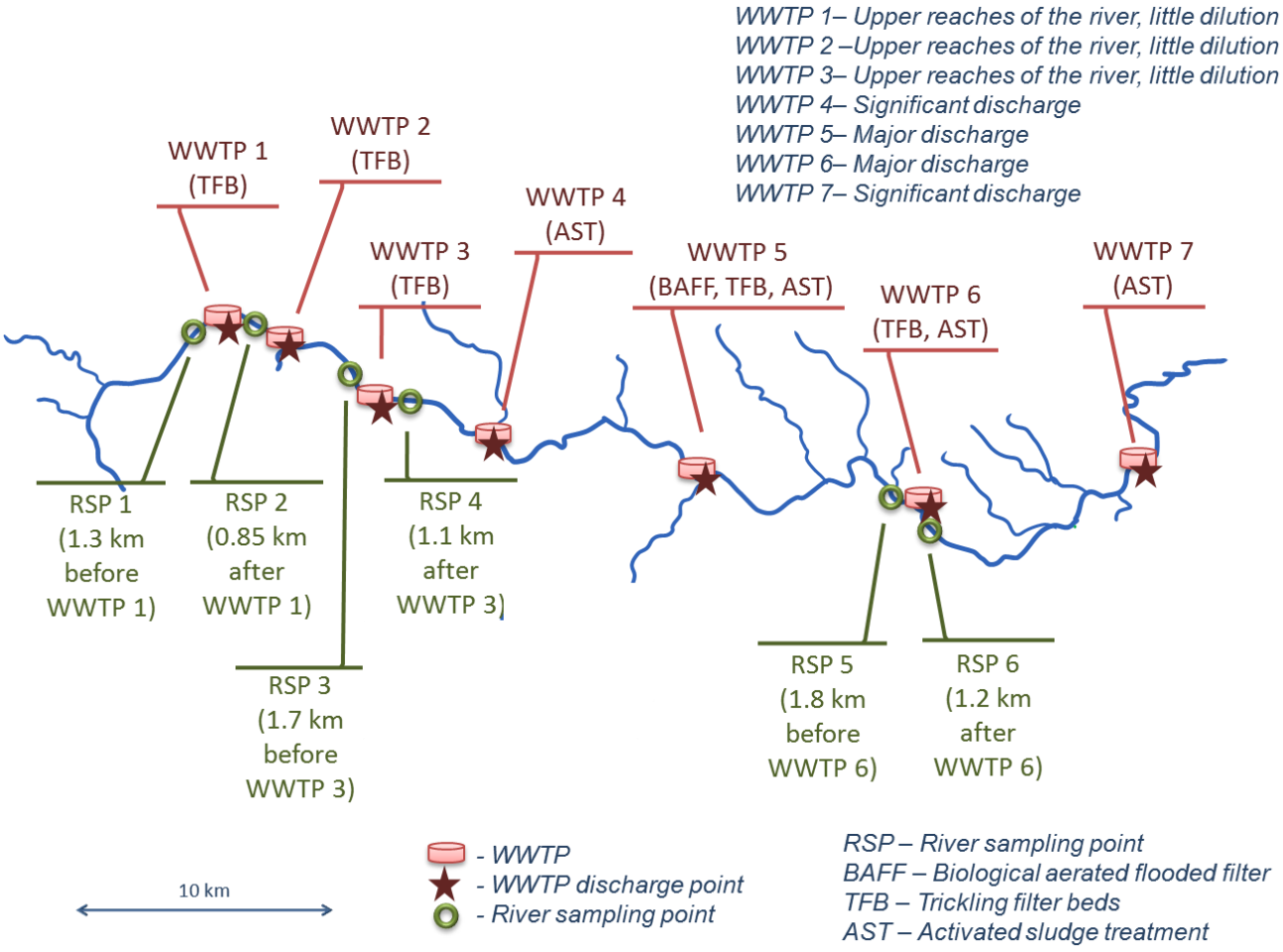


Figure S1. Location of WWTPs and river sampling points.

Table S2. General information on the studied WWTPs and receiving waters.

WWTP	Parameter				
	Population served (thousands)	Flows [L s^{-1}]	Wastewater (% industrial/ %domestic)	Treatment technology	Receiving waters Dilution factor
WWTP 1	15	89-212	10-15/85-90	Trickling filter beds	-
WWTP 2	10	32-75	10-15/85-90	Trickling filter beds	-
WWTP 3	11	40-115	10-15/85-90	Trickling filter beds	16-27
WWTP 4	190	366-1300	30/70	Activated sludge	-
WWTP 5	240	603-1231	30/70	30% BAFF, 15% trickling filter beds, 55% activated sludge	-
WWTP 6	244	476-1378	30/70	75% activated sludge, 25% trickling filter beds	9-20
WWTP 7	190	395-563	20/80	Activated sludge	-

Table S3. Optimised MRM conditions for the analysis of chiral drugs by UPLC/MS/MS (CV-cone voltage [V]; CE-collision energy [eV]).

Analyte	CV/CE	MRM1 (quantification)	CV/CE	MRM2 (confirmation)
R/S(±)-Amphetamine	18/8	136.16>119.10	18/16	136.16>91.10
1R,2S(-)/*1S,2R*(+)-Ephedrine/ *1S,2S*(+)/*1R,2R*(-)-Pseudoephedrine	23/12	166.09>148.10	23/21	166.09>133.00
R/S(±)-MDA	21/11	180.03>163.10	21/22	180.03>105.10
R/S(±)-MDMA	24/13	194.09>163.10	24/24	194.09>105.10
R/S(±)-Methamphetamine	24/10	150.20>119.05	24/19	150.20>91.10
R/S(±)-Venlafaxine	27/12	278.15>260.10	27/32	278.15>121.00
R/S(±)-Atenolol	34/19	266.9>190.10	34/25	266.9>145.00
R/S(±)-Amphetamine-d11	18/8	147.16>130.10	-	-
R/S(±)-MDA-d5	21/11	185.09>168.10	-	-
R/S(±)-MDMA-d5	26/13	199.1>165.10	-	-
R/S(±)-Methamphetamine-d14	24/19	164.16>98.10	-	-

Table S4. Validation parameters for SPE-Chiral LC-MS/MS method.

Analyte	Method parameters							
	EF_o*	R_s**	R^2***	RSD% (n=3)		MQL**** [ng/L]		
				10 [ng/L]	50 [ng/L]	WWTP influent	WWTP effluent	River
R/S(±)-Amphetamine (IS: *R/S*-(±)-AMPH-d11)	0.52±0.02	2.1	0.999	6.5	3.7	-	-	-
1R,2S(-)-Ephedrine (IS: *R/S*-(±)-MDMA-d5)	0.52±0.03*****	1.2	0.998	3.6	5.2	-	-	-
1S,2S(+)-Pseudoephedrine (IS: *R/S*-(±)-MDMA-d5)		2.3	0.999	8.7	6.8	-	-	-
R/S(±)-MDA (IS: *R/S*-(±)-MDA-d5)	0.48±0.01	3.0	0.998	7.8	3.3	-	-	-
R/S(±)-MDMA (IS: *R/S*-(±)-MDMA-d5)	0.48±0.01	1.9	0.999	7.5	2.6	-	-	-
R/S(±)-Methamphetamine (IS: *R/S*-(±)-METH-d14)	0.47±0.01	0.95	0.999	4.9	3.7	-	-	-
E1/E2(±)-Venlafaxine (IS: *R/S*-(±)-MDMA-d5)	0.49±0.01	0.95	0.997	4.9	4.7	-	-	-
R/S(±)-Atenolol (IS: *R/S*-(±)-MDMA-d5)	0.48±0.03	8.0	0.996	-	-	1.7	1.7	0.3

* - EF_o – enantiomeric fraction in standard solution spiked with racemic chiral drug (concentrations: 1-500 ng/L)

** - R_s – resolution

***- Studied linearity range: 1-500ng/L

****- *MQL* – method quantification limit

***** the value refers to *DF* (diastereomeric fraction) in standard solution spiked with *1R,2S*(-)-ephedrine and *1S,2S*(+)-pseudoephedrine

IS – Internal standard

Note: Deuterated ISs were not available for ephedrine, pseudoephedrine, venlafaxine and atenolol. For these compounds quantitation results should be considered on semi-quantitative basis.

Table S5. Validation parameters for SPE-UPLC-MS/MS method.

Analyte	Method parameters						
	Linearity range [ng/L]	R^2	RSD%		MQL [ng/L]		
			0.5 [ng/L]	50 [ng/L]	WWTP influent	WWTP effluent	River
Amphetamine (IS: Amphetamine-d11)	0.5-1000	0.999	<MQL*	10	5.1	2.1	1.0
Ephedrine/ Pseudoephedrine (IS: Amphetamine-d11)	0.5-1000	0.999	<MQL	10.7	5.6	5.2	10
MDA (IS: MDA-d5)	0.1-1000	0.999	11	2.1	4.2	4.2	0.5
MDMA (IS: MDMA-d5)	0.1-1000	0.999	9.4	3.9	0.7	0.7	0.5
Methamphetamine (IS: Methamphetamine-d14)	0.05-1000	0.999	8.3	4.1	0.6	0.6	0.1
Venlafaxine (IS: Fentanyl-d5)	0.1-250	0.999	9.6	3.8	3.5	3.6	0.5

* - MQL – method quantification limit

IS – Internal standard

Note: Deuterated ISs were not available for ephedrine, pseudoephedrine, venlafaxine and atenolol. For these compounds quantitation results should be considered on semi-quantitative basis.

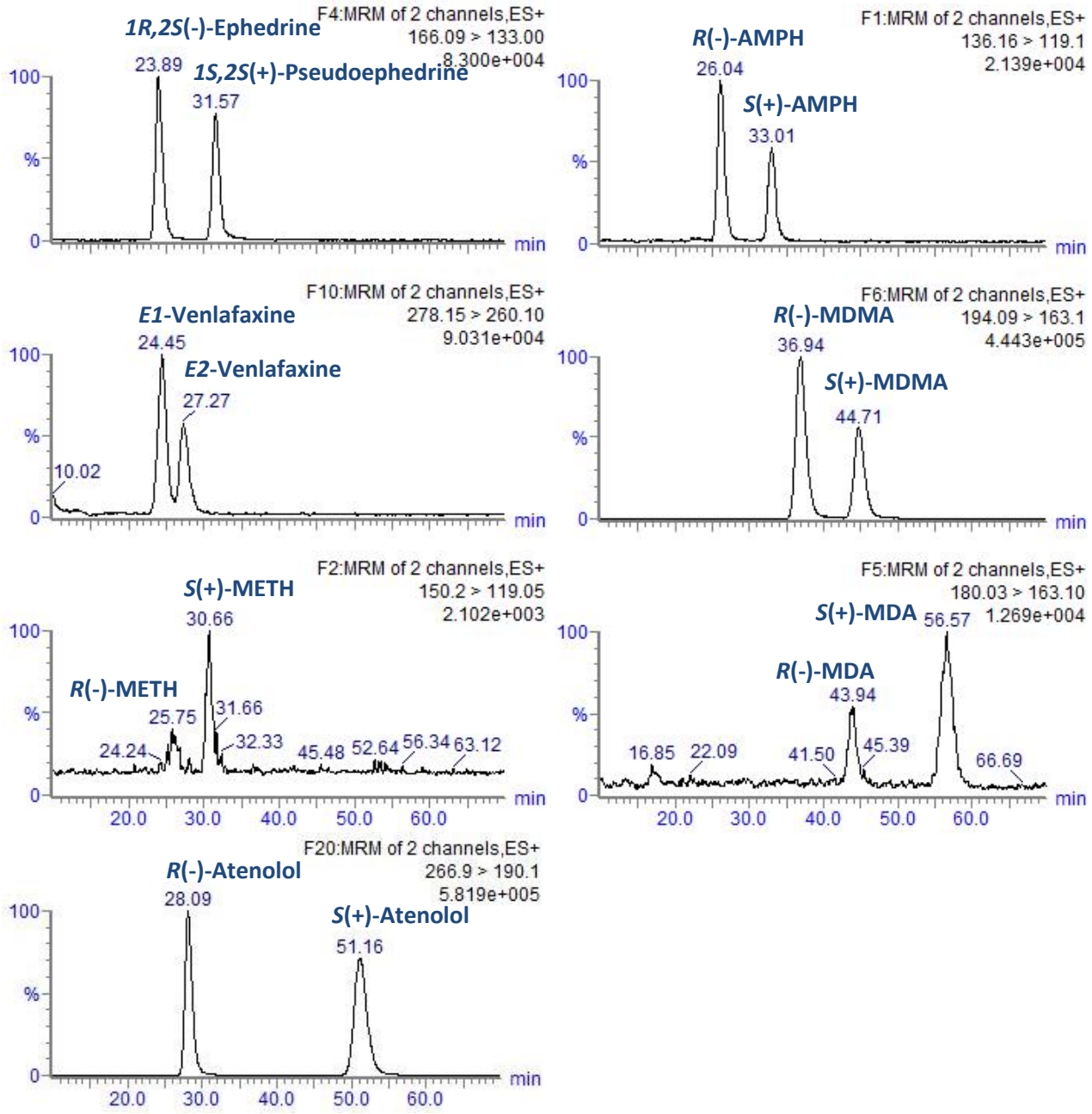


Figure S2. LC-MS/MS chromatograms of chiral drugs in wastewater influent collected from WWTP 2 during August sampling campaign.

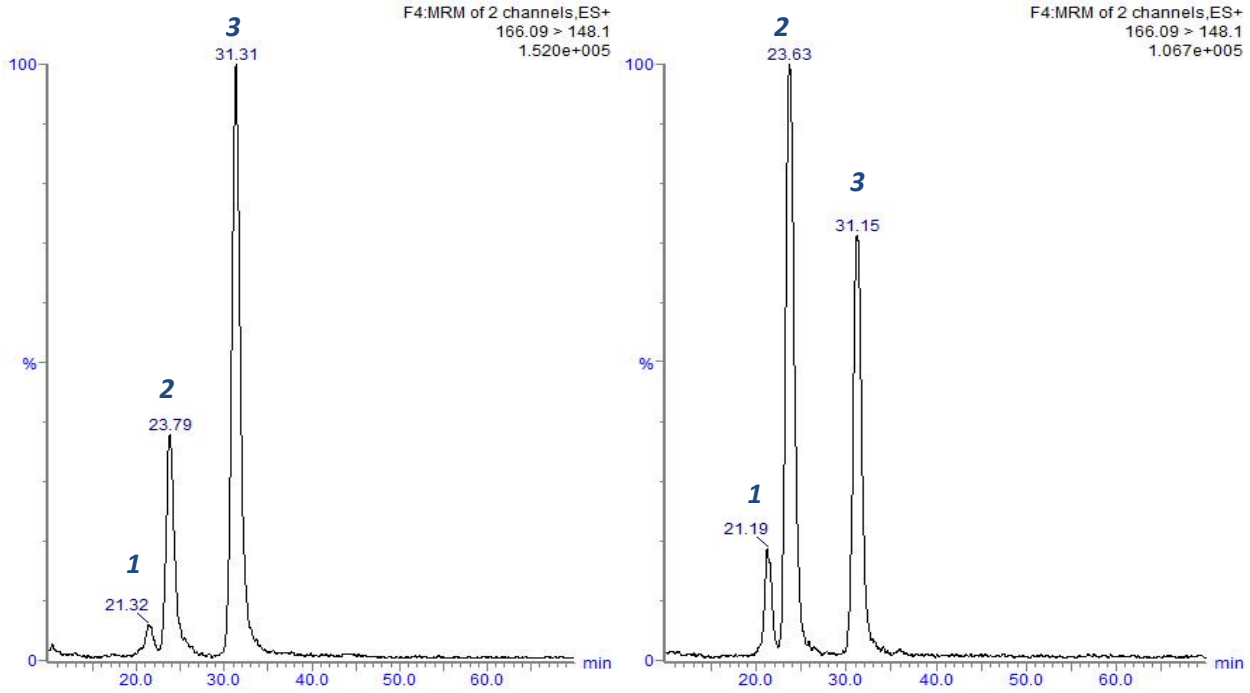


Figure S3. LC-MS/MS chromatograms of chiral drugs in wastewater influent and effluent collected from WWTP 3 during August sampling campaign (peak 1 – *1S,2R*(+)-ephedrine; peak 2 -*1R,2S*(-)-ephedrine and peak 3 – *1S,2S*(+)-pseudoephedrine).

Table S6. Concentrations and enantiomeric fractions of MDMA in WWTPs and receiving waters (Note: different methods were used for quantification of drugs and enantiomeric profiling; see Tabs. S4 and S5 for validation parameters; concentrations and enantiomeric fractions represent mean values for duplicate samples, each analyzed three times).

WWTPs		Concentration [ng/L]					
		JANUARY	FEBRUARY	MARCH	APRIL	AUGUST	
WWTP 1	INFL	<MQL	72.1 ± 3.0	39.8 ± 0.4	15.2 ± 0.1	76.7 ± 3.3	
	EFFL	<MQL	43.9 ± 0.2	30.8 ± 1.3	29.7 ± 0.3	115.4 ± 2.1	
WWTP 2	INFL	<MQL	180.1 ± 11.6	92.6 ± 4.7	91.0 ± 1.0	455.4 ± 12.3	
	EFFL	<MQL	65.3 ± 8.0	79.2 ± 4.8	22.0 ± 0.1	103.1 ± 0.8	
WWTP 3	INFL	<MQL	<MQL	<MQL	2.0 ± 0.4	42.9 ± 1.6	
	EFFL	<MQL	<MQL	<MQL	1.9 ± 0.1	53.7 ± 1.8	
WWTP 4	INFL	2.5 ± 0.2	1.6 ± 0.3	5.4 ± 0.4	31.2 ± 0.5	19.9 ± 1.6	
	EFFL	<MQL	<MQL	<MQL	3.7 ± 0.2	12.9 ± 0.7	
WWTP 5	INFL	<MQL	<MQL	<MQL	1.5 ± 0.0	5.7 ± 0.2	
	EFFL	<MQL	<MQL	<MQL	1.0 ± 0.0	8.8 ± 0.0	
WWTP 6	INFL	<MQL	<MQL	<MQL	2.5 ± 2.1	3.0 ± 0.0	
	EFFL	<MQL	<MQL	<MQL	1.8 ± 0.4	5.5 ± 0.0	
WWTP 7	INFL	<MQL	<MQL	4.3 ± 0.7	2.5 ± 0.0	25.4 ± 13.8	
	EFFL	<MQL	<MQL	<MQL	2.3 ± 0.3	18.0 ± 0.1	

WWTPs		Enantiomeric Fraction					
		JANUARY	FEBRUARY	MARCH	APRIL	AUGUST	
WWTP 1	INFL	0.61 ± 0.01	0.67 ± 0.02	0.58 ± 0.01	0.69 ± 0.01	0.74 ± 0.00	
	EFFL	0.68 ± 0.01	0.71 ± 0.03	0.64 ± 0.02	0.68 ± 0.02	0.65 ± 0.00	
WWTP 2	INFL	0.64 ± 0.05	0.66 ± 0.00	0.80 ± 0.02	0.61 ± 0.01	0.64 ± 0.00	
	EFFL	0.69 ± 0.00	0.69 ± 0.00	0.71 ± 0.00	0.71 ± 0.02	0.69 ± 0.00	
WWTP 3	INFL	0.76 ± 0.01	0.65 ± 0.02	0.56 ± 0.06	0.63 ± 0.04	0.68 ± 0.02	
	EFFL	0.74 ± 0.01	0.73 ± 0.01	0.77 ± 0.00	0.71 ± 0.05	0.69 ± 0.00	
WWTP 4	INFL	0.58 ± 0.01	0.80 ± 0.02	0.52 ± 0.03	0.75 ± 0.02	0.69 ± 0.02	
	EFFL	0.73 ± 0.01	0.75 ± 0.03	0.69 ± 0.02	0.81 ± 0.01	0.75 ± 0.00	
WWTP 5	INFL	0.63 ± 0.12	-	-	-	0.63 ± 0.02	
	EFFL	-	-	-	-	0.84 ± 0.01	
WWTP 6	INFL	0.50 ± 0.02	-	0.63 ± 0.03	0.61 ± 0.03	0.72 ± 0.07	
	EFFL	0.75 ± 0.03	-	0.88 ± 0.00	0.82 ± 0.02	0.91 ± 0.00	
WWTP 7	INFL	0.75 ± 0.02	-	0.52 ± 0.04	0.77 ± 0.02	0.64 ± 0.01	
	EFFL	0.85 ± 0.00	-	0.91 ± 0.01	0.83 ± 0.05	0.78 ± 0.01	

River	Concentration [ng/L]														
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	<MQL			<MQL			<MQL			<MQL			<MQL			0.6	±	0.0
After WWTP 1	<MQL			<MQL			5.8	±	0.6	2.8	±	0.1	5.0	±	0.2	16.3	±	0.2
Before WWTP 3	<MQL			<MQL			1.8	±	0.1	<MQL			1.9	±	0.3	7.4	±	0.3
After WWTP 3	<MQL			<MQL			2.0	±	0.1	<MQL			1.9	±	0.3	5.4	±	0.2
Before WWTP 6	<MQL			<MQL			<MQL			<MQL			0.7	±	0.1	4.1	±	0.1
After WWTP 6	<MQL			<MQL			<MQL			<MQL			0.6	±	0.0	4.6	±	0.1

River	Loads [g/day]														
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	-			-			-			-			-			-		
After WWTP 1	-			-			-			-			-			-		
Before WWTP 3	<MQL			<MQL			0.2	±	0.0	<MQL			0.2	±	0.0	0.5	±	0.0
After WWTP 3	<MQL			<MQL			2.3	±	0.0	<MQL			0.3	±	0.0	0.4	±	0.0
Before WWTP 6	<MQL			<MQL			<MQL			<MQL			0.5	±	0.1	1.6	±	0.0
After WWTP 6	<MQL			<MQL			<MQL			<MQL			0.3	±	0.0	2.1	±	0.0

River	Enantiomeric fraction														
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	-			-			0.58	±	0.02	-			0.62	±	0.04	0.56	±	0.01
After WWTP 1	-			-			0.68	±	0.01	0.65	±	0.10	0.69	±	0.00	0.65	±	0.00
Before WWTP 3	0.68	±	0.05	-			0.67	±	0.01	0.67	±	0.03	0.67	±	0.03	0.62	±	0.01
After WWTP 3	0.63	±	0.02	-			0.67	±	0.00	0.67	±	0.00	0.65	±	0.00	0.61	±	0.00
Before WWTP 6	-			-			0.72	±	0.07	0.75	±	0.00	0.81	±	0.03	0.79	±	0.01
After WWTP 6	-			-			0.72	±	0.01	0.76	±	0.00	0.80	±	0.03	0.80	±	0.01

Table S7. Concentrations and enantiomeric fractions of MDA in WWTPs and receiving waters (Note: different methods were used for quantification of drugs and enantiomeric profiling; see Tabs. S4 and S5 for validation parameters; concentrations and enantiomeric fractions represent mean values for duplicate samples, each analyzed three times).

WWTPs		Concentration [ng/L] AUGUST
WWTP 1	INFL	13.8 ± 1.1
	EFFL	17.0 ± 0.7
WWTP 2	INFL	45.8 ± 3.8
	EFFL	19.7 ± 0.3
WWTP 3	INFL	11.8 ± 1.8
	EFFL	12.3 ± 0.4

WWTPs		Enantiomeric fraction AUGUST
WWTP 1	INFL	0.47 ± 0.04
	EFFL	0.58 ± 0.02
WWTP 2	INFL	0.26 ± 0.04
	EFFL	0.38 ± 0.03
WWTP 3	INFL	0.30 ± 0.03
	EFFL	0.40 ± 0.01

River	Concentration [ng/L] AUGUST
Before WWTP 1	<MQL
After WWTP 1	3.4 ± 0.3
Before WWTP 3	2.1 ± 0.1
After WWTP 3	1.9 ± 0.0
Before WWTP 6	<MQL
After WWTP 6	<MQL

River	Enantiomeric fraction AUGUST
Before WWTP 1	-
After WWTP 1	0.56 ± 0.02
Before WWTP 3	0.58 ± 0.08
After WWTP 3	0.57 ± 0.02
Before WWTP 6	-
After WWTP 6	-

River	Load [g/day] AUGUST
Before WWTP 1	-
After WWTP 1	-
Before WWTP 3	0.1 ± 0.0
After WWTP 3	0.1 ± 0.0
Before WWTP 6	<MQL
After WWTP 6	<MQL

Table S8. Concentrations and enantiomeric fractions of methamphetamine in WWTPs (Note: different methods were used for quantification of drugs and enantiomeric profiling; see Tabs. S4 and S5 for validation parameters; concentrations and enantiomeric fractions represent mean values for duplicate samples, each analyzed three times).

WWTPs		Concentration [ng/L]		
		AUGUST		
WWTP 1	INFL	0.9	±	0.1
	EFFL	<MQL		
WWTP 2	INFL	<MQL		
	EFFL	<MQL		
WWTP 4	INFL	1.8	±	0.0
	EFFL	<MQL		

WWTPs		Enantiomeric fraction		
		AUGUST		
WWTP 1	INFL	0.53	±	0.05
	EFFL	0.87	±	0.19
WWTP 2	INFL	0.22	±	0.00
	EFFL	1.00	±	0.00
WWTP 4	INFL	0.28	±	0.04
	EFFL	0.70	±	0.06

Table S9. Concentrations and enantiomeric fractions of amphetamine in WWTPs and receiving waters (Note: different methods were used for quantification of drugs and enantiomeric profiling - see Tabs. S4 and S5 for validation parameters. Due to high variability of MS ion ratios in the case of amphetamine quantitation results should be considered on semi-quantitative basis; concentrations and enantiomeric fractions represent mean values for duplicate samples, each analyzed three times).

WWTPs		Concentration [ng/L]				
		JANUARY	FEBRUARY	MARCH	APRIL	AUGUST
WWTP 1	INFL	101.2 ± 2.5	263.3 ± 13.0	195.4 ± 13.2	1091.8 ± 103.6	332.2 ± 18.4
	EFFL	<MQL	<MQL	<MQL	<MQL	19.7 ± 1.5
WWTP 2	INFL	3.5 ± 1.3	80.5 ± 8.7	114.0 ± 7.1	50.7 ± 1.8	107.0 ± 8.3
	EFFL	<MQL	<MQL	<MQL	<MQL	<MQL
WWTP 3	INFL	<MQL	<MQL	<MQL	92.6 ± 0.4	79.1 ± 2.5
	EFFL	<MQL	<MQL	<MQL	10.4 ± 0.2	<MQL
WWTP 4	INFL	80.3 ± 6.2	349.6 ± 44.1	360.5 ± 94.0	517.3 ± 49.9	518.3 ± 83.1
	EFFL	<MQL	<MQL	<MQL	<MQL	<MQL
WWTP 5	INFL	<MQL	<MQL	<MQL	187.0 ± 2.0	159.3 ± 0.2
	EFFL	<MQL	<MQL	<MQL	5.9 ± 0.2	<MQL
WWTP 6	INFL	102.5 ± 17.0	120.0 ± 60.2	60.2 ± 13.2	317.0 ± 27.1	501.8 ± 20.9
	EFFL	<MQL	<MQL	<MQL	<MQL	<MQL
WWTP 7	INFL	3112.5 ± 2047.1	<MQL	121.9 ± 13.5	824.7 ± 83.3	391.8 ± 1.0
	EFFL	<MQL	<MQL	<MQL	<MQL	<MQL

WWTPs		Enantiomeric fraction				
		JANUARY	FEBRUARY	MARCH	APRIL	AUGUST
WWTP 1	INFL	0.65 ± 0.00	-	0.67 ± 0.02	0.69 ± 0.00	0.67 ± 0.03
	EFFL	-	-	-	-	0.84 ± 0.01
WWTP 2	INFL	0.62 ± 0.00	0.70 ± 0.00	0.67 ± 0.05	0.69 ± 0.04	0.60 ± 0.03
	EFFL	-	-	-	-	-
WWTP 3	INFL	0.68 ± 0.02	0.66 ± 0.01	0.70 ± 0.01	0.65 ± 0.00	0.64 ± 0.02
	EFFL	-	0.77 ± 0.02	0.77 ± 0.03	0.71 ± 0.03	-
WWTP 4	INFL	0.71 ± 0.02	0.52 ± 0.01	0.59 ± 0.02	0.59 ± 0.04	0.59 ± 0.04
	EFFL	-	-	-	0.78 ± 0.01	0.68 ± 0.04
WWTP 5	INFL	0.63 ± 0.00	0.70 ± 0.00	-	0.84 ± 0.01	0.59 ± 0.04
	EFFL	-	-	-	1.00 ± 0.00	-
WWTP 6	INFL	0.56 ± 0.02	-	0.57 ± 0.00	0.58 ± 0.00	0.59 ± 0.01
	EFFL	-	-	0.70 ± 0.04	0.75 ± 0.02	-
WWTP 7	INFL	0.69 ± 0.00	-	0.58 ± 0.04	0.57 ± 0.01	0.59 ± 0.01
	EFFL	-	-	-	-	-

River	Concentration [ng/L]					
	DECEMBER	JANUARY	FEBRUARY	MARCH	APRIL	AUGUST
Before WWTP 1	<MQL	<MQL	<MQL	<MQL	<MQL	2.6 ± 0.1
After WWTP 1	<MQL	<MQL	<MQL	<MQL	<MQL	4.3 ± 0.0
Before WWTP 3	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
After WWTP 3	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
Before WWTP 6	<MQL	<MQL	<MQL	<MQL	1.7 ± 0.1	<MQL
After WWTP 6	<MQL	<MQL	<MQL	<MQL	2.0 ± 2.0	<MQL

River	Load [g/day]					
	DECEMBER	JANUARY	FEBRUARY	MARCH	APRIL	AUGUST
Before WWTP 1	-	-	-	-	-	-
After WWTP 1	-	-	-	-	-	-
Before WWTP 3	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
After WWTP 3	<MQL	<MQL	<MQL	<MQL	<MQL	<MQL
Before WWTP 6	<MQL	<MQL	<MQL	<MQL	1.3 ± 0.0	<MQL
After WWTP 6	<MQL	<MQL	<MQL	<MQL	1.7 ± 1.7	<MQL

River	Enantiomeric fraction					
	DECEMBER	JANUARY	FEBRUARY	MARCH	APRIL	AUGUST
Before WWTP 1	-	-	-	-	-	0.86 ± 0.01
After WWTP 1	-	-	-	-	-	0.81 ± 0.01
Before WWTP 3	-	-	-	-	-	-
After WWTP 3	-	-	-	-	-	-
Before WWTP 6	-	-	-	-	-	-
After WWTP 6	-	-	-	-	-	-

Table S10. Concentrations and enantiomeric/diastereomeric fractions of ephedrine in WWTPs and receiving waters (Note: different methods were used for quantification of drugs and enantiomeric profiling; see Tabs. S4 and S5 for validation parameters; concentrations and enantiomeric fractions represent mean values for duplicate samples, each analyzed three times).

WWTPs		Concentration [ng/L]					
		JANUARY	FEBRUARY	MARCH	APRIL	AUGUST	
WWTP 1	INFL	8.7 ± 0.3	290.2 ± 50.2	139.4 ± 11.5	69.7 ± 1.0	192.0 ± 9.7	
	EFFL	<MQL	<MQL	<MQL	<MQL	15.5 ± 7.5	
WWTP 2	INFL	276.9 ± 2.5	784.7 ± 1.4	81.2 ± 7.0	295.7 ± 30.4	913.2 ± 23.1	
	EFFL	<MQL	<MQL	<MQL	35.0 ± 0.2	<MQL	
WWTP 3	INFL	<MQL	497.1 ± 32.6	215.8 ± 0.6	119.8 ± 1.5	137.6 ± 7.6	
	EFFL	<MQL	<MQL	<MQL	61.6 ± 0.8	20.3 ± 3.8	
WWTP 4	INFL	87.4 ± 1.1	479.9 ± 25.6	451.6 ± 36.1	542.5 ± 25.2	383.8 ± 12.1	
	EFFL	<MQL	<MQL	<MQL	25.2 ± 1.7	15.7 ± 8.4	
WWTP 5	INFL	333.9 ± 21.8	265.1 ± 12.8	1174.5 ± 74.5	176.4 ± 10.5	295.8 ± 1.2	
	EFFL	<MQL	<MQL	84.1 ± 21.6	<MQL	<MQL	
WWTP 6	INFL	370.1 ± 2.5	712.7 ± 20.7	803.7 ± 16.3	513.7 ± 10.1	525.2 ± 12.6	
	EFFL	<MQL	<MQL	<MQL	59.2 ± 7.2	<MQL	
WWTP 7	INFL	15171.0 ± 7678.0	1625.3 ± 15.1	785.1 ± 65.9	648.6 ± 31.2	235.3 ± 22.1	
	EFFL	<MQL	<MQL	<MQL	5.3 ± 2.3	<MQL	

WWTPs		Enantiomeric Fraction					
		JANUARY	FEBRUARY	MARCH	APRIL	AUGUST	
WWTP 1	INFL	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	
	EFFL	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	
WWTP 2	INFL	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	
	EFFL	0.55 ± 0.00	0.22 ± 0.00	0.89 ± 0.00	1.00 ± 0.00	0.91 ± 0.01	
WWTP 3	INFL	1.00 ± 0.00	1.00 ± 0.00	-	1.00 ± 0.00	0.95 ± 0.07	
	EFFL	1.00 ± 0.00	1.00 ± 0.00	-	1.00 ± 0.00	0.90 ± 0.02	
WWTP 4	INFL	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.96 ± 0.01	
	EFFL	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.78 ± 0.00	0.83 ± 0.05	
WWTP 5	INFL	1.00 ± 0.00	1.00 ± 0.00	-	1.00 ± 0.00	1.00 ± 0.00	
	EFFL	1.00 ± 0.00	1.00 ± 0.00	-	1.00 ± 0.00	1.00 ± 0.00	
WWTP 6	INFL	1.00 ± 0.00	-	1.00 ± 0.00	1.00 ± 0.00	0.81 ± 0.01	
	EFFL	1.00 ± 0.00	-	1.00 ± 0.00	1.00 ± 0.00	0.76 ± 0.02	
WWTP 7	INFL	1.00 ± 0.00	-	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	
	EFFL	1.00 ± 0.00	-	1.00 ± 0.00	1.00 ± 0.00	0.72 ± 0.03	

WWTPs		Diastereomeric Fraction														
		JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
WWTP 1	INFL	0.09	±	0.00	0.44	±	0.00	0.23	±	0.01	0.08	±	0.00	0.44	±	0.01
	EFFL	0.07	±	0.05	0.10	±	0.00	0.29	±	0.01	0.33	±	0.09	0.82	±	0.00
WWTP 2	INFL	0.02	±	0.00	0.03	±	0.00	0.37	±	0.01	0.03	±	0.00	0.56	±	0.01
	EFFL	0.09	±	0.02	0.13	±	0.13	0.53	±	0.01	0.18	±	0.01	0.34	±	0.01
WWTP 3	INFL	0.28	±	0.05	0.07	±	0.01	0.25	±	0.01	0.09	±	0.01	0.26	±	0.04
	EFFL	0.04	±	0.00	0.16	±	0.03	0.19	±	0.00	0.22	±	0.01	0.55	±	0.00
WWTP 4	INFL	0.12	±	0.01	0.26	±	0.01	0.16	±	0.05	0.36	±	0.06	0.51	±	0.04
	EFFL	0.21	±	0.04	0.40	±	0.04	0.28	±	0.04	0.51	±	0.00	0.47	±	0.00
WWTP 5	INFL	0.25	±	0.01	0.27	±	0.00		-		0.12	±	0.02	0.47	±	0.00
	EFFL	0.60	±	0.01	0.52	±	0.00		-		0.30	±	0.01	0.66	±	0.02
WWTP 6	INFL	0.19	±	0.01		-		0.18	±	0.01	0.20	±	0.03	0.44	±	0.00
	EFFL	0.34	±	0.01		-		0.35	±	0.04	0.33	±	0.03	0.68	±	0.02
WWTP 7	INFL	0.07	±	0.02		-		0.38	±	0.04	0.33	±	0.04	0.66	±	0.00
	EFFL	0.16	±	0.02		-		0.43	±	0.08	0.45	±	0.03	0.65	±	0.06

River	Concentration [ng/L]																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	<MQL			<MQL			<MQL			<MQL			<MQL			<MQL		
After WWTP 1	<MQL			<MQL			<MQL			<MQL			<MQL			<MQL		
Before WWTP 3	<MQL			<MQL			<MQL			<MQL			<MQL			<MQL		
After WWTP 3	<MQL			<MQL			<MQL			<MQL			<MQL			<MQL		
Before WWTP 6	<MQL			<MQL			<MQL			18.0	±	1.3	8.4	±	0.2	6.8	±	0.1
After WWTP 6	<MQL			<MQL			<MQL			19.5	±	1.4	12.5	±	0.6	7.7	±	0.3

River	Load [g/day]																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	-			-			-			-			-			-		
After WWTP 1	-			-			-			-			-			-		
Before WWTP 3	<MQL			<MQL			<MQL			<MQL			<MQL			<MQL		
After WWTP 3	<MQL			<MQL			<MQL			<MQL			<MQL			<MQL		
Before WWTP 6	<MQL			<MQL			<MQL			16.1	±	1.2	6.5	±	0.2	2.6	±	0.0
After WWTP 6	<MQL			<MQL			<MQL			19.0	±	1.4	10.5	±	0.5	3.5	±	0.1

River	Enantiomeric fraction																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	0.86	±	0.00
After WWTP 1	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	0.98	±	0.00
Before WWTP 3	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	0.79	±	0.01	0.98	±	0.00
After WWTP 3	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	0.87	±	0.00	0.93	±	0.00
Before WWTP 6	-			1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	0.86	±	0.00
After WWTP 6	-			1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	1.00	±	0.00	0.80	±	0.00

River	Diastereomeric fraction																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	0.00	±	0.00	1.00	±	0.00	1.00	±	0.00	0.82	±	0.11	1.00	±	0.00	0.93	±	0.03
After WWTP 1	0.22	±	0.01	0.59	±	0.02	0.52	±	0.05	0.63	±	0.05	0.59	±	0.06	0.85	±	0.00
Before WWTP 3	0.17	±	0.00	0.50	±	0.09	0.71	±	0.04	0.74	±	0.04	0.60	±	0.03	0.89	±	0.01
After WWTP 3	0.00	±	0.00	0.32	±	0.00	0.51	±	0.01	0.54	±	0.03	0.61	±	0.00	0.71	±	0.00
Before WWTP 6	-			0.48	±	0.09	0.40	±	0.11	0.28	±	0.00	0.31	±	0.01	0.44	±	0.01
After WWTP 6	-			0.35	±	0.03	0.33	±	0.01	0.33	±	0.00	0.33	±	0.05	0.33	±	0.03

Table S11. Concentrations and enantiomeric fractions of venlafaxine in WWTPs and receiving waters (Note: different methods were used for quantification of drugs and enantiomeric profiling; see Tabs. S4 and S5 for validation parameters; concentrations and enantiomeric fractions represent mean values for duplicate samples, each analyzed three times).

WWTPs		Concentration [ng/L]														
		JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
WWTP 1	INFL	30.0	±	2.0	63.4	±	3.1	28.8	±	0.0	52.0	±	1.7	57.6	±	1.1
	EFFL	25.0	±	0.1	52.1	±	4.1	45.0	±	2.2	74.8	±	0.4	71.2	±	4.2
WWTP 2	INFL	159.9	±	2.0	304.5	±	3.5	227.2	±	5.2	286.1	±	7.6	164.6	±	1.7
	EFFL	75.6	±	1.0	117.6	±	9.4	90.4	±	3.1	167.4	±	2.6	141.6	±	1.2
WWTP 3	INFL	29.5	±	0.4	72.4	±	0.6	179.3	±	0.6	287.9	±	8.0	187.6	±	6.4
	EFFL	43.1	±	0.2	99.6	±	3.2	101.1	±	3.2	171.7	±	0.1	176.1	±	1.5
WWTP 4	INFL	67.3	±	0.1	104.9	±	3.7	115.4	±	9.9	184.9	±	0.8	145.8	±	7.2
	EFFL	54.7	±	3.2	102.1	±	0.6	84.0	±	3.6	142.1	±	2.2	128.8	±	10.7
WWTP 5	INFL	53.9	±	0.2	57.5	±	2.2	62.2	±	6.1	90.1	±	2.1	87.7	±	4.1
	EFFL	46.5	±	1.0	76.3	±	3.3	94.9	±	5.1	109.8	±	6.5	93.6	±	2.3
WWTP 6	INFL	115.9	±	3.4	148.8	±	6.6	114.4	±	6.1	178.0	±	2.9	172.2	±	10.7
	EFFL	64.4	±	4.1	100.7	±	0.6	84.2	±	1.6	143.2	±	1.0	119.2	±	1.6
WWTP 7	INFL	115.9	±	1.8	139.4	±	1.8	257.2	±	2.1	210.3	±	2.0	325.5	±	7.1
	EFFL	82.0	±	2.1	222.6	±	11.1	135.8	±	8.5	180.6	±	0.2	139.7	±	3.9

WWTPs		Enantiomeric Fraction														
		JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
WWTP 1	INFL	0.47	±	0.00	0.49	±	0.01	0.47	±	0.03	0.35	±	0.01	0.46	±	0.02
	EFFL	0.47	±	0.01	0.46	±	0.03	0.48	±	0.01	0.50	±	0.00	0.51	±	0.01
WWTP 2	INFL	0.51	±	0.01	0.43	±	0.02	0.46	±	0.00	0.42	±	0.00	0.65	±	0.01
	EFFL	0.52	±	0.01	0.52	±	0.00	0.50	±	0.01	0.52	±	0.02	0.52	±	0.01
WWTP 3	INFL	0.47	±	0.00	0.47	±	0.00	0.56	±	0.03	0.43	±	0.00	0.39	±	0.03
	EFFL	0.52	±	0.00	0.51	±	0.01	0.51	±	0.01	0.52	±	0.01	0.52	±	0.00
WWTP 4	INFL	0.47	±	0.01	0.43	±	0.03		-			-		0.48	±	0.03
	EFFL	0.51	±	0.00	0.51	±	0.01	0.50	±	0.01	0.52	±	0.01	0.51	±	0.00
WWTP 5	INFL	0.47	±	0.03	0.52	±	0.00		-		0.56	±	0.01	0.62	±	0.01
	EFFL	0.58	±	0.01	0.55	±	0.01		-		0.56	±	0.00	0.69	±	0.00
WWTP 6	INFL	0.51	±	0.05		-		0.51	±	0.02	0.49	±	0.04	0.50	±	0.00
	EFFL	0.49	±	0.00		-		0.51	±	0.01	0.50	±	0.00	0.51	±	0.00
WWTP 7	INFL	0.45	±	0.00		-			-			-		0.47	±	0.00
	EFFL	0.48	±	0.00		-		0.49	±	0.00	0.51	±	0.00	0.50	±	0.01

River	Concentration [ng/L]																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	1.1	±	0.1	<MQL			<MQL			<MQL			0.8	±	0.1	1.2	±	0.2
After WWTP 1	2.8	±	0.1	<MQL			7.6	±	0.6	6.3	±	0.1	14.9	±	0.1	14.4	±	1.1
Before WWTP 3	1.5	±	0.1	<MQL			3.5	±	0.0	2.6	±	0.1	9.3	±	4.4	7.6	±	0.8
After WWTP 3	3.2	±	0.2	1.2	±	0.1	6.9	±	0.6	5.9	±	0.4	9.4	±	4.6	11.4	±	1.2
Before WWTP 6	<MQL			8.4	±	1.8	18.5	±	0.6	21.7	±	0.3	34.3	±	0.3	40.6	±	0.6
After WWTP 6	<MQL			10.2	±	1.3	26.5	±	1.0	28.8	±	0.3	39.1	±	0.3	63.0	±	5.8

River	Load [g/day]																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	-			-			-			-			-			-		
After WWTP 1	-			-			-			-			-			-		
Before WWTP 3	0.4	±	0.0	<MQL			0.5	±	0.0	0.4	±	0.0	1.2	±	0.6	0.6	±	0.1
After WWTP 3	0.9	±	0.1	2.2	±	0.2	1.0	±	0.1	0.9	±	0.1	1.3	±	0.6	0.9	±	0.1
Before WWTP 6	<MQL			20.4	±	4.5	18.7	±	0.7	19.4	±	0.3	26.4	±	0.3	15.8	±	0.2
After WWTP 6	<MQL			25.8	±	3.2	28.9	±	1.1	28.1	±	0.3	32.7	±	0.3	28.7	±	2.6
River	Enantiomeric fraction																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	0.40	±	0.02	-			-			-			0.51	±	0.03	0.50	±	0.00
After WWTP 1	0.47	±	0.03	0.47	±	0.08	0.47	±	0.02	0.52	±	0.00	0.50	±	0.02	0.49	±	0.00
Before WWTP 3	0.50	±	0.02	0.45	±	0.00	0.53	±	0.01	0.59	±	0.04	0.51	±	0.01	0.50	±	0.01
After WWTP 3	0.49	±	0.02	0.48	±	0.00	0.50	±	0.00	0.51	±	0.01	0.50	±	0.01	0.52	±	0.00
Before WWTP 6	-			0.53	±	0.00	0.55	±	0.03	0.54	±	0.00	0.55	±	0.00	0.65	±	0.01
After WWTP 6	-			0.50	±	0.07	0.51	±	0.02	0.52	±	0.00	0.55	±	0.00	0.62	±	0.01

Table S12. Concentrations and enantiomeric fractions of atenolol in WWTPs and receiving waters (Note: chiral-LC-MS/MS method was used for both quantification of drugs and enantiomeric profiling; see Tab. S4 for validation parameters; concentrations and enantiomeric fractions represent mean values for duplicate samples, each analyzed three times).

WWTPs		Concentration [ng/L]					
		JANUARY	FEBRUARY	MARCH	APRIL	AUGUST	
WWTP 1	INFL	7631.8 ± 136.0	4288.3 ± 809.0	16909.3 ± 1767.0	8997.3 ± 229.0	18446.8 ± 1660.6	
	EFFL	2007.0 ± 40.0	8280.3 ± 1814.0	18831.0 ± 0	3893.5 ± 233.0	3445.8 ± 464.9	
WWTP 2	INFL	7831.5 ± 710.0	15721.3 ± 480.0	7389.5 ± 491.0	14706.5 ± 1552.8	11216.3 ± 94.4	
	EFFL	4424.0 ± 345.0	3811.0 ± 0.0	4459.8 ± 422.0	3284.5 ± 58.0	2873.5 ± 299.8	
WWTP 3	INFL	8543.8 ± 734.0	12032.0 ± 162.0	8196.8 ± 162.0	12135.8 ± 230.9	19160.5 ± 352.1	
	EFFL	4854.5 ± 338.0	7605.5 ± 363.0	7140.0 ± 363.0	3761.5 ± 0.0	4521.0 ± 419.3	
WWTP 4	INFL	6524.3 ± 907.0	11216.3 ± 3.9	7705.5 ± 313.0	8325.5 ± 166.9	11362.0 ± 1465.4	
	EFFL	4505.5 ± 236.0	4628.3 ± 499.0	5921.5 ± 1353.0	2625.5 ± 601.7	3399.0 ± 201.5	
WWTP 5	INFL	6805.8 ± 95.0	7193.8 ± 20.0	-	5101.5 ± 242.5	5858.0 ± 176.1	
	EFFL	3833.5 ± 197.0	4580.5 ± 67.0	-	5770.3 ± 503.8	1480.3 ± 23.0	
WWTP 6	INFL	9995.5 ± 1826.0	-	13838.0 ± 1699.0	16560.5 ± 1540.6	16958.5 ± 301.9	
	EFFL	3790.0 ± 882.0	-	7229.5 ± 1464.0	5900.3 ± 1136.0	2079.0 ± 117.4	
WWTP 7	INFL	16745.5 ± 472.0	-	12907.3 ± 1528.0	13371.3 ± 1402.1	13717.5 ± 691.6	
	EFFL	11238.0 ± 0.0	-	5894.8 ± 1348.0	7403.3 ± 1512.0	4814.0 ± 771.5	

WWTP		Enantiomeric Fraction					
		JANUARY	FEBRUARY	MARCH	APRIL	AUGUST	
WWTP 1	INFL	0.43 ± 0.00	0.44 ± 0.01	0.43 ± 0.00	0.42 ± 0.01	0.44 ± 0.01	
	EFFL	0.48 ± 0.01	0.41 ± 0.03	0.43 ± 0.01	0.45 ± 0.07	0.44 ± 0.00	
WWTP 2	INFL	0.43 ± 0.01	0.40 ± 0.01	0.42 ± 0.00	0.41 ± 0.01	0.44 ± 0.01	
	EFFL	0.44 ± 0.00	0.42 ± 0.00	0.41 ± 0.00	0.41 ± 0.02	0.44 ± 0.02	
WWTP 3	INFL	0.44 ± 0.01	0.41 ± 0.00	0.38 ± 0.01	0.44 ± 0.02	0.43 ± 0.01	
	EFFL	0.47 ± 0.01	0.44 ± 0.00	0.45 ± 0.01	0.50 ± 0.07	0.43 ± 0.02	
WWTP 4	INFL	0.47 ± 0.06	0.42 ± 0.06	0.35 ± 0.02	0.35 ± 0.02	0.41 ± 0.02	
	EFFL	0.47 ± 0.01	0.45 ± 0.00	0.49 ± 0.02	0.48 ± 0.04	0.45 ± 0.02	
WWTP 5	INFL	0.41 ± 0.00	0.40 ± 0.01	-	0.39 ± 0.01	0.38 ± 0.01	
	EFFL	0.42 ± 0.01	0.43 ± 0.01	-	0.40 ± 0.01	0.45 ± 0.01	
WWTP 6	INFL	0.30 ± 0.04	-	0.33 ± 0.04	0.31 ± 0.05	0.42 ± 0.00	
	EFFL	0.47 ± 0.01	-	0.49 ± 0.01	0.45 ± 0.01	0.45 ± 0.01	
WWTP 7	INFL	0.42 ± 0.00	-	0.33 ± 0.03	0.32 ± 0.02	0.41 ± 0.01	
	EFFL	0.48 ± 0.00	-	0.61 ± 0.00	0.52 ± 0.02	0.46 ± 0.01	

River	Concentration [ng/L]																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	49.0	±	12.9	16.3	±	1.4	52.5	±	3.9	156.4	±	58.4	299.4	±	76.1	319.8	±	3.9
After WWTP 1	224.2	±	27.4	203.8	±	35.3	349.5	±	12.7	315.1	±	59.0	309.5	±	19.7	3048.5	±	0.7
Before WWTP 3	214.6	±	12.1	114.2	±	10.5	130.4	±	29.3	242.9	±	36.4	136.8	±	34.4	1311.8	±	86.6
After WWTP 3	111.0	±	2.1	123.9	±	0.0	451.6	±	85.1	599.7	±	67.2	603.9	±	106.4	1876.8	±	161.6
Before WWTP 6	-			747.0	±	297.0	1145.0	±	0.0	1129.8	±	0.0	1071.4	±	67.4	-		
After WWTP 6	-			-			1723.3	±	964.6	1402.0	±	0.0	2329.0	±	164.2	-		

River	Loads [g/day]																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	-			-			-			-			=			-		
After WWTP 1	-			-			-			-			=			-		
Before WWTP 3	57.7	±	3.3	202.3	±	18.6	17.8	±	4.0	37.6	±	5.6	18.1	±	4.5	95.2	±	6.3
After WWTP 3	30.9	±	0.6	220.7	±	0.0	65.2	±	12.3	96.3	±	10.8	83.8	±	14.8	145.1	±	12.5
Before WWTP 6	-			-			1021.3	±	0.0	868.2	±	0.0	823.3	±	126.2	-		
After WWTP 6	-			-			1678.5	±	939.5	1173.1	±	0.0	1300.2	±	137.4	-		

River	Enantiomeric fraction																	
	DECEMBER			JANUARY			FEBRUARY			MARCH			APRIL			AUGUST		
Before WWTP 1	0.46	±	0.11	0.46	±	0.09	0.49	±	0.03	0.56	±	0.09	0.48	±	0.01	0.47	±	0.03
After WWTP 1	0.48	±	0.02	0.46	±	0.05	0.43	±	0.01	0.46	±	0.01	0.41	±	0.00	0.43	±	0.00
Before WWTP 3	0.41	±	0.03	0.50	±	0.02	0.43	±	0.03	0.46	±	0.02	0.51	±	0.07	0.44	±	0.01
After WWTP 3	0.46	±	0.01	0.50	±	0.00	0.43	±	0.00	0.48	±	0.02	0.46	±	0.02	0.44	±	0.02
Before WWTP 6	-			0.46	±	0.02	0.46	±	0.03	0.43	±	0.00	0.39	±	0.00	0.38	±	0.03
After WWTP 6	-			0.47	±	0.03	0.46	±	0.01	0.43	±	0.00	0.39	±	0.00	0.39	±	0.00