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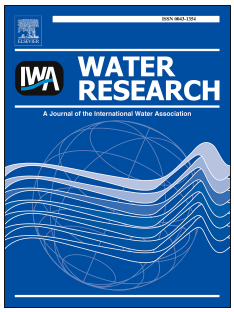

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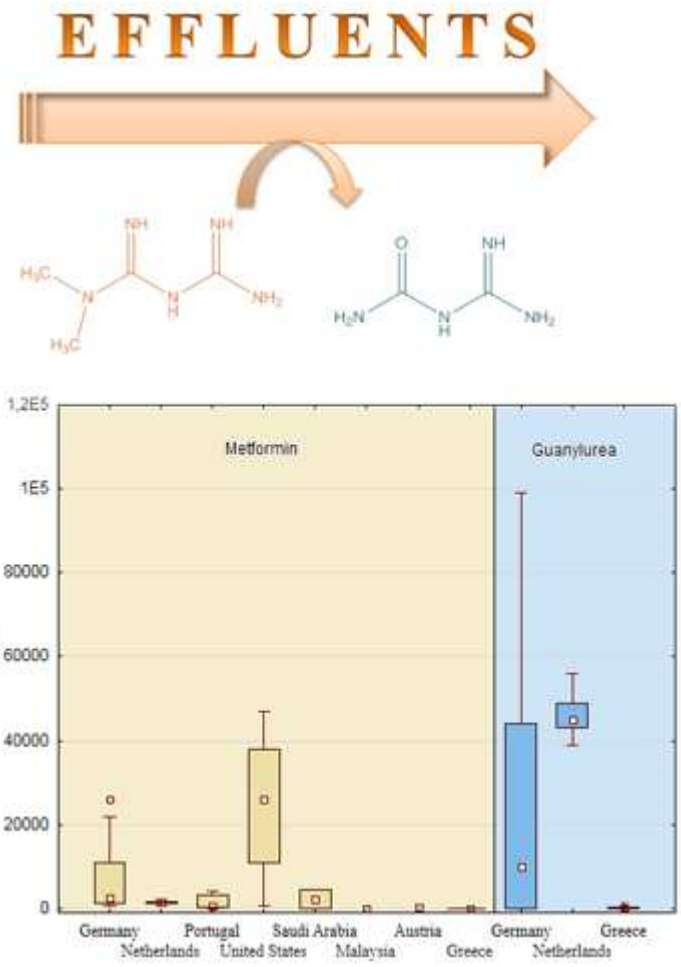

Comprehensive Study of the Antidiabetic Drug Metformin and its Transformation Product Guanylurea in Greek Wastewaters

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A B S T R A C T

Many pollutants such as pharmaceuticals and their transformation products (TPs) are not efficiently removed from wastewater treatment plants and enter into surface waters. The aim of this study was to investigate the occurrence and behavior of metformin, one of the most prescribed drugs worldwide, and its biological transformation product guanylurea, in eight wastewater treatment plants (WWTPs) of Greece. All WWTPs were equipped with conventional activated sludge treatment and the samples were taken from the influents and the effluents, over the four seasons of one year. The analytical method developed based on SPE followed by LC-UV/Vis-ESI/MS analysis, while positive findings were confirmed also by means of LTQ Orbitrap mass spectrometer. High polarity of both compounds led to the extraction with Oasis HLB and the use of the anionic surfactant SDS. The results showed that metformin dominated in the influents (bql-1167 ng/L), while guanylurea in the effluents (bql-627 ng/L) of the wastewater treatment plants, with Metformin/Guanylurea ratio ranging between 0.88 and 81.3 in the influents and between 0.005 and 0.78 in the effluents. Lack of a clear seasonal tendency in the occurrence and removal or formation was observed. Finally, an ecotoxicological risk assessment of metformin in effluent wastewaters took place by calculating the ratio between the environmental concentrations (MEC) and the predicted no effect concentrations (PNEC). Despite the fact that metformin presented low risk in all cases, an environmental concern is suspected for guanylurea since it is continuously released into the aquatic environment.

Keywords: Guanylurea, Metformin, Occurrence, Removals, Risk assessment, Wastewaters

1. Introduction

In recent years, there have been a number of research articles and reviews dealing with the environmental occurrence, distribution and transport at national, European and worldwide scale of a vast array of pharmaceuticals in aquatic environment. In this light, many high production volume medicines like β-blockers, analgesics, antibiotics, lipid regulators, anti-inflammatories, and X-ray contrasts etc have been increasingly documented in groundwater, surface waters and wastewaters (Al-Qaim et al., 2014; Carmona et al., 2014; Gros et al., 2013; Kosma et al., 2010; Kosma et al., 2014; Padhye et al., 2014; Stamatis et al., 2013; Stamatis and Konstantinou, 2013). However, antidiabetic drugs, which are among the most widely used pharmaceutical compounds, have received less attention. The number of people suffering from diabetes, a very common glucose-metabolic disease, accounts for more than 200 million on a worldwide scale. Hence, antidiabetic drugs are widely used for the treatment of this chronic disease and consequently, they are continuously being released into the environment through wastewater discharges from wastewater treatment plants. Interestingly, despite their high prescription rates and consumption volumes, up to date, little work has been conducted on the presence of antidiabetics in waters and wastewaters. Expectantly, the most attention has been given to metformin, which is the first line drug of choice used for the treatment of diabetes mellitus type II and is excreted non metabolized in the urine. In some countries Metformin is in the top twenty list of prescribed, produced and environmentally loaded pharmaceutically active compounds and it is ranked number one in mass loading (1.10×10^6 kg/yr) (Dong et al., 2013). Its consumption increased in Netherland and in Western Europe, with 26 % between 2008 and 2012 and is expected to grow in the near future (ter Laak and Baken, 2014). Thus, not surprisingly, metformin has been detected in

surface water, in concentrations up to 29 μg/L as well as in wastewater treatment plant influent and effluent up to 129 and 47 μg/L, respectively (Blair et al., 2013a; Blair et al., 2013b; ter Laak and Baken, 2014).

Until recently, relatively little work has been conducted on the environmental fate and distribution of metformin. Two recent works (Scheurer et al., 2012; Trautwein and Kümmerer, 2011), suggested that metformin is biologically transformed in activated sludge to the transformation product, known as guanylurea. Other studies demonstrated that because of their incomplete degradation and removal during wastewater treatment both compounds are expected in considerable amounts (up to several tens of mg/L) into effluents (see Fig.1 & Fig.S1 (Supplementary Data)) and consequently into receiving waters. Furthermore, ter Laak and Baken (2014) reported that concentrations of metformin and guanylurea together, account for more than half of the total load of pharmaceuticals in surface waters. Due to the high environmental loading and the limited number of investigations on the environmental fate and occurrence of this group of bioactive compounds, increased knowledge is strongly recommended. Therefore, local research studies will be a key source of data to provide a better understanding of their behavior in wastewaters and water resources.

In view of the scarce data on the occurrence of antidiabetics in the aquatic environment, the aim of this work was: (1) to comprehensively investigate the simultaneous occurrence of metformin and its aerobic, bacterial dead-end TP, guanylurea, in eight WWTPs in Greece, by means of LC-UV/Vis-ESI/MS and LC-MS/LTQ Orbitrap systems, during one year monitoring program, (2) to evaluate the removal efficiencies across various types of WWTPs, (3) to provide a risk analysis in order to assess and compare the potential environmental risk of various types of wastewaters (hospital and municipal effluents) towards different aquatic organisms,

(algae, daphnids fish) through the calculation of risk quotients (Gros et al., 2010; Kosma et al., 2014; Valcárcel et al., 2011; Zhao et al., 2010).

To the present state of knowledge this is the first time that the simultaneous occurrence of these substances, is investigated in Greek aquatic environment and especially in conventional wastewater treatment plants. In addition, only a limited amount of information has been currently produced on the occurrence of guanylurea in WWTPs worldwide (Blair et al., 2013b; Oosterhuis et al., 2013; Scheurer et al., 2009, 2012; Trautwein and Kümmerer, 2011).

2. Materials and Methods

2.1 Chemicals and reagents

Metformin hydrochloride was purchased from Promochem (Wesel, Germany), while guanylurea was purchased from Sigma-Aldrich (Steinheim, Germany). Methanol and acetone were obtained from Pestiscan (Labscan, Ltd., Dublin, Ireland) and anhydrous sodium sulfate from Merck (Darmstadt, Germany). Acetonitrile (ACN) and water (for chromatographic analysis, LC-MS grade) were received from Fisher Scientific (Leicestershire, UK). Formic acid (purity, 98-100 %), was obtained from Merck KGaA (Darmstadt, Germany) and Sodium Dodecyl Sulfate (SDS), was obtained from Sigma-Aldrich (Steinheim, Germany). Oasis HLB (200 mg, 6 cc) were purchased from Waters Corporation (Milford, MA, U.S.A.). Stock 1000 mg/L solutions of each pharmaceutical were prepared in methanol and stored at -20 ^{o}C. A mixture of the compounds was prepared by appropriate dilution of individual stock solutions in methanol-water (50-50 v/v). Physicochemical properties of metformin hydrochloride and guanylurea are listed in Table 1.

2.2 Sampling and sample preparation

Influent and effluent samples were collected along 2010-2011. They were obtained from eight WWTPs (7 municipal WWTPs and 1 hospital WWTP), from various cities (Ioannina City, Ioannina Hospital, Arta, Preveza, Agrinio, Grevena, Kozani, Veroia), in N.W. Greece (Epirus, Macedonia, Aitoloakarnania). All municipal WWTPs investigated are equipped with primary treatment (grit removal) and conventional activated sludge secondary treatment with denitrification/nitrification, removal of phosphorous and a final disinfection step. The solid retention times (SRTs) and the hydraulic retention times (HRTs) of the WWTPs vary between 1-28 days and 1.5-39 hours, respectively. Ioannina City WWTP is the biggest plant, servicing a municipal area with 100,000 inhabitants, and uses sand filters as a polishing step before disinfection. Ioannina City receives also industrial wastewaters as well as the effluent wastewaters from the WWTP of the Hospital which has a capacity of 800 beds. The rest of the plants are smaller and serve populations between 20,000 and 90,000 inhabitants. Detailed description of the eight WWTPs can be found elsewhere (Kosma et al., 2014).

All samples were taken over a 24-hour period (composite samples). Amber glass bottles, which were prerinsed with deionized water, were used for the collection of the samples and after the sampling the bottles were immediately transport to the laboratory and filtered with 1 μm glass fiber filters GF/B (Whatman, UK). Then the samples were kept in the dark at 4 °C and extracted within 48 h (Kosma et al., 2014).

Initially, a previous SPE procedure was applied in order to evaluate the efficiency of this process. Briefly, Oasis HLB cartridges were precondiotioned with 5 mL of methanol and 5 mL of HPLC-grade water. Sample aliquots of 400 mL, without pH adjustment, were loaded into the cartridge at a flow rate of 10 mL/min and washed

with 5 mL of HPLC-grade water. The cartridges were dried under vacuum for 10 min. Elution step was done with 2 × 5 mL of methanol at 1 mL/min. Next, the extracts were dried over anhydrous sodium sulfate and under a gentle stream of nitrogen until dryness and finally, refilled with 0.5 mL of methanol:water, 50:50 (v/v) (Kosma et al., 2014).

According to the results, very low recoveries were obtained for metformin and guanylurea, respectively. This fact may be attributed to their physicochemical properties (high polarity, low molecular weight, high pKa), which may lead in low recoveries, when applying conventional methods. Metformin recoveries were also found to be very low in previous studies by performing similar preparation methodologies (Al-Odaini et al., 2010; Cahill et al., 2004).

So, taking into consideration all the above and in order to achieve better recoveries for these two compounds, aqueous solution of anionic surfactant SDS (Sodium Dodecyl Sulfate) was used. In general, in order to improve the recoveries of polar organic compounds, ion-pair (IP) reagent is used during the solid phase extraction analysis (AbuRuz et al., 2005; Gaurav et al., 2008; Keal and Somogyi, 1986; Martín et al., 2012).

For this purpose, Oasis HLB cartridges were pre-conditioned with 5 mL of methanol, 5 mL of HPLC-grade water and 5 mL of aqueous 2mM SDS solution. Next, 400 mL of the sample, without pH adjustment, were loaded into the cartridge, at a flow rate of 10 mL/min, washed with 5 ml of HPLC-grade water and dried under vacuum for 10 min. The analytes were eluted with 2 x 5 mL of methanol at 1 mL/min. The extracts were dried over anhydrous sodium sulphate and then under a gentle stream of nitrogen until dryness and finally, reconstituted with 0.5 mL of methanol:water, 50:50 (v/v) and stored at -20 °C until being analyzed.

2.3 Instrumentation

A LC-UV/Vis-ESI/MS system from Shimadzu (Kyoto, Japan), was used for the determination of metformin and guanylurea in wastewaters. A SIL 20A autosampler (injection volume, 20 μL) and a LC-20AB pump were used, while separation of the compounds was achieved with a C_{18} (Restek, USA) analytical column (150 × 4.6 mm, 5 μm). The SPD 20A UV–vis detector was coupled in series with the LC-MS 2010EV mass selective detector, which was equipped with an atmospheric pressure ionization source electrospray (ESI) interface. For the analysis of the samples, ESI interface in positive ionization (PI) mode was used and the mobile phase contained of solvent (A) water with 0.1% formic acid and solvent (B) acetonitrile with 0.1% formic acid using gradient as follows: Initial conditions 70 % A, kept constant for 1 min, decreased to 40 % in 4 min, decreased to 0 % in 4 min, returns to the initial conditions after 1 min and re-equilibration time was set at 2 min. The flow rate was 0.5 mL/min and the column temperature was set at 40 °C. Mass parameters such as drying gas, nebulizing pressure, capillary voltage and fragmentation voltage were 10 L/min at 200 °C, 100 psi, 4500 V and 5 V, respectively. Quantitative analysis was performed in selected ion monitoring (SIM) mode and for each compound the precursor molecular ion [M + H], and at least one confirming ion was acquired (Table 2). For the identification of the analytes both the retention time (within 2.5 %) and mass spectrum were matched with the standards (Kosma et al., 2014).

Next, for further confirmation of positive findings of metformin and guanylurea, liquid chromatography coupled to a high resolution Orbitrap mass spectrometer was used (Thermo Fischer Scientific, Bremen, Germany). Accela AS autosampler, Accela Pump and a Thermo hypersil gold column (150 × 2.1 mm, 5 μm) were used for the

separation of the compounds. Capillary voltage, tube lens voltage, sheath gas flow rate, auxiliary gas flow rate and capillary temperature were set at 40 V, 110 V, 60 arbitrary units (au), 20 au and 350 °C, respectively. Full scan acquisition over a mass range of 120–500 Da was performed at spray voltage 3.2 kV and a resolution of 60,000. Data analysis was done by the Thermo Xcalibur 2.1 software package. For the identification of the compounds, their retention time, relative to that of the standards, as well as their accurate mass (theoretical mass was matched with the observed mass, with mass window <5ppm) were taken into consideration. For the analysis in PI mode the mobile phase contained of solvent (A) water with 0.1% formic acid and solvent (B) methanol with 0.1% formic acid using gradient as follows: Initial conditions 100% A, decreased to 90% in 2 min, decreased to 60% in 5 min, then decreased to 0% in 2 min, remain 0% for 1 min and finally returns to the initial conditions after 2 min with the re-equilibration of the column set at 3 min. The flow rate was set at 0.45 mL/min (Kosma et al., 2014). Elemental composition, retention time, exact and measured accurate mass of the two compounds studied, as well as their MS/MS fragment ions obtained, are given in Table 3. MS/MS experiments were carried out at 35 eV.

2.4 Validation study

All the validation and quality assurance/quality control studies performed according to Kosma et al. (2014) and are given in Tables 4 and 5. Matrix matched calibration curves were used for quantification, using influent and effluent SPE extracts. Firstly, blank samples of effluent and influent wastewaters was analysed and positive findings were subtracted from the spiked samples. Calibration curves were prepared with each batch of 12 samples. In addition, in every batch of samples, two quality control (QC)

samples were injected which were blank wastewater samples (previously analyzed) spiked at LOQ and 10 times the LOQ level, respectively. QC recoveries were considered satisfactory for all the tested samples and were used for concentration calculation.

Limits of detection (LODs), limits of quantification (LOQs), precision and signal suppression are given in Table 4. LODs were 3.9 ng/L and 4.6 ng/L in distilled water, 7.8 ng/L and 6.2 ng/L in influent wastewater and 5.1 ng/L and 9.8 ng/L, in effluent wastewater, for metformin and guanylurea, respectively. For the evaluation of ion suppression or enhancement in the effluents, Eq. (1) was applied. According to Eq (1), the peak areas from the native analytes in the sample (area blank) were extracted from the peak areas of spiked wastewater extracts (area matrix) and compared to the peak areas resulting from the solvent (area solvent: methanol–water 50:50, v/v) spiked at the same level (Kosma et al., 2014). Both compounds presented signal suppression, which means that the signal intensity of the analytes decreased due to the ionization of various coeluting substances (see Table 4).

$$\% \text{ suppression/enhancement} = 100 - (((\text{area matrix} - \text{area blank}) * 100)/\text{area solvent}) \quad (1)$$

Mean recoveries are given in Table 5. Recoveries of the target compounds were determined for different matrixes (distilled, influent and effluent wastewaters) by spiking the samples with the analytes at concentrations of 0.2 and 2 μg/L. As influents and effluents might contain already the target analytes, concentrations of the respective unspiked samples were subtracted from concentrations of the spiked samples and then divided by the spiked level. Mean recoveries ranged between 38.3 % in distilled water for metformin and 59.4 % in effluent wastewater for guanylurea,

when spiking with 0.2 μg/L and between 40.7 % in distilled water for metformin and 62.2 % in influent wastewater for metformin, when spiking with 2 μg/L.

3. Results and discussion

3.1 Occurrence of metformin and guanylurea in WWTPs and removal efficiencies

In the present study, the occurrence of metformin, one of the most prescribed drugs worldwide, in 64 influent and effluent wastewater samples was investigated. Furthermore, the detection of the polar transformation product of metformin, guanylurea, took place, since it is known to be very recalcitrant and formed during wastewater treatment (Oosterhuis et al., 2013; Sheurer et al. 2012). The samples were collected over the four seasons of a year (autumn 2010, winter 2011, spring 2011 and summer 2011) in eight Greek WWTPs. Table 6 indicates the percentage of positive findings, as well as the range and average concentrations of the selected compounds in wastewaters.

As it can be seen it Table 6 both compounds were present in the influents. Metformin was a very abundant compound in the influents found in the 97 % of the samples analyzed. Influent concentrations of metformin ranged in municipal WWTPs from bql to 573 ng/L, presenting maximum concentrations in the WWTPs of Veroia (496 ng/L) and Arta (357 ng/L), while the lowest concentrations were observed in the WWTPs of Preveza (38 ng/L) and Agrinio (54 ng/L). In the Hospital WWTP concentrations were found between bql and 1167 ng/L demostrating that hospitals can be considered hotspot sources for pharmaceutical emission in the aquatic environment. The levels obtained are low compared to other WWTPs with higher urban, industrial or medical pressure (Blair et al., 2013b; Oosterhuis et al., 2013; Sheurer et al., 2009, 2012; Trautwein and Kümmerer, 2011; Van Nuijs et al., 2010). In general, the concentration variability of metformin from site-to-site could be attributed to regional prescribing

customs and usage fands, population density, contexts of water treatment technology capabilities as well as seasonal and environmental climatic conditions. For example, in German WWTPs which serve much more population (i.e capacity of 220,000 to 600,000 habitants-equivalent) than the WWTPs studied in the present work (i.e capacity of 15,000 to 100,000 habitants-equivalent), the concentration of metformin ranged between 18000 and 129000 ng/L (Sheurer et al. 2009; Sheurer et al. 2012; Trautwein and Kümmerer, 2011). In Netherlands, average detected concentrations of metformin were up to 79000 ng/L in the influents of two WWTPs (Oosterhuis et al. 2013). According to Oosterhuis et al. (2013) >3.5 % of the Dutch population consumes metformin and its high daily dose is 2000 mg/d, while the consumption of metformin in Netherlands exceeds the average European consumption. In Belgium, metformin influent concentrations (18 WWTPs) measured between 20331 and 94311 ng/L (Van Nuijs et al., 2010). In Virginia-USA, Ottmar et al. (2010) developed a model in order to estimate pharmaceutical loadings and concentrations in influent and effluent wastewaters of five WWTPs, taking into consideration the highest predicted mass loadings and the total population of United States as of 2008 (304 million). According to the model, predicted concentrations of metformin ranged between 3490 and 88000 ng/L in the influents of five WWTPs. In addition, Blair et al. (2013b), found in the influent of a South Shore Water Reclamation Facility (SSWRF) in the town Oak Creek, in Milwaukee, United States, metformin concentrations ranging between 3200 and 100000 ng/L. SSWRF services the greater area of Milwaukee, has a treatment capacity of 1,135,000 m^3/day with an average flow of approximately 379,000 m^3/day and it consists of preliminary treatment (7 bar screens/grit channels), primary treatment (16 primary clarifiers), activated sludge treatment (28 aeration basins and 24 secondary clarifiers) and chlorine disinfection (25-pass contact

channels) (Blair et al., 2013b). As a consequence the concentration of metformin is correlated to the wastewater treatment technology capability, the daily effluent discharge flow (L/day) and the population density of the given site.

Furthermore, Shraim et al. (2012) reported higher average concentrations of metformin (15200 ng/L), in the influent of the WWTP of Almadinah Almunawarah, in Saudi Arabia. It is worth noticing that Almadinah Almunawarah WWTP has a total capacity of 300,000 m^3/day and in addition to domestic sewage sometimes receives partially treated industrial and medical wastewaters (Shraim et al., 2012).

Conversely to previous mentioned findings, comparable concentrations of metformin (between <bql and 1568 ng/L) to our study was reported in a recent investigation conducted in influents of WWTPs of Coimbra (Portugal) which serves a population close to Ioannina city (~140,000) (Santos et al., 2013).

On the other hand, guanylurea was present in the 69 % of the influent samples, at relatively low concentrations ranging between bql and 59 ng/L. This may indicate, in most of the cases, the fact that the long distance between the cities and the WWTPs results in the transformation of metformin by bacteria that already exist in the sewer system (Trautwein and Kümmerer, 2011). As it was reported elsewhere (Trautwein and Kümmerer, 2011), guanylurea is formed by twofold dialkylation at position 1 and oxidative deamination at position 2 of metformin. Similarly to metformin, guanylurea presented lower concentrations in the influents than those reported in previous studies (Sheurer et al., 2012; Trautwein and Kümmerer, 2011). The occurrence of metformin and guanylurea in the influents was also confirmed by the Orbitrap mass analyzer (Fig. 2).

In the effluents, metformin was present in 66 % of the analyzed samples, while guanylurea was present in higher percentage (78 %). Concentrations of metformin

ranged between bql and 26 ng/L, while guanylurea concentrations ranged between bql and 627 ng/L. In general, metformin dominated in the influents and guanylurea in the effluents, highlighting the strong degree of metformin biotransformation in WWTPs. Effluent ratios of Metformin/Guanylurea ranged from 0.005 to 0.78, whereas influent ratios were inversely related and ranged from 0.88 to 81.3 (Fig. S2). Comparatively to previous findings, metformin was also found in Greek wastewater effluents at much lower concentration values than those in wastewater effluents of other sites. For instance, metformin was present in the effluents of WWTPs in Germany in concentrations ranging from 760 to 26000 ng/L (Sheurer et al., 2009; Sheurer et al., 2012; Trautwein and Kümmerer, 2011). Oosterhuis et al. (2013) reported 1500 ng/L of metformin in the effluents of two WWTPs in Netherlands, whereas Ottmar et al. (2010) estimated that concentrations of metformin ranged between 870 and 44000 ng/L in the effluents of five WWTPs. Furthermore, Blair et al. (2013b) detected metformin in concentrations between 640 and 47000 ng/L, in the effluents of a South Shore Water Reclamation Facility (SSWRF) in United States. High average concentrations (3190 ng/L) were also reported by Shraim et al. (2012), in the effluent of the WWTP of Almadinah Almunawarah, in Saudi Arabia. Finally, in accordance to our study, low concentration or even traces were reported in effluent wastewaters in Austria (253 ng/L, Martín et al., 2012) and in Kajang town in Malaysia, (16 ng/L, Al-Odaini et al., 2010), respectively.

As regards the hospital influents, in a recent big survey, Santos et al. (2013) detected metformin in the effluents of four different hospital WWTP (a university, a general, a pediatric and a maternity) at concentrations ranging from 4 to 4040 ng/L. The highest concentration was detected in the maternity hospital and this fact may be attributed,

according to the authors, to the high consumption rate of metformin among Portuguese population.

To sum up, the above mentioned data about occurrence of metformin, translate its consumption pattern and number of the population served by each WWTP and removal efficiencies of each WWTP, and as expected higher metformin values were obtained for WWTPs that serve higher populations.

As far as guanylurea concerns, in a recent study (Oosterhuis et al., 2013) it was detected in the effluents of one small WWTP in Netherlands, in average concentrations of 48000 ng/L. Furthermore, in the tallest WWTP in Southern Germany, Trautwein and Kümmerer (2011) detected in the influents and in the effluents 400 ng/L and 1860 ng/L of guanylurea, respectively.

From the results obtained in the present study, in most of the cases, the increase of guanylurea concentrations in the effluents, matches the decrease of metformin concentration in the effluents. This statement is in agreement with those reported by Scheurer et al. (2012) were the removal of metformin in five WWTPs in Germany was directly related to the formation of guanylurea. Guanylurea was found to be very stable in photolysis and as a consequence in sunlight or technical irradiation in advanced water treatment, indicating that is not expected to be eliminated in wastewater and advanced water treatment (Trautwein and Kümmerer, 2011).

Box plots indicating removal efficiencies of metformin and the formation of guanylurea in the eight WWTPs during the monitoring period are calculated according to Kosma et al. (2014) and are shown in Fig. 3. Removal of metformin in many cases was not feasible to be estimated due to the fact that it was not detected in the effluents. In the cases that metformin and guanylurea concentrations were below the limit of quantification, the removal/formation was calculated by matching the

concentration with LOQ/2. Removal efficiencies of metformin ranged between 78 % and 99 %. The WWTPs were operating normally during all sampling events, and generally comparable removal efficiencies were observed in almost all of them, indicating small variations with respect to different capabilities of the treatment technologies (clarification, activated sludge, P removal etc) and hydraulic retention times (HRT) (1.5-39 h depending on the technology). The lowest removal was observed in Preveza WWTP in winter, while the highest in Grevena WWTP, also in winter period. These values were similar to those reported for WWTPs sited in Europe and USA that included primary and biological treatment (Santos et al., 2013; Scheurer et al., 2009, Trautwein and Kümmerer, 2011). Concerning the WWTP of Preveza, the average removal efficiencies were around 80%. It is difficult to reach a clear quantitative conclusion on removal efficiency of this WWTP compared to the others, since as Verlicchi et al., (2012) have previously pointed out, many factors such as configuration of the specific WWTP (C, N and P removal, biological reactor shape), operating conditions (SRT, HRT, pH, T, redox conditions, etc.) and feeding mode were shown to have pronounced effects on the efficiency of WWTPs. Despite of being less efficient than the other tested WWTPs, metformin removal values were still much higher than those cited by Blair et al. (2013b) (median removal efficiencies 24 %) for North American WWTPs that included preliminary, primary and biological treatment and chlorine disinfection.

On contrary to removals of metformin, noticeable differences were observed for the formation of guanylurea through the tested WWTPs. Formation ranged between 17 and 95%, observing the highest in hospital of Ioannina city, Grevena and Veroia WWTPs. The lowest values were observed in Agrinio WWTP which was about a half of those obtained in almost all the rest of WWTPs. Variability ranges of guanylurea in

the effluents reflected that, in general, its formation throughout the WWTPs can be affectively minimized or promoted by a number of factors (i.e temperature changes, microbial activity and biological reactions, HRT, sludge retention etc) and therefore it is difficult to explain pointedly the observed differences.

In general, the high abundance of guanylurea in effluent samples demonstrates the high degree of metformin biotransformation in WWTPs. However, besides biodegradation, adsorption to sludge (although it is expected to be low for polar compounds such as metformin) or some other abiotic elimination pathways of metformin may exist in WWTPs. For instance, Donia and Awad (1995), reported the faculty of metformin to form stable complexes with heavy metals under specific occasions, while Collin et al. (2004) reported the formation of eleven oxidation end-products in radical induced oxidation of metformin in aqueous aerated and deaerated solutions. Furthermore, as in the case of guanylurea, the elimination of metformin could not be attributed in photolysis process (Trautwein and Kümmerer, 2011). Another important issue for the removal of metformin is the passage through the chlorination step, where it has been proven to be very effective (Scheurer et al., 2012). In the present study, the use of chlorine in the final disinfection step is applied in all WWTPs, even in the WWTP of Kozani, were basically three ultraviolet disinfection filters are used, but are supported also by the addition of NaClO. Finally, we could say that high metformin concentrations could be an index for untreated wastewaters, while low metformin and high guanylurea concentrations could be an index for treated wastewaters (Scheurer et al., 2012).

Oosterhuis et al. (2013) related the consumption of metformin, and as a consequence of guanylurea, to recovery in wastewaters (influent and effluent) and surface waters, in the city Ootmarsum of Netherlands. They concluded that 82±52 % of the degraded

metformin can be recovered as guanylurea in the effluents, considering the molecular weight ratio of guanylurea and metformin. Furthermore, 87 % and 187 % of the effluent load of guanylurea and metformin can be recovered in the surface water, respectively. This may attributed to the fact that daily concentrations of metformin reach four days retention in surface waters and as a consequence its concentrations come from emissions with different ratio of metformin and guanylurea the previous four days.

3.2. Seasonality

In order to examine seasonal trends in metformin and guanylurea concentrations, samples taken in autumn, winter, spring and summer were compared. According to Fig. 4A, the highest values were observed in hospital influent of Ioannina city and Grevena WWTP influent in winter and in Veroia WWTP in autumn (1167, 573 and 496 ng/L, respectively). On the opposite, the minimum values were found in autumn, winter and spring for Arta WWTP, Preveza WWTP and Kozani WWTP, respectively. Considering all the measurements during the four sampling periods (Fig. 4A), a clear tendency in metformin concentrations of the untreated wastewaters could not be established. These data are in agreement with the prescription and use patterns of metformin since antidiabetics are used for therapeutic reasons all over the year. Occurrence of guanylurea in effluents (Fig. 4B) was proportionally correlated with the concentrations of metformin in the influents, observing the highest value in hospital influents in winter.

As regards the removal efficiencies, seasonal variations in treatment of metformin throughout the WWTPs were minimal in almost all the tested WWTPs (Fig. 5A). Considering the exact date of sampling, average day temperatures were 13 ^{0}C, 9 ^{0}C,

19 ^{0}C and 26 ^{0}C for autumn, winter, spring and summer, respectively. Generally speaking, removal of many pharmaceuticals could be less effective in lower temperatures since at these conditions microbial activity and biological reactions are reduced (Verlicchi et al., 2012). This trend was clearly observed in Preveza WWTP, which achieved a removal close to 80% for both autumn and winter seasons but was not noticed in the other WWTPs.

On the other hand, discrepancies were observed for formation of guanylurea between the four sampling periods (Fig. 5B). For example, although, formation of guanylurea decreased notably (~20%) in effluents of Agrinio WWTP and hospital WWTP in autumn, this pattern was not observed for the same WWTPs in winter season. In addition, higher formation was found for Veroia WWTP and Grevena WWTP in winter and autumn and lower in spring and summer. The aforementioned data showed a lack of clear seasonal tendency. However, further investigations would be interesting in order to complete the conclusions here achieved and evaluate if there are any environmental conditions (i.e temperature changes) that could affect their occurrence and removal or formation.

3.3 Environmental Risk Assessment

The occurrence of metformin and guanylurea in surface waters (Al-Odaini et al., 2010; Blair et al., 2013a; Kolpin et al., 2002; Martín et al. 2012; Vulliet and Cren-Olivé, 2011) comprises necessary to estimate their potential ecotoxicological risk in environment. For this reason, risk quotients (RQs) were determined in the effluent wastewaters, for fish, daphnids and algae. Available information regarding ecotoxicological effects of metformin and guanylurea is very limited (ter Laak and Baken, 2014). For guanylurea it was not feasible to estimate RQs due to the fact that

there are no available data about its acute or chronic toxicity. RQs of metformin calculated based on the acute toxicity data since chronic toxicity data were not available (Table 7). RQs are defined by the US EPA. Further details on the calculation of RQs can be found elsewhere (Kosma et al., 2014).

According to literature, no algal growth and microbial respiration or change in bacterial communities in streams biofilms, was provoked by metformin (Rosi-Marshall et al., 2013). Metformin and guanylurea found to be absorbed into plants, leafs, seeds, grains, fruits, vegetables, and beans and produced negative effects on growth and development of carrots in soil concentration between 6 and 10 mg/kg dw (Eggen and Lillo, 2012).

Blair et al. (2013a) found that metformin posed low ecological risk in surface water. In another study, Carlsson et al. (2006) reported that there are no acute environmental effects for metformin, while no conclusion can be made for chronic toxicity effects since there is a lack of experimental data. In addition, Escher et al. (2011) reported no ecotoxicological effect for metformin. In another recent study, Santos et al. (2013) calculated the hazard quotients (HQs) for metformin in effluent wastewaters for fish, daphnids and algae and resulted to pose low risk. In contrast, Hanisch et al. (2004) resulted in a potential risk for metformin hydrochloride in the aquatic environment due to the fact that the ratio between PEC/PNEC was about 1 or higher.

Trautwein and Kümmerer (2011) reported that guanylurea presented no toxic effects on the bacterial community in a manometric respiratory test at a concentration of 11.9 mg/L. Furthermore, Gartiser et al. (2012) studied the mutagenic activity of metformin and guanylurea after testing inherent biodegrability and/or after drinking water ozonation experiments and concluded that none of the compounds indicated mutagenic effect.

Fig. 6 represents RQ values calculated for metformin for acute toxicity, in three trophic levels. As it can be seen in Fig. 6, in all effluents RQ < 0.001 which means that low risk is suspected for the three trophic levels in the receiving water bodies of the eight WWTPs. This was due to the fact that low effluent concentrations of metformin were detected in all cases since its removal rates were very high. To the authors knowledge this was the first time that ecotoxicology of metformin in the effluents of WWTPs was assessed in Greek aquatic ecosystem. Furthermore, since concentrations of guanylurea in wastewater effluents are known to be higher than metformin levels, information about its biological activity and ecotoxic effects will be very useful for further research.

4. Conclusions

Application of an analytical method based on SPE and followed by LC-UV/Vis-ESI/MS, provided wider knowledge about the occurrence and fate of metformin and its transformation product guanylurea, in the influents and effluents of eight WWTPs in Greece. Further confirmation of positive findings of the two compounds took place by means of LTQ Orbitrap mass analyzer. Both compounds have gained little attention until now and this was the first time that their occurrence was investigated in Greek aquatic system. According to the results the following conclusions can be reached:

- The extensive work demonstrated the occurrence of these two compounds in the influents and the effluents of all WWTPs.
- Concentrations ranged between bql and 1167 ng/L in the influents and between bql and 627 ng/L, in the effluents.

- High concentrations of metformin in the influents resulted in high concentrations of guanylurea in the effluents.
- Removal efficiencies of metformin were up to 99 %, while formation of guanylurea reached 95 %.
- Lack of a clear seasonal tendency in the occurrence and removal or formation was observed. However, further research in these issues is warranted and needed.
- Elimination of metformin can be mainly related to its biological formation to guanylurea but also some other abiotic elimination pathways may exist such as the faculty of metformin to stable complexes with heavy metals. In addition, further transformation of metformin or guanylurea may also occur following different transformation routes.
- Ecotoxicology assessment of metformin in the effluents took place and the results indicated low acute risk for water bodies. Moreover, further studies are strongly recommended in order to estimate acute (EC_{50} or LC_{50}) and chronic (NOEC) toxicity data of guanylurea, since it is continuously released in water bodies and in many cases in relatively high concentrations with unknown effects.

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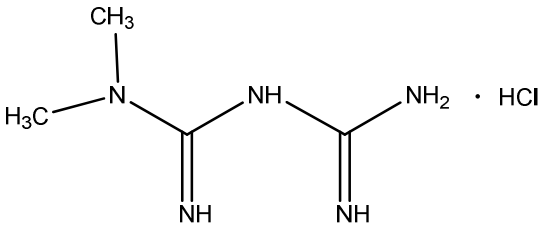
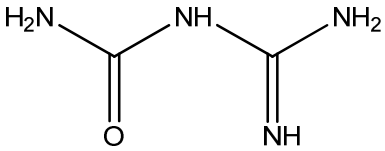
Table 1 - Characteristics of metformin hydrochloride and gualynurea

Compound	Molecular formula	Chemical formula	MW	pKa	LogK_{ow}	Henry's law constant (atm/m^3/M)[c]	Aqueous solubility (g/L) (25 °C)[c]
Metformin hydrochloride	$C_4H_{11}N_5 \cdot HCl$		165.6	10.4[a]/12.3[a]/12.1[b]	-1.43[b] /-4.3[c] (at pH 7.4)	7.6 x 10^{-16}	1000
Gunylurea	$C_2H_6N_4O$		102.1	8.0[a]/13.5[a]	-2.51[c] (at pH 7.4)	2.47 x 10^{-15}	1000

[a]Sheurer e al., 2012, [b]Thomas et al., 2010, [c]ter Laak and Kirsten Baken, 2014

Table 2 - Instrumental parameters for metformin and guanylurea in LC-ESI-MS

Compounds	Polarity (ES)	Time (min)	m/z ions	Relative ion intensity %
Metformin	+	4.162	**130**, 60	100, 38.67
Guanylurea	+	4.261	**103**, 60	100, 25.12

Quantitation ions in bold

Table 3 - Retention time, exact and accurate mass information, mass error deviation, double bond equivalent number (DBE) and MS/MS data of metformin and guanylurea in Orbitrap MS.

Compounds	RT (min)	Elemental Composition	Exact mass (theoritical)	Accurate/nominal mass (detected)	Error (ppm)	DBE
Guanylurea	0.83	$C_2H_7N_4O$	103.0614	103.0610	0.437	1.5
Frag. Ion 1		$C_2H_4N_3O$	86.0349	86.0345	-4.514	2.5
Metformin	0.94	$C_4H_{12}N_5$	130.1087	130.1092	3.674	1.5
Frag. Ion 1		$C_4H_9N_4$	113.0822	113.0820	-1.529	2.5
Frag. Ion 2		$C_3H_{10}N_3$	88.0869	88.0872	3.134	0.5

Table 4 - Limits of detection (LODs) and limits of quantification (LOQs) in distilled water, influent and effluent wastewater. Precision (RSD in the same day (RSD_r) and RSD in different days (RSD_R)) and signal suppression/enhancement (%), in effluent wastewater.

Compound	LOD (ng/L)			LOQ (ng/L)			RSD_r (%)	RSD_R (%)	% signal
	DW	WWI	WWE	DW	WWI	WWE	(n=5)	(n=5)	suppression (+)
Metformin	3.9	7.8	5.1	12.9	25.1	16.7	9.6	13.3	9.9
Guanylurea	4.6	6.2	9.8	14.1	19.6	28.3	7.1	7.9	5.8

Table 5 - Mean recoveries (%) and RSD (%) in distilled water, influent and effluent wastewater after spiking with 0.2 and 2 µg/L (n=3).

Compound	% Recoveries after spiking with 0.2 µg/L (RSD %)			% Recoveries after spiking with 2 µg/L (RSD %)		
	DW	WWI	WWE	DW	WWI	WWE
Metformin	38.3 (7.7)	58.4 (9.0)	53.1 (8.3)	40.7 (7.1)	62.2 (8.9)	57.0 (8.0)
Guanylurea	44.4 (5.8)	57.9 (5.0)	59.4 (6.3)	47.3 (6.8)	60.1 (9.5)	57.4 (12.0)

Table 6 - Ranges of concentrations and corresponding mean values in brackets of metformin and guanylurea found in the influents and effluents of the eight WWTPs.

Compound	Influent wastewater (n=32) (ng/L)								
	% PF	Ioannina Hospital	Ioannina City	Arta	Preveza	Agrinio	Grevena	Kozani	Veroia
MFM	97	bql-1167 (346)	103-249 (174)	bql-357 (217)	n.d.-38 (bql)	bql-54 (bql)	bql-573 (207)	bql-251 (130)	85-496 (321)
GNL	69	n.d.-84 (29)	bql-39 (bql)	n.d.-33 (bql)	n.d.-bql	n.d.-bql	n.d.-33 (bql)	n.d.-28 (bql)	n.d.-59 (bql)
	Effluent wastewater (n=32) (ng/L)								
	% PF	Ioannina Hospital	Ioannina City	Arta	Preveza	Agrinio	Grevena	Kozani	Veroia
MFM	66	n.d.-26 (bql)	bql-23 (bql)	n.d.-18 (bql)	n.d.-bql	n.d.-bql	n.d.-bql	n.d.-19 (bql)	n.d.-bql
GNL	78	bql-627 (188)	83-154(96)	n.d.-136 (51)	n.d.-26 (bql)	n.d.-33 (bql)	bql-328 (116)	n.d.-84 (50)	n.d.-237 (133)

P.F: Positive findings, MFM: Metformin, GNL: Guanylurea

Table 7 - Acute toxicity (mg/L) data of metformin on fish, daphnids and algae (lower values indicated in bold fonds).

Compound	Fish			Daphnids			Algae		
	EC50 (mg/L)	**test organism**	**References**	**EC50 (mg/L)**	**test organism**	**References**	**EC50 (mg/L)**	**test organism**	**References**
Metformin	3,32x10^4		Sanderson et al., 2003	1345	*Daphnid*	Sanderson et al., 2003	511		Sanderson et al., 2003
	>100* (<96h)		Sanderson and Thomsen, 2009	**64*** (48h)	*Daphnid*	Sanderson and Thomsen, 2009	320* (24h)		Sanderson and Thomsen, 2009
	>982*	*L. macrochirus*	Montforts, 2005	130	*D. magna*	Montforts, 2005	**110**		Santos et al., 2013

*indicates LC50 (mg/L) value

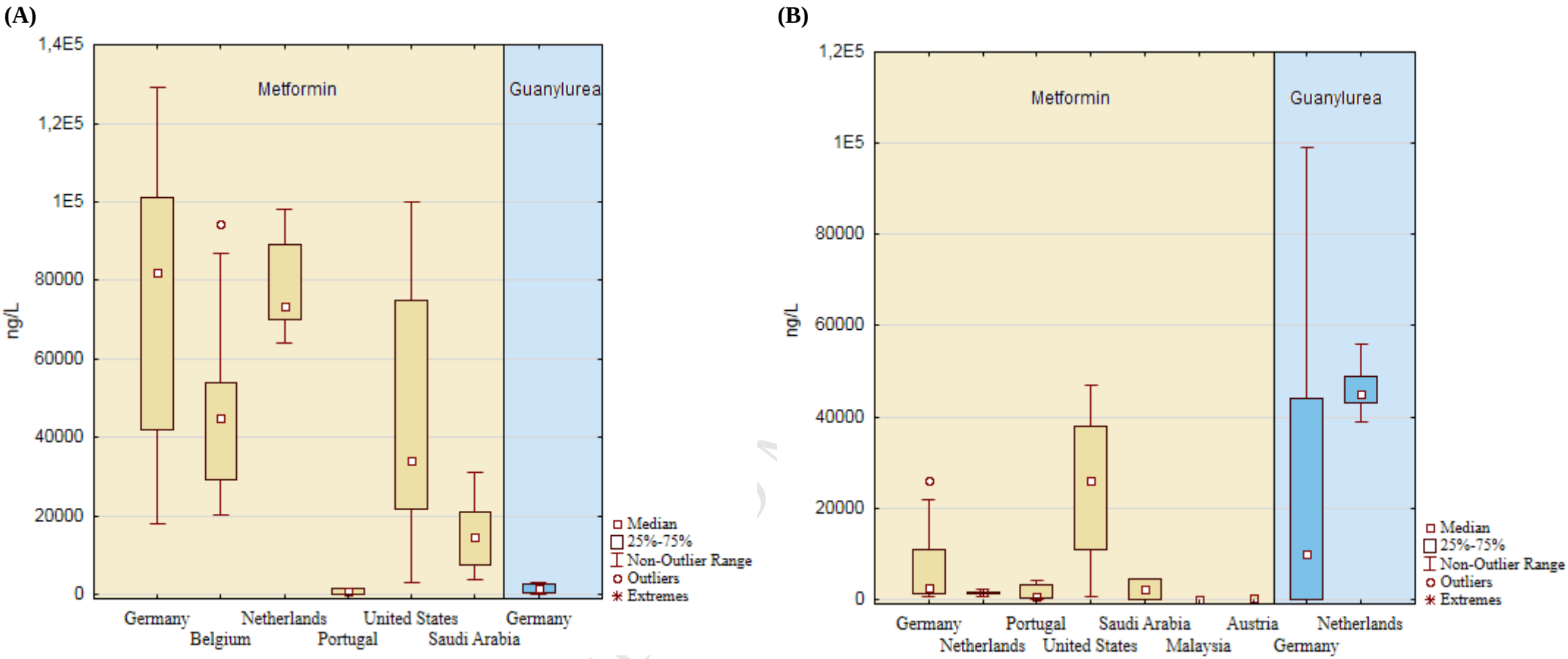


Fig. 1 - Concentration levels of metformin and guanylurea in (A) the influents and (B) the effluents of WWTPs, worldwide (Germany: Scheurer et al., 2009; Trautwein and Kümmerer, 2011; Scheurer et al., 2012, Belgium: Van Nuijs et al., 2010, Netherlands: Oosterhius et al., 2013, Portugal: Santos et al., 2013, United States: Blair et al., 2013b, Saudi Arabia: Shraim et al., 2012, Malaysia: Al-Odaini et al., 2010, Austria: Martín et al., 2012).

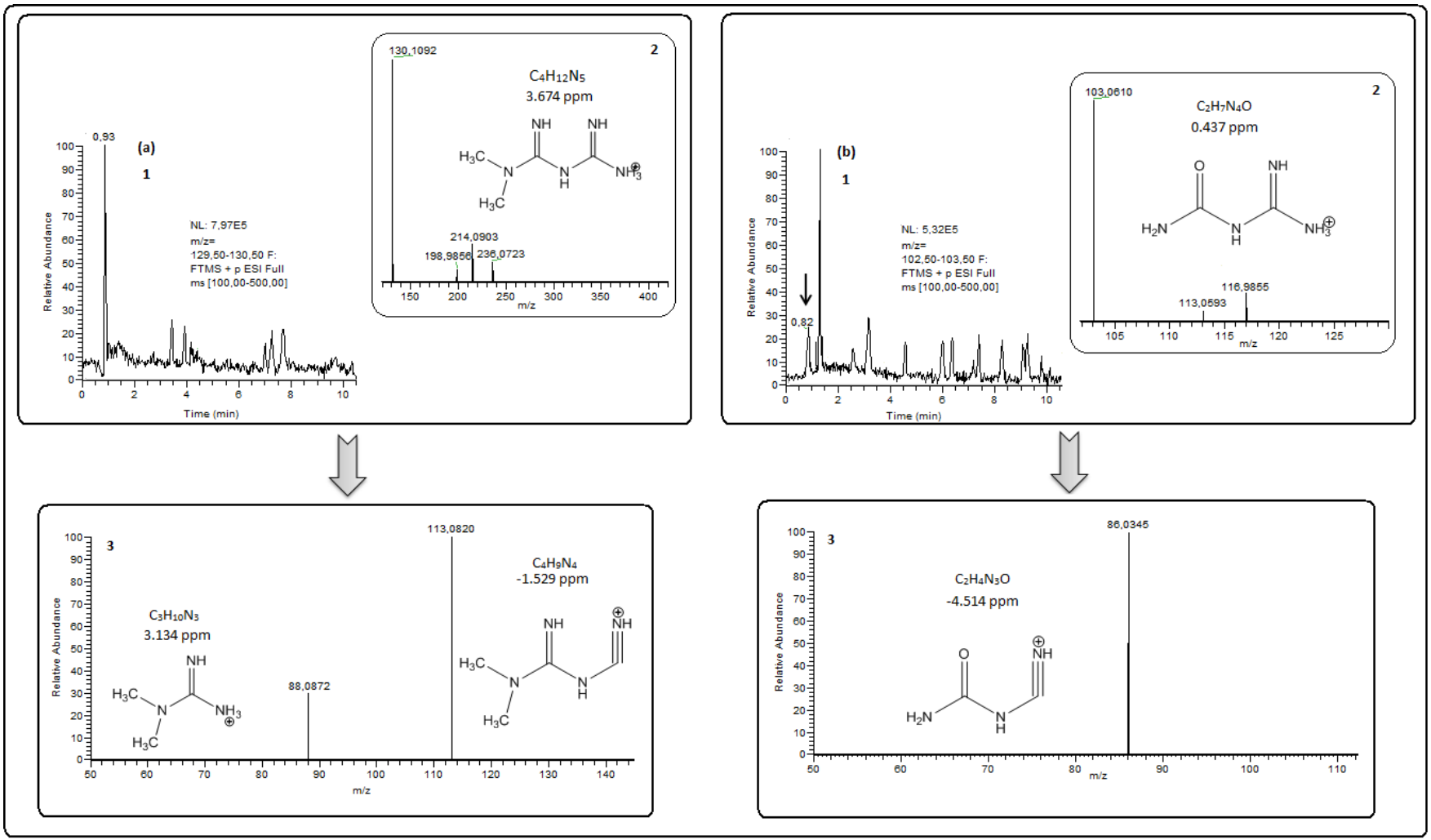


Fig. 2 - (1) Chromatogram, (2) full scan accurate mass product ion spectrum and (3) MS/MS data obtained using Orbitrap MS targeting the corresponding ions, for (a) metformin and (b) guanylurea, respectively, found in the influent of Ioannina hospital WWTP in winter.

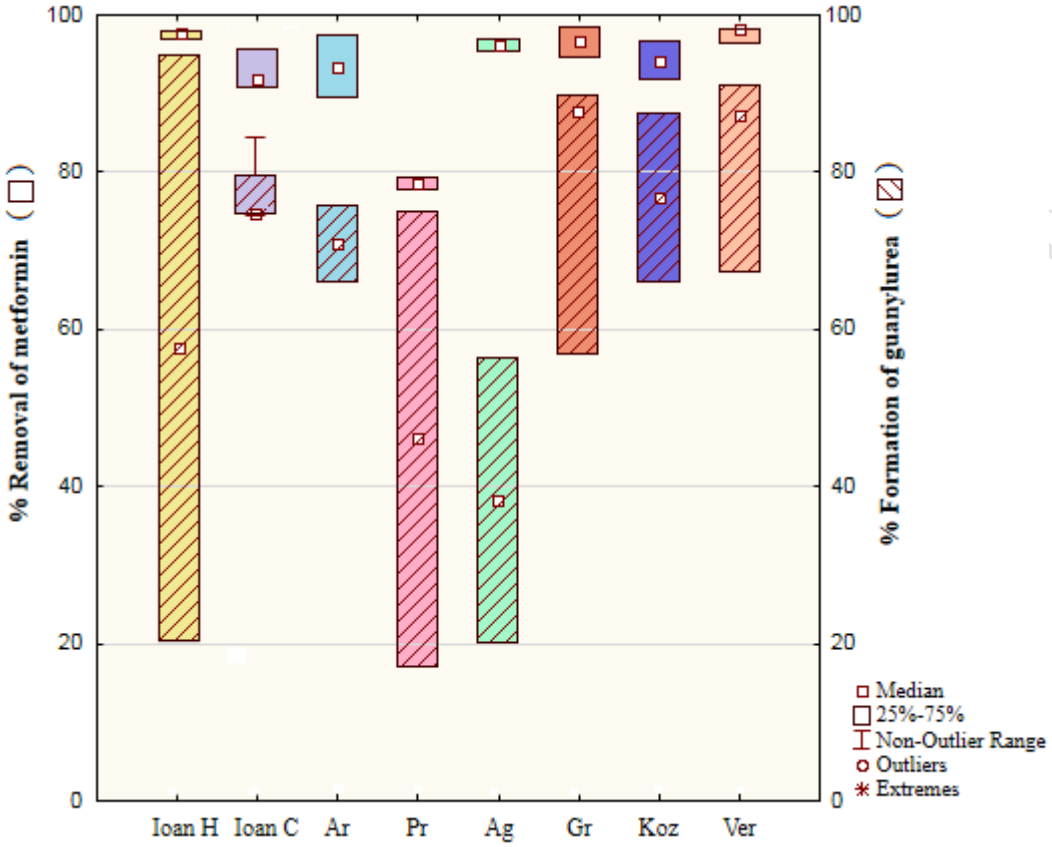


Fig. 3 - Box and Whisker graphs showing the removal efficiencies (%) of metformin and the formation of guanylurea (%), in the eight WWTPs (Ioan C: Ioannina City, Ioan H: Ioannina Hospital, Ar: Arta, Pr: Preveza, Ag: Agrinio, Gr: Grevena, Koz: Kozani, Ver: Veroia).

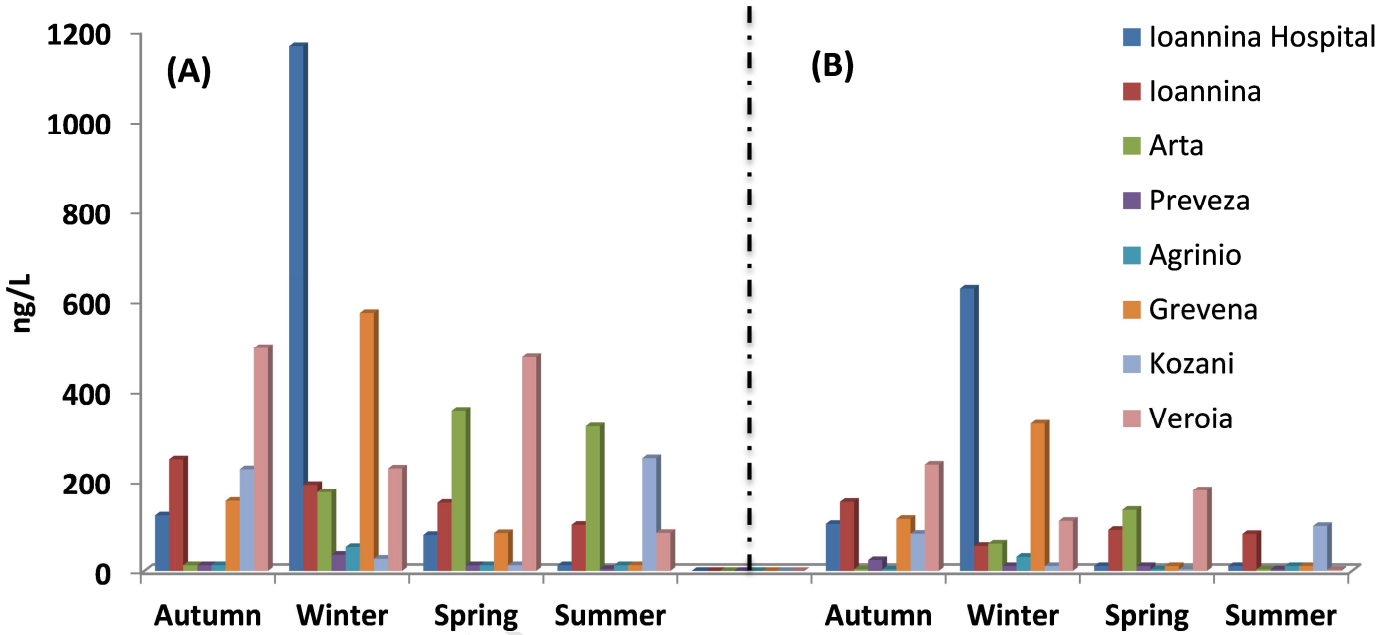


Fig. 4 - Seasonal occurrence of (A) metformin in the influents and (B) guanylurea in the effluents.

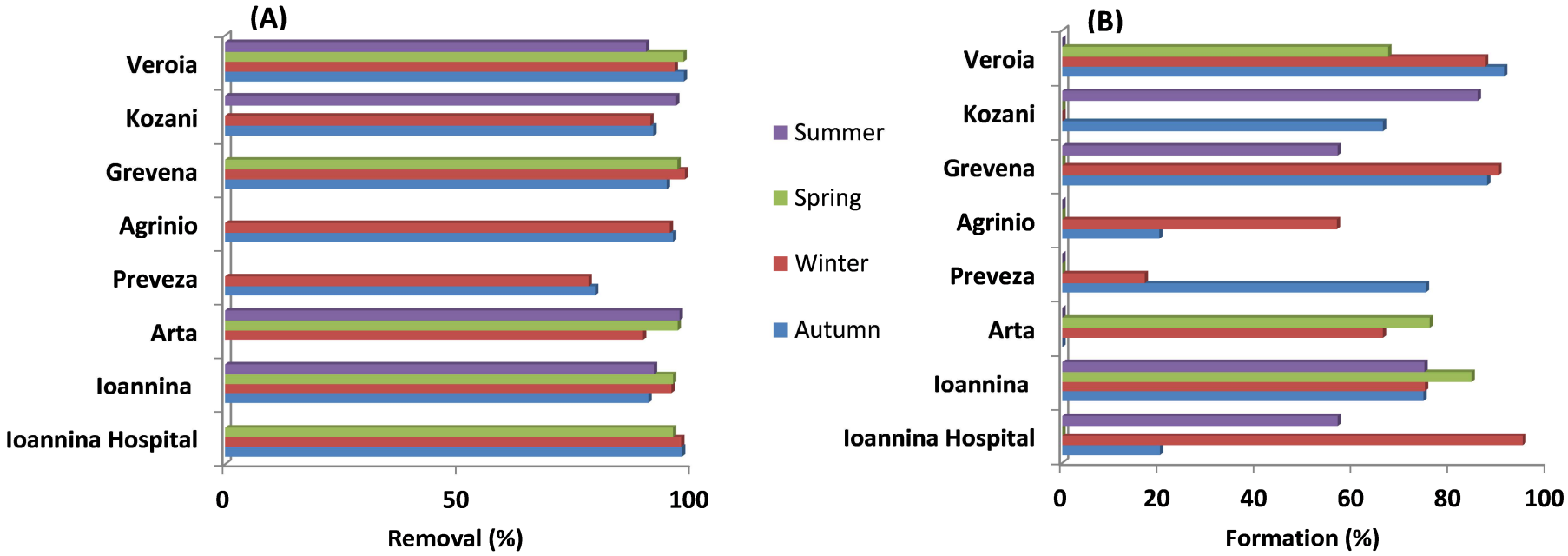


Fig. 5 - (A) Seasonal removal efficiency (%) of metformin and (B) Seasonal formation (%) of guanylurea.

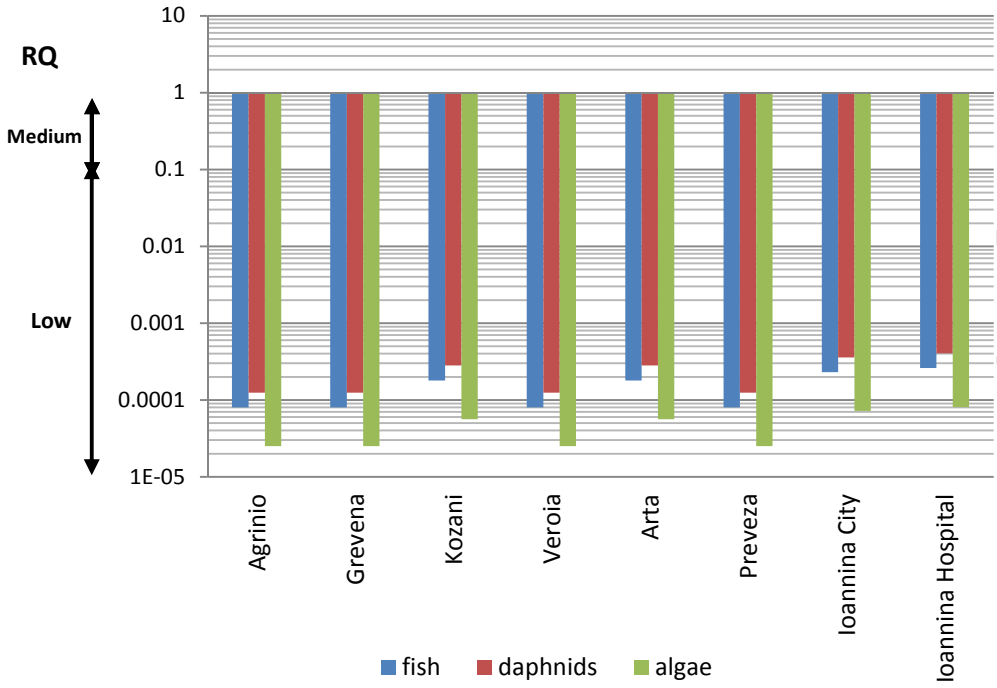


Fig. 6 - Risk quotients for metformin in effluent wastewaters of the eight WWTPs, for acute toxicity.

H I G H L I G H T S

- Comprehensive study of metformin and guanylurea in eight WWTPs in Greece.
- Further confirmation by means of LTQ Orbitrap MS.
- The Metformin/ Guanylurea ratio appears to be variable.
- Concentration of guanylurea mostly exceeds its parent metformin in the effluents.
- Risk quotients for metformin were lower than 1.

Supplementary Data (SD)

Comprehensive Study of the Antidiabetic Drug Metformin and its Transformation Product Guanylurea in Greek Wastewaters

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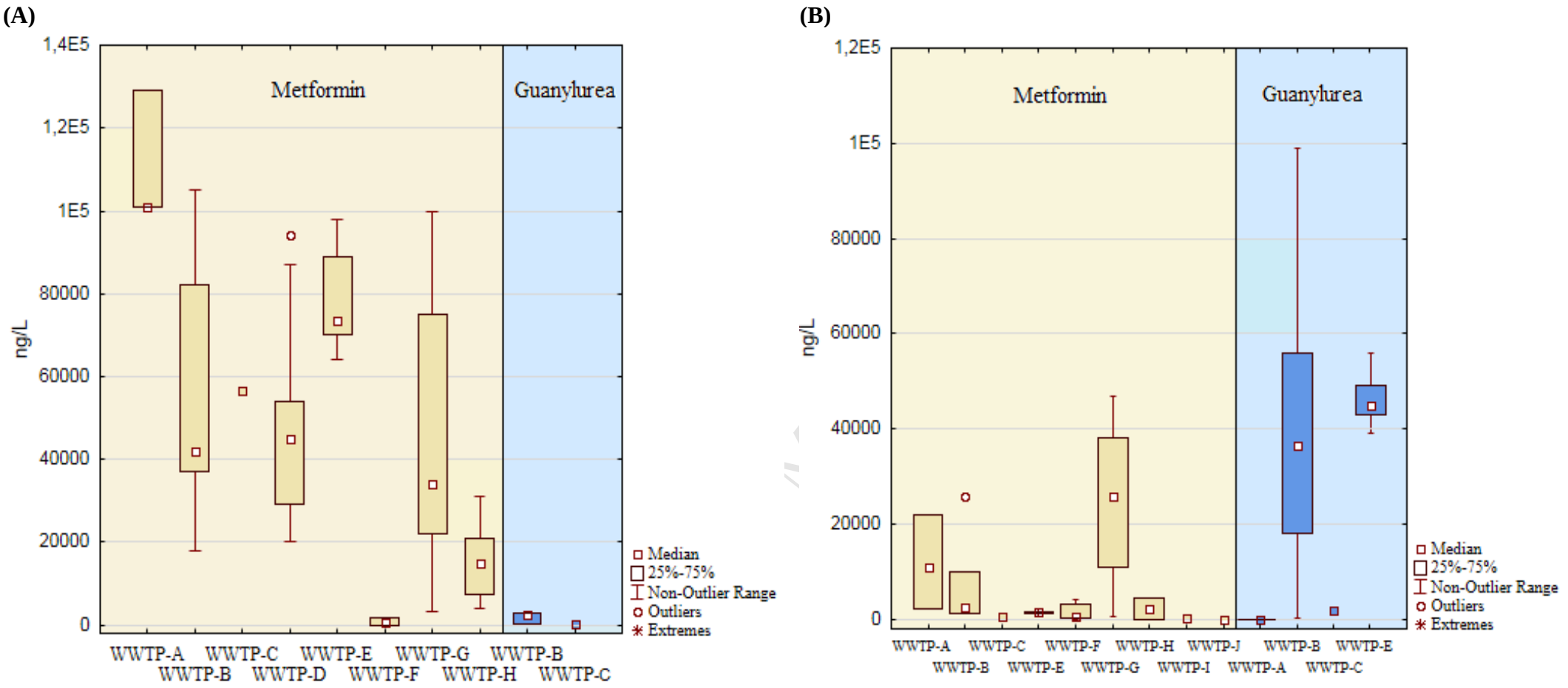


Fig. S1 - Concentration levels of metformin and guanylurea in (A) the influents and (B) the effluents of WWTPs, worldwide.

WWTP-A: Three WWTPs in Germany (Scheurer et al., 2009).

WWTP-B: Five WWTPs in Germany. The first (capacity 20,000 Population Equivalent-PE) applies mechanical and biological wastewater treatment and serves about 15,000 inhabitants. The second (capacity 875,000 PE) applies mechanical treatment with additional phosphate

precipitation, followed by biological treatment with a denitrification/nitrification unit with downstream trickling filters and serves about 350,000 inhabitants. The third (PE 75,000) and the fourth (12,000 PE) serve about 35,000 and 12,000, respectively. The fifth treats 380,000 PE with 220,000 residents living in the catchment area and the conventional mechanical and biological treatment is followed by the addition of 5-20 mg/L powdered activated carbon (Scheurer et al., 2012).

WWTP-C: Tallest STP in southern Germany with 600,000 PE (Trautwein and Kümmerer, 2011).

WWTP-D: Eighteen WWTPs in Belgium (Van Nuijs et al., 2010).

WWTP-E: Two WWTPs in Netherlands. A village with 7,220 inhabitants and a city with 157,052 inhabitants. Both consist of a conventional activated sludge system (CAS) with biological phosphate removal and nitrogen removal via nitrification/denitrification (Oosterhius et al., 2013).

WWTP-F: Influents from a WWTP in Portugal (213,000 PE) with primary and secondary treatment operating with tricking filters (receiving urban wastewaters including domestic wastewaters and hospital effluents). Effluents from four hospitals: University hospital with 1456 beds (serves approximately 430,000 inhabitants), general hospital with 350 beds (serves approximately 369,000 inhabitants), pediatric hospital with 110 beds (serves approximately 90,000 inhabitants) and a maternity hospital with 96 beds (serves approximately 507,000 inhabitants) (Santos et al., 2013).

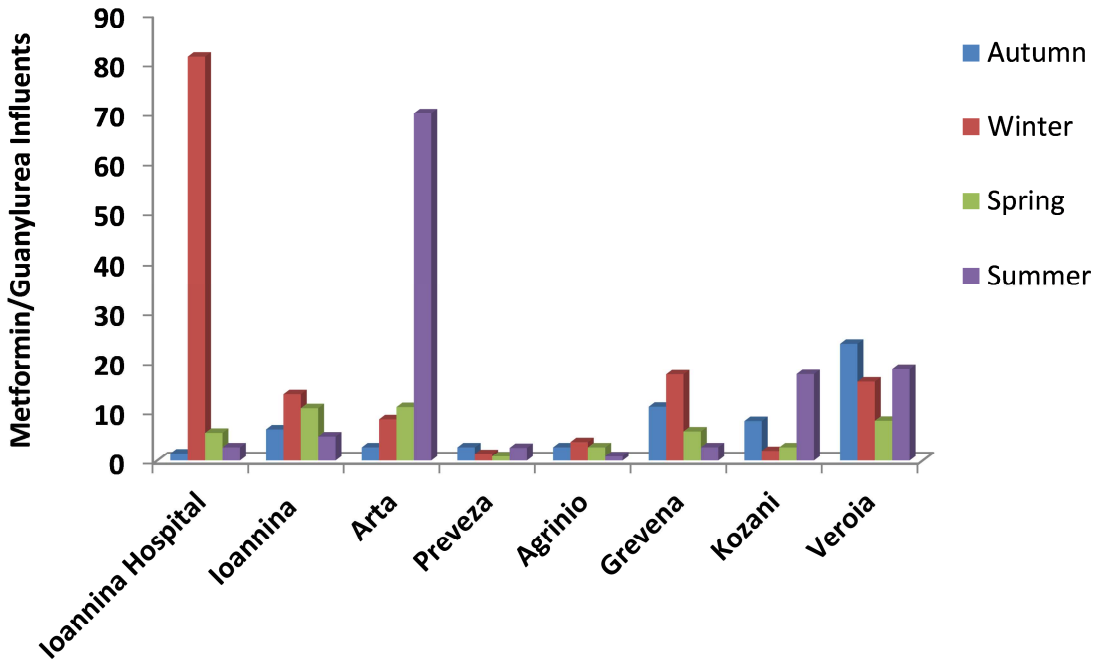
WWTP-G: A SSWRF services the greater area of Milwaukee (United States), has a treatment capacity of 1,135,000 m^3/day and it consists of preliminary treatment (7 bar screens/grit channels), primary treatment (16 primary clarifiers), activated sludge treatment (28 aeration basins and 24 secondary clarifiers) and chlorine disinfection (25-pass contact channels) (Blair et al., 2013b).

WWTP-H: STP of Almadinah Almunawarah (Saudi Arabia) with total capacity of 300,000 m^3/day. The plant undertakes tertiary treatment and sometimes in addition to domestic sewage, receives partially-treated industrial and medical wastewater (Shraim et al., 2012).

WWTP-I: WWTP in the region of Linz in Austria (Martín et al., 2012).

WWTP-J: STP in Kajang town (Malaysia) operating an extended aeration with 2,785 EP (Al-Odaini et al., 2010).

(A)



(B)

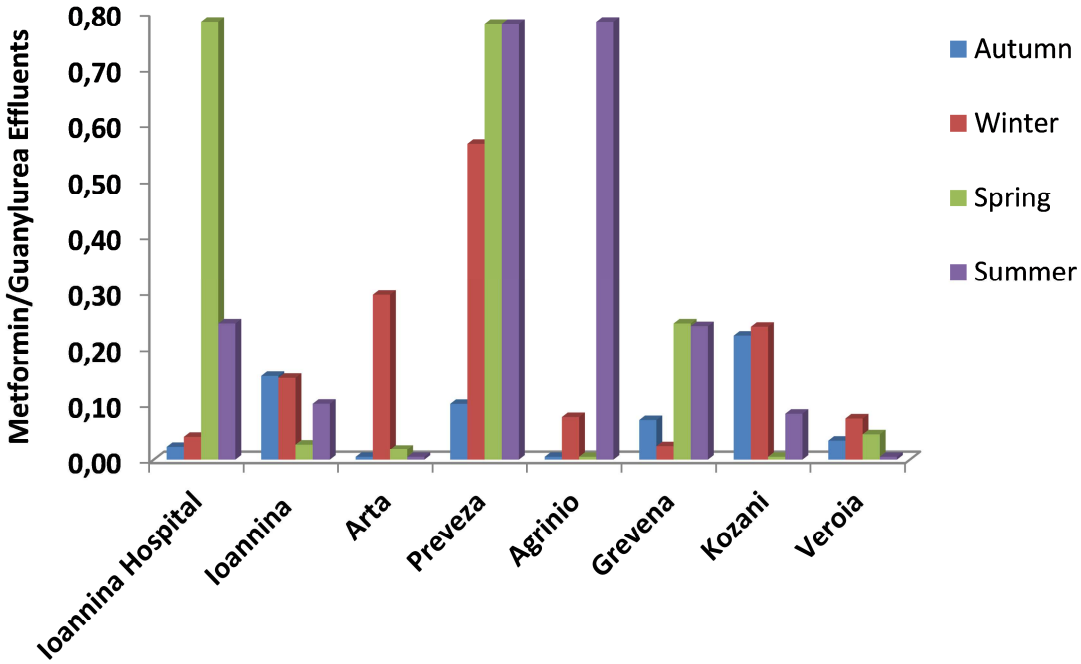


Fig. S2 - Ratios of Metformin/Guanylurea in (A) the influents and (B) the effluents of the WWTPs.

R E F E R E N C E S

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