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Abstract: Comprehensive study on the occurrence and fate of several classes of antimicrobials, including sulfonamides, trimethoprim, fluoroquinolones and macrolides, in Croatian municipal wastewaters was performed using an integrated approach, which comprised analysis of both dissolved and particulate fractions. A nation-wide screening showed ubiquitous occurrence of human-use antimicrobials in raw wastewater samples with the total concentrations ranging from 2 to 20 μg/L, while veterinary antimicrobials were typically present in much lower concentrations (<100 ng/L). The percentage of the particulate fraction in raw wastewater varied significantly depending on the type of the antimicrobial and the load of suspended solids. A detailed study of the mass flows of dissolved and particulate antimicrobials, performed in the wastewater treatment plant of the city of Zagreb, allowed an improved assessment of the biological and physico-chemical removal mechanisms of investigated compounds during the conventional activated sludge treatment. The overall removal efficiencies of antimicrobials from the water phase were rather variable, ranging from 0% for trimethoprim to 85% for norfloxacin. A significant percentage of fluoroquinolones (norfloxacin and ciprofloxacin) and macrolides (azithromycin and clarithromycin) was associated with the primary and excess secondary sludge, explaining 14 to 77% of the total removal. The removal, which could be attributed to biological transformation, was relatively poor for all antimicrobials, exceeding 30% only for SMX (32%) and clarithromycin (55%).

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Zagreb, 12 June 2012

Dear dr. Loosdrecht,

please find enclosed our manuscript on Occurrence and fate of dissolved and particulate antimicrobials in municipal wastewater treatment by Ivan Senta, Senka Terzic and Marijan Ahel, which we would like to be considered for publication in Water Research as a full length research article. The paper represents a comprehensive study on the occurrence and fate of several classes of antimicrobials in municipal wastewaters and provides new insights into the contributions of physico-chemical and biological processes to the overall removal of these compounds during the conventional wastewater treatment. We hope that you will find the manuscript suitable for publication in your journal.

The submitted files include the main text (6629 words), 6 tables, 3 Figures, Supplementary Information and Highlights.

Please send all further correspondence to me (ahel@irb.hr).

Sincerely,

Prof. Marijan Ahel

Occurrence and fate of dissolved and particulate antimicrobials in municipal wastewater treatment

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Highlights:

- Dissolved and particulate antimicrobials were studied in municipal wastewater
- Improved assessment of the removal mechanisms in sewage treatment was achieved
- A significant percentage of fluoroquinolones and macrolides was removed by sorption
- Removal that could be attributed to biological transformation was relatively poor

OCCURRENCE AND FATE OF DISSOLVED AND PARTICULATE ANTIMICROBIALS IN MUNICIPAL WASTEWATER TREATMENT

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Abstract

Comprehensive study on the occurrence and fate of several classes of antimicrobials, including sulfonamides, trimethoprim, fluoroquinolones and macrolides, in Croatian municipal wastewaters was performed using an integrated approach, which comprised analysis of both dissolved and particulate fractions. A nation-wide screening showed ubiquitous occurrence of human-use antimicrobials in raw wastewater samples with the total concentrations ranging from 2 to 20 μg/L, while veterinary antimicrobials were typically present in much lower concentrations (<100 ng/L). The percentage of the particulate fraction in raw wastewater varied significantly depending on the type of the antimicrobial and the load of suspended solids. A detailed study of the mass flows of dissolved and particulate antimicrobials, performed in the wastewater treatment plant of the city of Zagreb, allowed an improved assessment of the biological and physico-chemical removal mechanisms of investigated compounds during the conventional activated sludge treatment. The overall removal efficiencies of antimicrobials from the water phase were rather variable, ranging from 0% for trimethoprim to 85% for norfloxacin. A significant percentage of fluoroquinolones (norfloxacin and ciprofloxacin) and macrolides (azithromycin and clarithromycin) was associated with the primary and excess secondary sludge, explaining 14 to 77% of the total removal. The removal, which could be attributed to biological transformation, was relatively poor for all antimicrobials, exceeding 30% only for SMX (32%) and clarithromycin (55%).

Keywords: antimicrobials; fluoroquinolones; macrolides; sulfonamides; trimethoprim; wastewater treatment

1. Introduction

Pharmaceutical compounds represent one of the most important classes of emerging contaminants. In the last decade, they have been detected in different environmental compartments all over the world (Segura et al., 2009). Traces of pharmaceuticals in the environment raised concerns due to their high biological activity and there is an accumulating evidence in the literature that they can cause different adverse effects in non-target species (Fent et al., 2006).

Among different classes of pharmaceuticals, antimicrobial agents are under special scrutiny due to the possible formation of the resistant bacterial strains, which can pose a serious threat to the human health (da Costa et al., 2006). Antimicrobials represent the third biggest group among all pharmaceuticals in human medicine and the most prominent group in veterinary medicine (Thiele-Bruhn, 2003), with the estimated annual world consumption between 100,000 and 200,000 tons (Wise, 2002). Antimicrobials with the highest consumption are β-lactams, followed by tetracyclines, macrolides, fluoroquinolones and sulfonamides. However, due to the instability of the β-lactam ring, which is easily hydrolyzed, either chemically or microbiologically, penicilins and cephalosporins are not considered to be a potential threat for the environment (Cha, 2006). On the contrary, due to their comparatively higher stability, sulfonamides, fluoroquinolones and macrolides are widely recognised as important classes of environmental contaminants (Segura et al., 2009).

The most important global sources of antimicrobials in the environment are municipal wastewaters. After the intake, antimicrobials are only partially metabolised and their residues, excreted via urine or feces, reach municipal wastewater treatment plants (WWTPs). In addition, it was suggested that a significant percentage of pharmaceuticals in the sewage may derive from the illegal disposal of unused medications down the drain (Daughton and Ruhoy, 2009). Numerous reports throughout the world (Hirsch et al., 1999; Miao et al., 2004;

Lindberg et al., 2005; Karthikeyan and Meyer, 2006; Xu et al., 2007; Gulkowska et al., 2008; Watkinson et al., 2009; Zuccato et al., 2010; Gros et al., 2010; Gracia-Lor et al., 2012) showed that antimicrobials represent ubiquitous contaminants of municipal wastewaters. Recent study on the occurrence and fate of emerging contaminants in wastewaters of the Western Balkan Region, showed widespread occurrence of different classes of emerging contaminants, including many representatives of antimicrobials (Terzic et al., 2008).

Most of the literature reports suggested that the current design of conventional WWTPs does not assure a complete removal of pharmaceuticals, including several classes of antimicrobials (Castiglioni et al., 2006; Watkinson et al., 2007; Xu et al., 2007; Kasprzyk-Hordern et al., 2009; Gros et al., 2010). Moreover, treatment of municipal and industrial wastewaters in many less developed countries, including Croatia, is often incomplete and consists only of the mechanical treatment (Kastelan-Macan et al., 2007). As a consequence, a large percentage of pharmaceuticals introduced into WWTPs is released into the aquatic environment and pose a significant threat to the receiving ambient waters. Despite a growing number of studies on pharmaceutical in WWTPs, only few studies addressed the issue of particle bound antimicrobials (Göbel et al. 2005; Okuda et al., 2009; Jelic et al., 2011). The reason for that is a general perception that most of the pharmaceuticals belong to comparatively polar compounds, which are primarily expected to occur in the dissolved phase. Moreover, determination of organic micropollutants in the complex solid matrices is often analytically more challenging than their determination in the dissolved fraction. Nevertheless, comprehensive approach, which includes both dissolved and particulate fraction, is the key prerequisite for the accurate assessment of the pharmaceutical behaviour in the wastewater treatment and for understanding their ultimate fate in the aquatic environment.

The aim of this work was to investigate the occurrence of several important classes of antimicrobials, including sulfonamides, trimethoprim, fluoroquinolones and macrolides, in raw municipal wastewaters, and their fate in conventional wastewater treatment. Unlike most of the reports in the literature, our study involved simultaneous determination of these compounds in both dissolved and particulate fraction, thus providing a basis for a comprehensive assessment of the physico-chemical and biological removal processes.

2. Experimental

2.1. Target compounds

This study was focused on antimicrobials with the extensive usage in human medicine in Croatia, but some additional compounds, including several representatives of veterinary antimicrobials, and the main human metabolite of sulfamethoxazole *N*-acetylsulfamethoxazole, were also included. The list of all target compounds, together with their abbreviations and usage is presented in Table 1.

2.2. Sampling

All samples were collected in clean amber glass bottles pre-rinsed with methanol and ultrapure water. The nation-wide screening was performed in April and May 2005. Raw wastewater (RW) samples were collected in the largest Croatian cities (Table S1; Supplementary information). Additionally, where available, effluents from wastewater treatment plants were also collected. During this sampling campaign only a few cities (Bjelovar, Cakovec, Varazdin, Velika Gorica and Vinkovci) had facilities for full mechanical and biological treatment, while wastewaters of the cities of Rijeka and Split were treated only mechanically. For those cities, which did not have any wastewater treatment facilities, grab samples were collected directly from the sewerage system. In WWTPs of the cities Cakovec and Bjelovar 24-hour flow-proportional composite samples were collected using automatic devices, while on some other locations (Rijeka, Split, Varazdin) composite samples were prepared by mixing grab samples taken over a diurnal cycle.

A detailed study on the occurrence and fate of antimicrobials during conventional wastewater treatment was performed in the central WWTP of the city of Zagreb, which is fully operational since 2007. This WWTP receives a combined municipal and industrial sewage from the entire city and includes full mechanical and biological treatment based on conventional activated sludge (CAS) treatment. It has a designed capacity of 1000000 population equivalents and currently serves about 750,000 inhabitants (Schröder et al., 2001). The average hydraulic load of raw wastewater is about 250,000 m^3/day. The details about the WWTP are summarized in Table S2 (Supplementary information). During the study in this WWTP, several sampling campaigns in the period from March to September 2009 were performed, in which twenty-four-hour composite samples of both RW and biologically treated wastewater (secondary effluent, SE) were collected. In addition, for the investigation of the partitioning behaviour of the selected antimicrobials during CAS treatment, activated sludge samples were taken directly from the aeration tank.

2.3. Sample pre-treatment and instrumental analysis

All samples were filtered through 2.7 μm glass fiber filters (GF/D, Whatman, USA) immediately after being brought back to the laboratory, typically within 3 hrs after sampling. Filtrates were stored in the dark at 4 °C and extracted as soon as possible, typically within 24 hours. Filters containing particulate fraction were kept frozen at -18 ºC until the extraction. Dissolved fraction was enriched using solid-phase extraction (SPE) on Oasis HLB cartridges using the protocol described in our previous paper (Senta et al., 2008). Briefly, samples were acidified with formic acid to obtain pH 3. After percolation of the samples, adsorbed analytes were eluted from the cartridges with 4 mL of 1% ammonia solution in methanol. After evaporation to dryness, residue was dissolved in 0.1% formic acid just before the instrumental analysis. Absolute recoveries of individual antimicrobials from wastewater samples were between 49% and 119%, with good repeatabilities (1% – 18%).

For the determination of antimicrobials incorporated in particulate fraction, pressurized solvent extraction (PLE) followed by subsequent extract cleanup on Oasis HLB columns were used (Senta, 2009). Briefly, samples were extracted using PLE with the mixture of 50 mM *o*-phosphoric acid and methanol (50:50). Temperature was 100 °C and pressure was 138 bars. 3 cycles were performed with static time of 5 minutes. Preheating time was 5 minutes and flush volume 120%. PLE extracts were diluted to approximately 300 mL with ultrapure water and additionally extracted on Oasis HLB cartridges using the same protocol as for the dissolved fraction. Absolute recoveries for the analysis of the particulate antimicrobials were between 35% and 65%, with repeatabilities between 1% and 12%. The analyte losses during the extraction procedures and extract work-up, as well as matrix effects, were compensated by using several surrogate standards (at least one for each antimicrobial class), which were added in the samples prior the extraction (SPE or PLE). Instrumental standards sulfamerazine and josamycin were added in the final extracts just prior to analysis as a control of instrument performance.

Our previously described method (Senta et al., 2008) based on liquid chromatography – tandem mass spectrometry (LC-MS/MS) was used for determination of the selected antimicrobials. Target compounds were separated on C_{18} column in reversed-phase system using gradient elution with water and methanol both acidified with 0.1% of formic acid. The mass spectrometric analyses were performed on a TSQ Quantum triple quadrupole instrument (Thermo Electron, San Jose, USA). Electrospray ionization in positive mode was used for the production of ions. Detection and quantification of all analytes were performed using selected reaction monitoring (SRM). Instrumental detection limits were between 1 and 17 pg, while method detection limits were between 1.0 and 13.2 ng/L and 0.5 and 6.6 ng/L for raw wastewater and secondary effluent samples, respectively. The original method was later slightly modified in order to include deuterated azithromycin as a surrogate standard for azithromycin and other macrolides, which significantly improved the reliability of quantification of these compounds in heavily loaded wastewater samples. Furthermore, during the study performed in the Zagreb WWTP, the major metabolite of SMX *N*-acetylsulfamethoxazole, was also included in the method.

In addition, ultrahigh performance liquid chromatography (UHPLC, Waters, USA) coupled to quadrupole-time of flight mass spectrometry (QToF MS; Micromass, Manchester, UK) was applied in this study for the confirmation purposes, such as confirmation of the presence of erythromycin and *N*-acetyl derivatives of sulfonamides.

3. Results and discussion

3.1. Screening of antimicrobials in Croatian municipal wastewaters

Occurrence of antimicrobials in raw municipal wastewater samples collected during the nation-wide campaign in Croatia is presented in Table 2 (dissolved fraction) and Table 3 (particulate fraction). As can be seen, several antimicrobials were detected in all of the analysed samples. The most prominent compounds in the dissolved fraction were SMX, TMP, NOR, CIP, AZI, SPY and ERY-H_2O. The concentrations of major antimicrobials were typically found in concentration range between 100 and 1000 ng/L, with only few exceptions reaching into in the low ug/L range. The highest individual concentration was determined for SMX in the wastewater of Novi Zagreb (11.6 μg/L). These results show a strong predominance of antimicrobials used in the human medicine, while typical veterinary antimicrobials belong to the minor components. The observed levels of antimicrobials for human use are in a good agreement with the official reports on the consumption figures for top prescribed human-use antimicrobials in Croatia (Ferech et al., 2006). The only exception is SPY, which is rarely used as antimicrobial agent for human use. However, SPY is the main metabolite of sulfasalazine, which has a significant usage in human medicine in Croatia. ERY-H_2O is the metabolite and the degradation product of macrolide antibiotic erythromycin and represents its strongly predominant form in raw wastewater and wastewater effluents (Terzic et al., 2008). The results of preliminary screening are in a good agreement with the reports on the occurrence of antimicrobials in raw municipal wastewaters in other countries (Göbel et al., 2005; Lindberg et al., 2005; Watkinson et al., 2009; Zuccato et al., 2010, Gros et al., 2010), with some differences which can be explained with the different local consumption patterns. For example, relatively high concentrations of AZI in Croatian wastewaters can be explained with the fact that this antibiotic is among the top-prescribed pharmaceuticals in Croatia. On the other hand, ROX, which is frequently detected macrolide antibiotic in wastewaters (Göbel et al., 2004; Xu et al, 2007), was rarely detected in our samples due to its very limited usage in Croatia.

In contrast to human-use antimicrobials, compounds used exclusively in veterinary medicine (STZ, SMZ, ENR) were detected only occasionally and in much lower concentrations (usually below 100 ng/L). This was actually expected since this study was focused on municipal wastewaters, characterised by low inputs from agricultural sources. The only exception was the city of Belisce, located in an area characterised with the extensive livestock production. As a consequence, wastewater from this city contained enhanced concentration of veterinary antimicrobial SMZ (175 ng/L).

The most prominent antimicrobials associated with the particulate fraction were generally the same as in the dissolved fraction, with the exception of SPY, which could not be quantitatively determined in the particulate fraction (Table 3). However, distribution of antimicrobials in the particulate fraction was somewhat different than the distribution in the dissolved fraction, which can be explained by their different physico-chemical properties (Table 1). The percentage of antimicrobials associated with the particulate fraction, ranged from 1% for sulfonamides up to 35% for norfloxacin. The concentration of fluoroquinolones and azithromycin, expressed as mass fraction in the total suspended solids exceeded 1 µg/g (Table S3, Supplementary information).

The concentrations of individual antimicrobials in secondary effluents were generally of the same order of magnitude as their concentrations in raw wastewater, collected at the same location, indicating their incomplete removal during conventional wastewater treatment (Table S4, Supplementary information). Incomplete removal was observed even in the WWTPs with the full mechanical and biological treatment (Cakovec, Bjelovar), which emphasized recalcitrant nature of investigated compounds. It should be noticed that for the effluent samples only the concentration in the dissolved fraction was determined.

3.2. Occurrence of antimicrobials in WWTP of the city of Zagreb

Detailed study on the occurrence and fate of antimicrobials during conventional wastewater treatment was performed in the central WWTP of the city of Zagreb during 2009, after this WWTP became fully operational, including both mechanical and biological treatment. In three sampling campaigns 36 RW and 34 SE samples were collected. Summary of the results for the dissolved fraction of RW is presented in Table 4. As can be seen, 9 compounds were detected in all analyzed samples and the most prominent antimicrobials were generally the same as in the preliminary screening of the Croatian wastewaters. In addition, *N*-Ac-SMX, the most prominent metabolite of SMX (Baselt, 2008) and macrolide antibiotic CLA were also included in the method during this study. The highest average concentration in RW was determined for *N*-Ac-SMX (656±87 ng/L), followed by AZI

(502±315 ng/L), SMX (484±189 ng/L) and fluoroquinolones NOR (339±181 ng/L) and CIP (333±197 ng/L). The average concentrations of SPY, TMP and CLA were also higher than 100 ng/L.

The most prominent compounds in the particulate fraction of RW samples from the Zagreb WWTP (Table 5) were NOR, CIP, AZI and CLA with the concentrations ranging from several hundreds ng/L to a few µg/L. Other human-use antimicrobials or their metabolites were also present, but in much lower concentrations, typically below 50 ng/L. As can be seen in Fig. 1A, the contributions of the particulate antimicrobials to the total concentrations in RW varied in a very wide range (from 0.4% to 47 %). All sulfonamides and trimethoprim were characterised by a strong predominance in the dissolved fraction (>95%). On the contrary, the percentage of particulate macrolides and fluoroquinolones was significant and varied from 7% for ERY-H_2O to 47% for NOR. As a consequence, ignoring contribution of their particulate fraction in raw wastewater, can lead to significant underestimation of the real input for these antimicrobials into a WWTP.

3.3. Partitioning of antimicrobials during wastewater treatment

Behaviour of organic contaminants during the conventional wastewater treatment can be significantly affected by physico-chemical processes, in particular sorption onto suspended solids (Ternes et al., 2004). Simultaneous determination of antimicrobials in dissolved and particulate fractions provided a basis for the determination of their distribution coefficients (K_d) and, consequently, the assessment of relative importance of the particulate fraction, which is usually overlooked in the studies of the occurrence and fate of antimicrobials in wastewater treatment. In this study we estimated the average distribution coefficients (K_d) of individual antimicrobials in two typical compartments of wastewater treatment, raw wastewater and mixed liquor from the aeration tank, using the following formula:

$$Kd = \frac{Cs}{Cd * SS}$$

where *Cs* is the concentration of the compound in the particulate fraction (suspended solids in RW or activated sludge in aeration tank) (μg/L), *Cd* is concentration of the compound in the dissolved fraction (μg/L) and *SS* is suspended solid concentration in raw wastewater or activated sludge concentration in the aeration tank (Ternes et al., 2004). The results are presented in Table 6. It should be noticed that sorption coefficients were calculated only for the major antimicrobials, which could be quantitatively determined in both fractions. Moreover, K_d values for RW shown in Table 6, represent an average value from 31 determinations, except for *N*-Ac-SMX (n = 18), while the average value for the aeration tank was calculated from 2 independent experiments. As expected, distribution coefficients of investigated antimicrobials varied in a broad range, from 16 L/kg for *N*-Ac-SMX to 6959 for NOR. It should be noted that K_d values are not positively correlated with octanol-water partition coefficients (Table 1; Fig.S1), indicating that mechanisms other that lipophilic partitioning played a predominant role for the strongly adsorbable antimicrobials (Okuda et al., 2009). However, it is well-known that these coefficients can be strongly affected by the type, composition and pH of the activated sludge (Ternes et al., 2004).

Calculated distribution coefficients for fluoroquinolones (NOR and CIP) were somewhat lower than the values reported by Golet et al. (2002), but were significantly higher than those for the other antimicrobial groups. As mentioned above, these enhanced values were not in correlation with their log K_{ow} values, indicating the predominant role of polar and/or ionic interactions for the adsorption process. Some authors suggested that sorption of these compounds onto the activated sludge may be accomplished by the electrostatic interactions with the cell membranes of the microorganisms forming activated sludge (Xu et al., 2007).

Similarly, it seems that pH-dependent speciation of sulfonamides and trimethoprim in wastewater played a role in their partitioning. At the pH, typically found in WWTP of the city of Zagreb (7.11−7.94), SMX (p$K_{a,2}$=5.6) is mainly present in the anionic form, while for trimethoprim (p$K_{a,2}$=6.8) and especially sulfapyridine (p$K_{a,2}$=8.4) non-ionic species are much more abundant. The results suggested a more efficient adsorption of non-ionic species. However the differences between K_d values for individual sulfonamides and TMP cannot be explained by their lipophilicity expressed as log K_{ow} values, indicating importance of polar interactions.

Unlike other antimicrobials in this study, macrolides are present in wastewater mainly as positively charged species (p$K_{a,1}$=8.7−8.9). Sorption coefficients for AZI (K_d=486 and 2156) and CLA (K_d=386 and 636) were found to be higher than sorption coefficients for sulfonamides, but lower than for fluoroquinolones. The K_d values determined in this study are in a good agreement with the literature data (Göbel et al., 2005). Higher sorption coefficient for AZI as compared with CLA is supported by its higher log K_{ow} value (4.0). Moreover, it should be taken into account that AZI has two basic sites (nitrogen atoms), while CLA has only one basic site. Such properties could promote the ionic interactions with the negatively charged suspended matter present in wastewater. The distribution coefficient for ERY-H_2O was significantly lower than the sorption coefficients for the other two macrolides, so that the concentration of this compound in the particulate fraction was usually close to or below the quantification limit. At this stage, we cannot find a plausible explanation for such behaviour.

It is generally expected that the percentage antimicrobials in the particulate fraction should be correlated with the concentration of suspended solids. In our samples this relationship was proven to be statistically significant for sulfonamides, trimethoprim and macrolides, but not for fluoroquinolones (Fig S2, Supplementary information). This suggests that for fluoroquinolones the variable character of particulate organic matter in RW must have played a significant role.

Taking into account a considerable variability of K_ds for all of the investigated antimicrobials among individual samples, it is interesting to note that the average K_d values for RW suspended solids and activated sludge were reasonably similar with no obvious trend regarding relationship between the two solid phases. However, it should be noted that the sorption coefficients for macrolides in mixed liquor were significantly lower than those in RW. Nevertheless, as a consequence of high concentration of suspended solids in the aeration tank (3.5 g/L), the percentage of particle-bound antimicrobials in mixed liquor is extremely high (Fig 1B). For example, more than 90% of fluoroquinolones in the aeration tank were associated with mixed-liquor suspended solids (MLSS). Even for the compounds with relatively low K_d values, such as SMX and TMP, the percentage of antimicrobials in the particulate fraction was 45 and 70%, respectively. However these percentages represent only a snapshot of the partitioning process in the aeration tank and should not be interpreted as the removal efficiencies because only the excess sludge contributes to the removal as it will be discussed below.

3.4. Occurrence in secondary effluents and assessment of removal mechanisms

The average concentrations of antimicrobials in secondary effluents of the Zagreb WWTP are presented in Table 4 along with the RW concentrations. It should be noted that only dissolved antimicrobials were determined in secondary effluents, since their estimated concentration in the particulate phase was less than 2% for all target analytes, including highly adsorbable fluoroquinolones, due to the very low concentration of suspended solids in SE (typically below 5 mg/L; Table S2; Supplementary information). As can be seen, all major antimicrobials found in RW were detected in all analysed SE samples indicating their incomplete removal. The most prominent compounds in SE were AZI (350±206 ng/L), SMX (323±135 ng/L) and *N*-Ac-SMX (214±177 ng/L).

The average removal efficiencies, presented in Fig. 2, were calculated using two different approaches. In the first approach, which is common in the literature (Watkinson et al., 2007; Vieno et al., 2007; Kasprzyk-Hordern et al., 2009; Gros et al., 2010), the removal efficiencies of individual antimicrobials were calculated by comparing secondary effluent concentrations with the input concentrations in the dissolved phase (Table 4). However, this approach can lead to significant errors for the compounds having high tendency for the sorption onto the solid particles (Deo and Halden, 2010). Along these lines, the overall removal efficiencies were alternatively calculated using a more realistic approach taking the total concentration in RW as a basis. The overall elimination varied significantly among different classes of antimicrobials. As expected, removal efficiencies using the second approach were significantly higher for strongly adsorbable compounds.

The highest removal rates (more than 80% using the second approach) were achieved for fluoroquinolones and they are in good agreement with most of the previously reported values for this group of antimicrobials (Golet et al., 2002; Lindberg et al., 2005; Castiglioni et al., 2006; Vieno et al. 2007; Watkinson et al., 2007; Xu et al., 2007).

It should be pointed out that literature data on the removal efficiencies for sulfonamides and macrolides are not as consensual as for fluoroquinolones. For example, the average removal rates for sulfonamides SMX and SPY in our study were 34%. This is comparable with the removal efficiency for SMX in Swedish WWTPs (Lindberg et al., 2005) and in WWTPs in the South China (Xu et al., 2007). However, Göbel et al. (2007) reported low and highly variable removal rates for both SMX and SPY during the secondary treatment in two Swiss WWTPs. In fact, concentrations of SMX and SPY in their study were up to two times higher in SE than in primary effluent, leading to the negative removal rates. Authors suggested that such results could be explained with the re-transformation of sulfonamide metabolites during the wastewater treatment. Our study confirmed the importance of *N*-Ac-SMX for the total load of SMX derived compounds, and the calculated removal efficiency for *N*-Ac-SMX (77%) was comparable with the removal rates reported by Göbel et al. (2007). Consequently, it is possible that re-transformation of this metabolite affected the overall removal rate for SMX, but not to such extent that would have lead to a negative elimination. It is reasonable to assume that similar mechanism could apply for SPY as well, however our attempt to identify *N*-acetylsulfapyridine in our wastewater extracts using UPLC-QToF technique, indicated no significant presence of this metabolite.

Regarding macrolides, the highest removal efficiency was observed for the macrolide antibiotic CLA (69%), which is in a very good agreement with the value reported by Zuccato et al. (2010). The removal efficiencies for the other two investigated macrolides, AZI and ERY-H_2O were significantly lower. Low removal efficiencies for macrolides, including observations on the negative elimination, have already been reported in the literature (Göbel et al., 2007; Xu et al., 2007, Gulkowska et al., 2008; Gros et al., 2010). Since the presence of de-conjugable metabolites of these compounds (Baselt, 2008) was never reported in the literature, some authors suggested that low removal rates could be explained with the fact that significant portion of these compounds are excreted from the body via bile and feces, so it is possible that overall content of these compounds that enters wastewater treatment plant is underestimated (Göbel et al., 2007). This assumption, however, was never experimentally verified. The results from this study showed that 30% of AZI in RW was adsorbed onto the solid particles. It should also be pointed out that the overall removal efficiency for AZI, based on the total concentration in RW was 51%, while the removal efficiency of ERY-H_2O was even lower (24%).

Virtually, no removal was observed for TMP. Other authors also reported low removal efficiencies for this compound, including some negative values as well (Lindberg et al., 2005; Göbel et al., 2007; Gulkowska et al., 2008; Gracia-Lor et al., 2012). It should be noted that 97% of TMP is excreted from the human body in the urine, mostly (80% – 90%) unchanged (Baselt, 2008), so that it is very difficult to speculate about possible reasons for the negative removal.

The overall removal is the result of combined biological and physicochemical elimination. In order to decouple this two mechanisms a simple mass balance was made by determining mass flows of major antimicrobials in the dissolved and particulate phase. The removal particle-bound fraction consisted of adsorption onto the primary sludge (150 g m^{-3}) and the adsorption onto the excess sludge (158 g m^{-3}). The result for the most abundant representatives of antimicrobials SMX, NOR and AZI are presented in Fig. 3, while for the other antimicrobials the results are shown in the supplementary information (Fig. S3; Supplementary information). The physico-chemical elimination was assessed from the mass flows in the primary sludge and excess activated sludge, assuming that the contribution of other physico-chemical processes, such as volatilization, was negligible (Senta et al., 2011). The physico-chemical removal varied from 2% for SMX to 78% for NOR and reflects the partitioning behaviour described above. The results are generally in agreement with the literature reports, which suggested that for compounds with sorption coefficients below 300 L/kg, sorption onto the secondary sludge particles is not relevant for their elimination (Joss et al., 2005).

The contribution of biological elimination was calculated as the difference between the overall elimination and physico-chemical elimination. The lowest biological elimination was obtained for TMP (-7%), suggesting its possible formation from yet unknown precursors. Moreover, low biological elimination of fluoroquinolones (8 − 22%) clearly confirmed earlier literature reports on the strong predominance of physico-chemical partitioning in their removal during the wastewater treatment (Lindberg et al., 2005). Compared to fluoroquinolones, the biological removal of sulfonamides and macrolides was more efficient however even for the most biodegradable compound CLA it reached only 55%.

Conclusion

This study showed a widespread occurrence of antimicrobials in raw municipal wastewater in Croatia, with strong predominance of human use antibiotics. Separate analysis of the dissolved and particulate fractions allowed an improved assessment of the biological and physico-chemical removal mechanisms of investigated compounds during the conventional activated sludge treatment. The results pointed out the importance of the sorption onto solid particles, especially for fluoroquinolones and macrolides. It was shown that the partitioning behaviour of antimicrobials cannot be predicted on the basis of simple parameters such as log K_{ow}, which warrants careful study of their mass flows in the wastewater treatment for each individual compound. The biological elimination in the conventional activated sludge treatment was rather modest for most of the antimicrobials, indicating that for a more efficient removal advanced treatment technologies have to be considered.

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Figure captions

Fig. 1 Percentage of antimicrobials associated with suspended solids in the WWTP of the city of Zagreb: (A) raw wastewater and (B) mixed liquor from aeration tank. SPY = sulfapyridine; SMX = sulfamethoxazole; N-Ac-SMX = *N*-acetylsulfamethoxazole; TMP = trimethoprim; NOR = norfloxacin; CIP = ciprofloxacin; AZI = azithromycin; ERY-H2O = dehydroerythromycin; CLA = clarithromycin.

Fig. 2 Overall removal efficiencies of antimicrobials in the WWTP of the city of Zagreb determined a) based on dissolved fraction only (1^{st} approach); b) based on the total concentration in raw wastewater (2^{nd} approach). SPY = sulfapyridine; SMX = sulfamethoxazole; *N*-Ac-SMX = *N*-acetylsulfamethoxazole; TMP = trimethoprim; NOR = norfloxacin; CIP = ciprofloxacin; AZI = azithromycin; ERY-H_2O = dehydroerythromycin; CLA = clarithromycin.

Fig. 3 Mass flows of dissolved and particulate antimicrobials in WWTP of the city of Zagreb and assessment of the biological elimination; d – dissolved fraction; p – particulate fraction; PC – primary clarifier; SC – secondary clarifier; RW – raw wastewater; SE – secondary effluent; PS – primary sludge; ES – excess sludge; SMX - sulfamethoxazole; NOR – norfloxacin; AZI – azithromycin.

Table 1
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Table 1. List of antimicrobials included in the present study along with their physico-chemical properties[1].

Antimicrobial class	**Analyte**	**Acronym**	**Usage**	**log K_{ow}**	**p$K_{a,1}$**	**p$K_{a,2}$**
Sulfonamides	Sulfadiazine	SDZ	Human + veterinary	-0.1	2.1	6.4
	Sulfathiazole	STZ	Veterinary	0	2.0	7.1
	Sulfapyridine	SPY	Human	0.4	2.6	8.4
	Sulfamethazine	SMZ	Veterinary	0.3	2.1	7.5
	Sulfamethoxazole	SMX	Human	0.9	1.9	5.6
	N-acetylsulfamethoxazole	*N*-Ac-SMX	Metabolite of SMX	1.5	5.0	-
	Trimethoprim[2]	TMP	Human + veterinary	0.9	3.2	6.8
Fluoroquinolones	Norfloxacin	NOR	Human	-1.0	3.1	8.6
	Ciprofloxacin	CIP	Human	0.3	3.0	8.7
	Enrofloxacin	ENR	Veterinary	0.9	3.9	7.6
Macrolides	Azithromycin	AZI	Human	4.0	8.7	9.5
	Dehydroerythromycin	ERY-H_2O	Human + veterinary[3]	3.0[4]	8.9[4]	-
	Clarithromycin	CLA	Human	1.8	8.9	-
	Roxithromycin	ROX	Human	nd	9.2	-

[1]Data on physico-chemical properties are from references: Vazquez et al., 2001; Göbel et al., 2004; Qiang and Adams, 2004; Batt and Aga, 2005; Stoob et al., 2005; Diaz-Cruz et al., 2006; Vieno et al, 2006; Choi et al., 2007; Gros et al., 2007; Sibley and Pedersen, 2008.

[2] used in combination with sulfonamides; [3] metabolite of erythromycin (ERY); [4] data for ERY

Table 2
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Table 2. Occurrence of antimicrobials in Croatian raw municipal wastewaters in April and May 2005 – dissolved fraction (concentrations in ng/L).

Location	SDZ	STZ	SPY	SMZ	SMX	TMP	NOR	CIP	ENR	AZI	ERY-H_2O	ROX
Belisce	12	nd	97	175	613	35	nd[2]	nd	nd	107	147	nd
Bjelovar	92	nd	385	nd	826	365	1231	196	16	122	66	nd
Cakovec	5	nd	370	nd	293	481	341	149	nd	281	58	nd
Karlovac	23	nd	364	nd	735	758	1843	169	nd	992	407	50
Novi Zagreb	29	4	809	7	11555	2551	2885	777	nd	799	122	nd
Osijek	16	nd	99	nd	1184	1817	1711	1079	nd	778	420	nd
Rijeka	15	nd	732	nd	1094	1045	1282	161	nd	352	171	nd
Sisak	96	nd	89	15	858	347	72	5	8	77	99	nd
Slavonski Brod	2	nd	292	nd	387	588	94	nd	nd	173	24	nd
Split	7	nd	567	nd	675	776	501	114	nd	482	213	nd
Split - sewer center	12	nd	507	nd	829	1068	1300	532	nd	1066	149	nd
Varazdin	132	1	376	2	640	3442	2009	496	18	1025	187	nd
Velika Gorica	28	nd	560	15	1944	2706	894	489	nd	501	275	nd
Vinkovci	12	nd	923	nd	943	659	1186	217	nd	317	48	nd
Zadar	8	nd	931	2	2033	2318	2937	2610	nd	1139	127	13
Zagreb	36	nd	313	17	720	840	581	227	nd	801	61	nd

nd – not detected

Table 3
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Table 3. Occurrence of antimicrobials in Croatian raw municipal wastewaters – particulate fraction (concentrations in ng/L).

Location	SMX	TMP	NOR	CIP	AZI	ERY-H_2O
Bjelovar	1.7	3.4	400	48	73	1.3
Cakovec	nd	1.4	147	21	66	0.7
Karlovac	3.6	2.9	278	40	41	0.9
Novi Zagreb	58	15	680	166	93	0.9
Osijek	6.2	5.0	202	111	37	5.2
Rijeka	9.3	23	949	139	29	2.2
Sisak	3.4	6.1	113	9.3	1.3	1.7
Slavonski Brod	3.8	22	852	67	31	1.5
Split	18	38	492	116	56	nd
Split - sewer center	6.6	12	547	152	22	1.2
Varazdin	1.1	11	22	4.6	76	nd
Velika Gorica	6.5	12	196	56	63	3.5
Vinkovci	16	52	4380	491	36	20
Zadar	21	47	1294	1046	47	1.2
Zagreb	23	16	159	28	67	1.7

nd – not detected

Table 4
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Table 4. Occurrence of antimicrobials in the wastewater treatment plant of the city of Zagreb – dissolved fraction.

	Raw wastewater				Secondary effluent			
Analyte	**n[1]**	**FD[2] (%)**	**Concentration (ng/L)**		**n[1]**	**FD[2] (%)**	**Concentration (ng/L)**	
			Range	**Average ± SD**			**Range**	**Average ± SD**
SPY	36	100	80 – 442	244 ± 111	34	100	48 – 288	161 ± 73
SMX	36	100	210 – 999	484 ± 189	34	100	119 – 544	323 ± 135
N-Ac-SMX	18	100	427 – 805	656 ± 87	17	100	14 – 486	214 ± 177
TMP	36	100	87 – 219	148 ± 34	34	100	75 – 245	155 ± 49
NOR	36	100	60 – 634	339 ± 181	34	100	24 – 175	97 ± 53
CIP	36	100	29 – 650	333 ± 197	34	100	11 – 168	87 ± 55
AZI	36	100	122 – 1634	502 ± 315	34	100	38 – 784	350 ± 206
ERY-H_2O	36	100	25 – 73	44 ± 12	34	100	15 – 59	36 ± 12
CLA	36	100	112 – 300	197 ± 61	34	100	25 – 133	71 ± 34

[1] Number of analyzed samples; [2] Frequency of detection

Table 5
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Table 5. Occurrence of antimicrobials in the municipal raw wastewater of the city of Zagreb – particulate fraction.

Analyte	**n**[1]	**FD**[2] **(%)**	**Concentration (ng/L)**		**Concentration (ng/g)**[3]	
			Range	**Average ± SD**	**Range**	**Average ± SD**
SPY	31	100	0.7 – 15.8	6.0 ± 3.5	2.1 – 49.4	23.1 ± 8.9
SMX	31	100	2.2 – 15.9	5.5 ± 2.9	8.1 – 41.6	21.1 ± 7.7
N-Ac-SMX	31	100	0.6 – 10.1	3.2 ± 2.1	1.9 – 26.5	12.5 ± 6.9
TMP	31	100	0.3 – 19.6	6.0 ± 4.4	0.8 – 52.8	22.2 ± 11.4
NOR	31	100	35 – 1924	324 ± 340	101 – 3026	1169 ± 630
CIP	30	100	22 – 887	180 ± 158	64 – 1395	670 ± 302
AZI	31	100	35 – 700	206 ± 149	103 – 1978	819 ± 409
ERY-H_2O	31	97	nd – 12.4	3.1 ± 2.7	nd – 38.6	12.0 ± 9.2
CLA	31	100	0.4 – 137	33.6 ± 31.9	1.2 – 322	125 ± 87.8

[1] Number of analyzed samples; [2] Frequency of detection; **[3]** Expressed as mass fraction in suspended solids;

Table 6. Distribution coefficients (K_d) of the selected antimicrobials in raw wastewater and aeration tank mixed liquor.

Compound	**Raw wastewater**	**Mixed liquor (aeration tank)**
	K_d **(L/kg)**[1]	K_d **(L/kg)**[2]
Sulfapyridine	132	454
Sulfamethoxazole	53	111
N-Acetylsulfamethoxazole	16	nd
Trimethoprim	163	316
Norfloxacin	6959	5974
Ciprofloxacin	4875	3934
Azithromycin	2156	486
Dehydrated erythromycin	325	114
Clarithromycin	636	386

K_d values were calculated as the ratio between the concentration of sorbed (in ng/kg) and dissolved (in ng/L) antimicrobials. [1] average of 31 independent determinations in 24-hr composite samples of raw wastewater; [2] measured in grab samples from the aeration tank − average of 2 independent determinations.

Figure 1

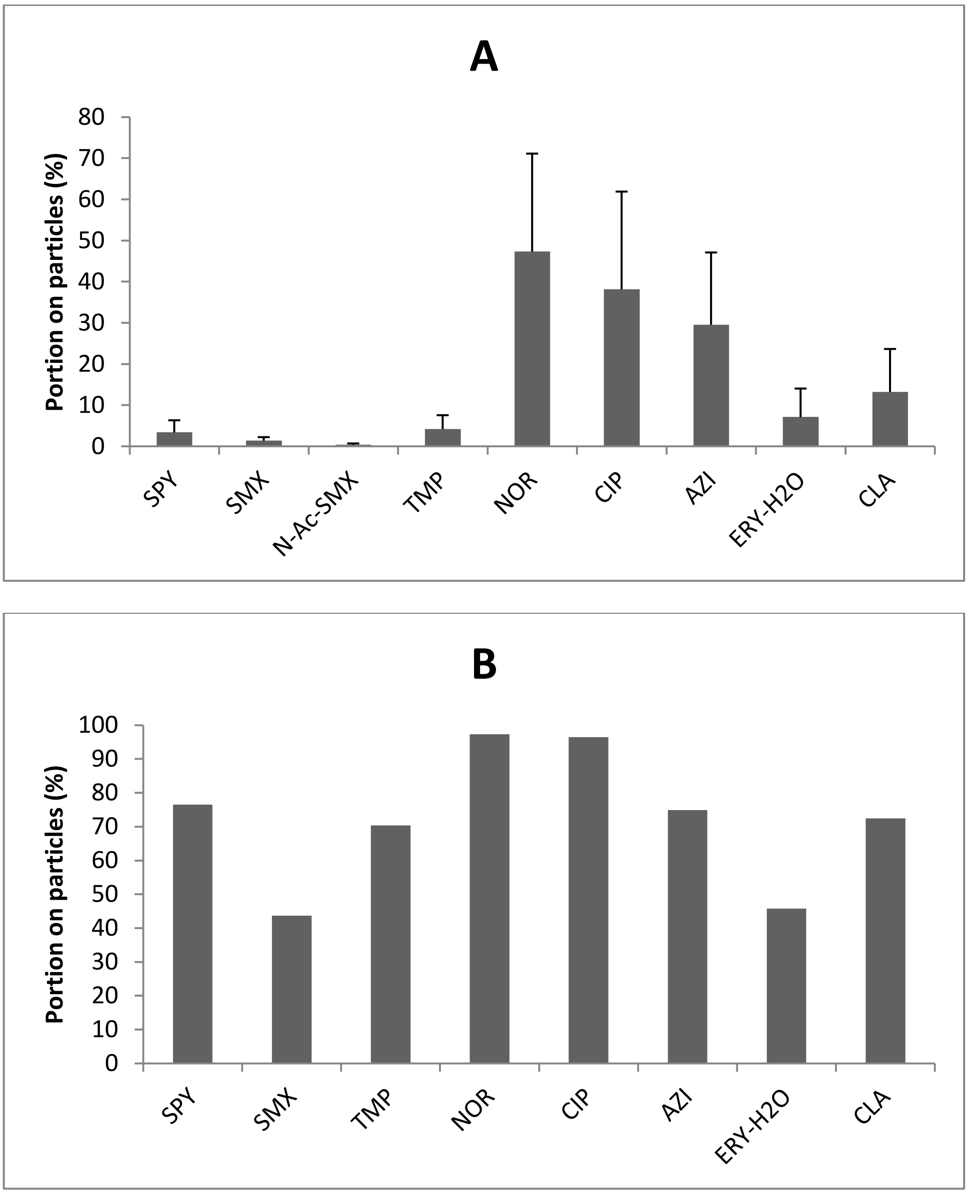

Fig. 1 Percentage of antimicrobials associated with suspended solids in the WWTP of the city of Zagreb: (A) raw wastewater and (B) mixed liquor from aeration tank. SPY = sulfapyridine; SMX = sulfamethoxazole; N-Ac-SMX = *N*-acetylsulfamethoxazole; TMP = trimethoprim; NOR = norfloxacin; CIP = ciprofloxacin; AZI = azithromycin; ERY-H2O = dehydroerythromycin; CLA = clarithromycin.

Figure 2

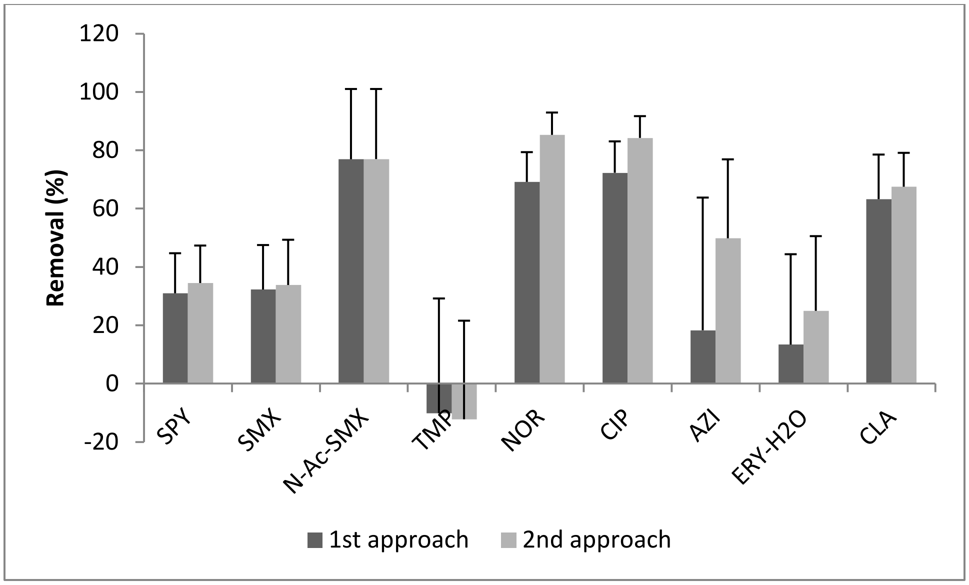

Fig. 2 Overall removal efficiencies of antimicrobials in the WWTP of the city of Zagreb determined a) based on dissolved fraction only (1st approach); b) based on the total concentration in raw wastewater (2nd approach). SPY = sulfapyridine; SMX = sulfamethoxazole; N-Ac-SMX = N-acetylsulfamethoxazole; TMP = trimethoprim; NOR = norfloxacin; CIP = ciprofloxacin; AZI = azithromycin; ERY-H2O = dehydroerythromycin; CLA = clarithromycin.

Figure 3

Fig. 3