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Occurrence of selected pharmaceuticals in wastewater treatment plants of Tuscany: An effect-based approach to evaluate the potential environmental impact

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ABSTRACT

Municipal wastewaters may pose a risk to the aquatic environment and ultimately to human kind. Their treatment is a fundament step but the actual WWTPs performances cannot be taken for granted, claiming instead for continuous evaluation campaigns. Our waters are indeed threatened by the continuous input of various persistent micropollutants that are part of human daily routine life; the potential effects of their presence in the receiving waters have to be quantified. The present paper reports data of a monitoring campaign focused on nine pharmaceuticals belonging to different therapeutic groups in three WWTPs in Tuscany (Italy). All the three WWTPs use conventional activated sludge process with pre-denitrification and no tertiary treatment. The analytical determination has been achieved through off-line solid phase extraction and analysis in liquid chromatography coupled with mass spectrometry. The overall ecotoxicological effect of effluents was evaluated through a battery of tests using organisms belonging to different trophic levels. All nine pharmaceuticals were detected in the influent of all WWTPs at least in one sampling campaign. The most concentrated compounds were acetaminophen, diclofenac and amoxicillin followed by atenolol, ketoprofen, clarithromycin, carbamazepine, doxycycline and E2; their average concentrations (considering all measurements from all plants) were, respectively: 3914 ± 2620; 2065 ± 739; 2002 ± 2170; 1223 ± 1042; 961 ± 1003; 356 ± 370; 233 ± 100; 196 ± 189; 4 ± 4 ng/L. The highest concentrations were found in the plant that treats urban and hospital wastewaters. Amoxicillin, atenolol and diclofenac were more concentrated in winter than in summer, while ketoprofen, doxycycline and 17-β-estradiol are higher in summer. These results are probably due to the different consumption of each drug during the year, depending on their therapeutic usage. Measured drugs can be divided into three categories: those ones that are generally well removed inside the WWTP (such as acetaminophen, ketoprofen and atenolol), the partly removed ones (doxycycline, clarithromycin and 17-β-estradiol) and the refractory ones to biodegradation during activated sludge process (carbamazepine, diclofenac and amoxicillin). Regarding ecotoxicological assays, the most sensitive organisms were *V. fisheri* and *R. subcapitata*, whereas *D. magna* almost never reacted to the wastewaters. Seasonal variability was not clearly observed among plants and collecting time. The toxicity score evaluated all the results coming from the bioassays battery, indicating that WWTPs treatments always determined a toxicity reduction, even though a residual toxicity was still measured. This observation, together with chemical data, clearly indicate WWTPs as an important source of pharmaceuticals in the Arno river with an important environmental toxicity; therefore, the reduction of pharmaceutical load originated from point source such us WWTPs would ask in the future the adoption of refinery steps in WWTPs able to increase RE of drugs.

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1. Introduction

Pharmaceuticals for human use represent a group of organic micropollutants widespread in the aquatic ecosystems (Rivera-Utrilla et al., 2013). Pharmaceutical compounds, especially when in mixtures, can present a threat to the aquatic ecosystems causing acute and chronic toxicity to aquatic organisms and undesired effects on human health (Vasquez et al., 2014). Major concerns arise from the direct exposure to the harmful molecules, but also from indirect effects associated to their transportation along the food chain; the consumption of food and water contaminated by pharmaceuticals residues is a potential health risk for humans (Barra Caracciolo et al., 2015).

Pharmaceuticals into water bodies can find their way into receiving bodies due to diffuse pollution (such as agricultural runoff or urban runoff) or point source pollution which is mainly caused by wastewater treatment plants (WWTPs) (Li, 2014). The first one has generally lower environmental loading, while WWTPs receive massive amounts of pharmaceuticals due to human and animal consumption and sewage discharge. Conventional chemical, physical and biological treatments are unable to completely remove micropollutants (Palli et al., 2017b; Rivera-Utrilla et al., 2013). Activated sludge processes, the most common wastewater treatment in worldwide, is inefficient against this emerging pollution issue. WWTPs performance can be enhanced by advanced techniques as activated carbon adsorption (Grover et al., 2011), advanced oxidation processes (Feng et al., 2013) but also bio-based systems. For instances, fungi have demonstrated their capability to remove pharmaceutical but they have not applied to full scale applications yet (Palli et al., 2017a). Being conventional WWTPs a weak containment barrier, pharmaceuticals are ultimately released in the aquatic basin causing the further contamination of river waters (Patrolecco et al., 2018; Praveena et al., 2018) or even drinking waters (Leusch et al., 2018).

This general background is unfortunately worsened by a missing strict worldwide legislation. In the European context, the quality standards are regulated by the Water Framework Directive, including a watch list of priority substances. In such list in 2015, the European Commission (EC) incorporated diclofenac, a commonly-used generic painkiller, and the hormones 17-α-ethinylestradiol (EE2) and 17-β-estradiol (E2), known as disruptors of the endocrine system in humans and fish (JRC Technical Report, 2015). Biological active compounds have indeed proved to threaten the ecosystems health, claiming for stricter control (inter alia Kidd et al., 2007): the most recent update enlarges the spectrum of chemicals to be monitored with few antibiotics (e.g. macrolide antibiotics, amoxicillin, ciprofloxacin) (Directive, 2018/840/EC). EC acknowledged warnings against pharmaceuticals occurrence in surface waters, asking also for additional information on the European water status.

Although recent researches are developing more and more sophisticated analytical methods (Appa et al., 2018) with limit of detection comparable to environmental concentration (Leusch et al., 2018), the chemical characterization of a real wastewater is still a challenge. Due to their multifaceted pollution source, several compounds or metabolites are unknown, influencing the actual possibility to characterise this complex matrix. Besides, not-complete chemical analyses alone are useless to predict the actual toxicity and the potential threat to aquatic organisms. Effect-based methods may instead overcome the problem, gaining a step forward to the evaluation of the possible effects of contaminated waters on the ecological status (Desbiolles et al., 2018; Tousova et al., 2017). Bioassays respond to the sample as a whole, detecting cumulative effects of known and undetected chemicals, insensitive to the environmental low concentration, e.g. below ng/L.

The main aim of the present paper is to monitor the occurrence of pharmaceuticals in both the influent and the effluent of three WWTPs in Tuscany (Italy) through a year sampling campaign, to estimate the removal efficiencies of the different plants and to assess the overall ecotoxicological effect of effluents. The concentrations of nine pharmaceutical compounds belonging to different therapeutic groups was evaluated: one hormone (E2), three non-steroidal anti-inflammatory drugs (diclofenac, ketoprofen, acetaminophen), three antibiotics (amoxicillin, doxycycline, clarithromycin), one β-blocker (atenolol) and one antiepileptic drug (carbamazepine). In order to provide a more comprehensive quality evaluation of the tested wastewaters, analytical data were coupled with an effect-based approach capable of describing the response of the potential perturbed ecosystem.

2. Materials and methods

2.1. Standards and chemicals

Diclofenac, ketoprofen, acetaminophen, carbamazepine, atenolol, clarithromycin, amoxicillin, doxycycline and E2 were purchased from Sigma–Aldrich. 1.2-dimethylimidazole-4-sulfonyl chloride (DMISC) was purchased from Oakwood Products. Isotopically labelled compound used as internal standard for both methods, bisphenol A-d16, acetaminophen-d4, Atenolol-d7, Carbamazepine-d10, Ketoprofen-d3 and E2-d5 was purchased from Sigma-Aldrich (St. Louis, MO, USA), Clarithromycin-N-methyl-d3, Diclofenac-d4, EE2-d4 was purchased from Giotto Biotech S.r.l. (Sesto Fiorentino, FI, Italy).The cartridges used for solid phase extraction (SPE) were Oasis HLB (60 mg, 3 mL) from Waters Corporation; SPEC C18 cartridge (3 mL) was purchased from Agilent. HPLC-grade acetonitrile was purchased from J.T. Baker. Analytical-grade acetone, hexane, and methanol were purchased from Mallinckrodt Baker. Water was purified with a MilliQ system.

2.2. Sampling sites and collection

Influents and effluents of three WWTPs in Tuscany were collected. WWTP1 treats urban wastewater (including hospital wastewater) and owns a potentiality of about 600,000 population equivalent (P.E.) while WWTP2, which owns a potentiality of 120,000 P.E., receives both sewer and hospital wastewater. WWTP3 is a medium-to-small plant (37,500 P.E.) and treats urban wastewater mainly of domestic origin with a negligible contribution from industrial activity. All the three WWTPs use conventional activated sludge process with pre-denitrification (Fig. 1). The treatment line of the three WWTPs in one of the most common in Italy: pre-treatment (bar screen and grit removal), primary treatment (primary sedimentation), secondary treatment (pre-denitrification, oxidation tank and secondary settler) and final disinfection. No tertiary treatments are present in none of the selected WWTP.

Composite 24 h samples were collected at the inflow and outflow of each WWTP and from the hospital flux in WWTP2, using high-density polyethylene containers previously rinsed with demineralized water and left to dry. Samples were immediately refrigerated during transport to the lab and then frozen and stored at −20 °C until chemical analysis.

Three sampling campaigns have been carried out: the first one in May 2016, the second one in December 2016 and the third in May 2017.

2.3. Analytical methods

The analytical determination of pharmaceuticals has been achieved through off-line solid phase extraction and analysis in liquid chromatography coupled with mass spectrometry, following a modified version of a previously reported protocol (Dugheri et al., 2018).

The isotope-labelled internal standards were added to the samples, and then samples were extracted by SPE off-line. For the determination of E2, samples were also derivatized with 1,2-dimethylimidazole-4-sulfonyl-chloride. LC-MS analysis have been performed with an AQUITY UPLC H-Class system-MS/MS Xevo TQ-S with StepWave ion guide. Separation of target compounds was obtained with column Cortecs C18 except for hormones which was separated with a CSH

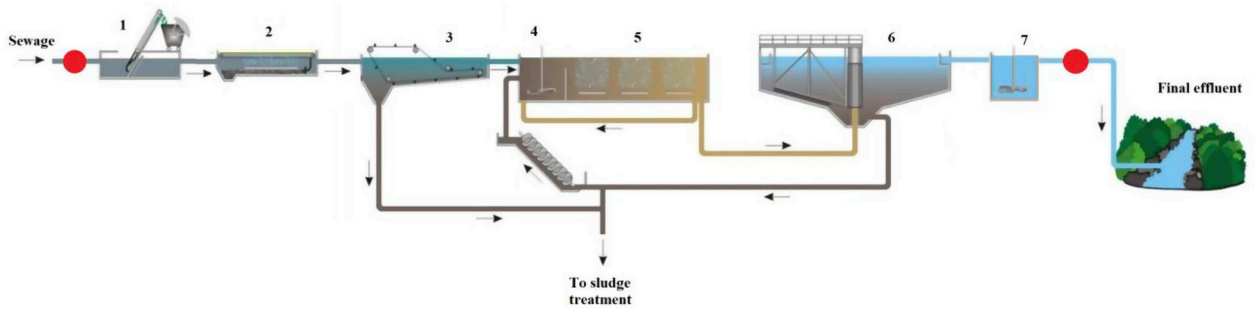

Fig. 1. Flow diagram of the WWTPs with indication of sample points (red circles). In WWTP2 also the hospital wastewater separately bestowed was sampled and analysed. 1: Bar screen; 2: Grit removal; 3: Primary settler; 4: Pre-denitrification; 5: Oxidation tank; 6: Secondary settler; 7: Disinfection. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Phenyl-Hexyl column. Limits of detection (LOD) of selected pharmaceuticals were calculated to be: 0.50 ng/L for diclofenac, 1.61 ng/L for ketoprofen, 29.54 ng/L for acetaminophen, 0.41 ng/L for carbamazepine, 1.84 ng/L for atenolol, 0.01 ng/L for clarithromycin, 59 ng/L for amoxicillin, 44 ng/L for doxycycline and for 0.02 ng/L for E2. Further information about the analytical method are published elsewhere (Dugheri et al., 2018).

2.4. Removal efficiency

Removal efficiency (RE) of the target pharmaceuticals was evaluated by dividing the difference between the influent and the effluent concentrations by the influent concentration.

2.5. Ecotoxicological analysis

For each test, serial dilutions were performed, from 100% to 3%. When the I% was above 50%, the EC_{50} and Toxic Units (TU) were calculated. By definition, EC50 is the concentration of the sample that gives the 50% of the effect, i.e. 50% of the algal growth inhibition. Positive controls were set up in the absence of wastewater samples.

Raphidocelis subcapitata (previously known as *Selenastrum capricornutum*) growth inhibition test was performed according to UNI EN ISO 692:2012. Samples were incubated with algae and concentrated nutrient solution for 72 ± 2 h at 23 ± 2 °C, 85% humidity and 8000 ± 2000 lux. After three days of incubation, algal growth was measured (TECAN Infinite M200) as the photosynthesis rate, evaluating the chlorophyll fluorescence yield (chlorophyll fluorometer ToxY-PAM, Walz Elektronik Gmbh, DEU). The inhibition percentage (I%) was calculated in comparison with the positive control. Negative controls were also set up in the absence of the algal inoculum to evaluate the wastewater absorbance.

Three terrestrial plants (e.g. *Sorghum commune*, *Lepidium sativum* and *Cucumis sativus*) were used for phytotoxicity evaluation, according to the standard UNI 11357:2010. The seeds were purchased from Ingegnoli S.p.A. (Milan). The seeds were incubated for 72 h in the dark at 25 ± 2 °C. Two different endopoints were analysed: the germination index (GI%) was indeed calculated measuring the germinated seeds and the average radical length.

Daphnia magna test was performed according to the UNI EN ISO 6341:2013, evaluating the development of wealthy individuals as endpoint of the test. Every sample was analysed with four replicates with five daphnids each. The organisms were incubated with a photoperiod of 16 h, luminous intensity of 1000 ± 200 lux at 20 ± 2 °C. After 48 h, immobile animals were counted and compared with the control.

Luminescent bacteria (*Vibrio fischeri*) test was performed according to the UNI EN ISO 11348–3, using the Microtox® toxicity system (Microtox M 500; Ecotox LDS SRL., ITA). *V. fischeri* strain NRRL B-11177 was incubated at oxygen ≥3 mg/L, 20–30 °C, pH 6–9 and salinity between 20 and 50‰. The luminescence intensity was measured after 30' and compared with the control.

2.6. Ecotoxicological toxicity score and statistical evaluation

The toxicity scores (TS) was developed modifying the model of Hartwell (1997), as already reported by Tigini and collaborators (2011). It considered several variables, such as severity of the effect, percentage of response, statistic relevance of the response (statistical correction coefficient), number of total endpoints and number of significant endpoints. Only the tests having a significant toxicity strongly contributed to the calculated TS. Seven endpoints were followed in the present battery of tests, but the weight of each endpoint was different accordingly to the effect severity: mortality = 5, bioluminescence inhibition = 4, growth/reproduction inhibition = 4.5, growth/development inhibition = 4, photosynthesis inhibition = 3. The statistical correction coefficient took into consideration the variability of each test and each sample: when sample data were not statistical different from the control, the coefficient helped to reduce the weight of this test in the TS calculation.

The toxicity assessment (TA) considered TS values, the consistency of the battery and the toxic units (TU) values obtained from the bioassays of the battery, providing a final toxicity output as follow: TA ≤ 10% = absent; 10% < TA ≤ 30% = low (or high if the consistency is < 0); 10% < TA ≤ 30% = moderate; 30% < TA ≤ 50% = high (or very high if TU ≥ 10, or extremely high if TU ≥ 100); 50% < TA ≤ 75% = very high (or extremely high if TU ≥ 100); TA > 75% = extremely high.

3. Results and discussions

3.1. Occurrence of pharmaceuticals in monitored WWTPs

Concentrations of selected pharmaceuticals measured in the influent and effluent of the three WWTPs during the three sampling campaigns are summarized in Table 1. All pharmaceuticals were detected in the influents of all WWTPs at least in one sampling campaign. The most concentrated compounds were acetaminophen, diclofenac and amoxicillin followed by atenolol, ketoprofen, clarithromycin, carbamazepine, doxycycline and E2; their average concentrations (considering all measurements from all plants) were, respectively: 3914 ± 2620; 2065 ± 739; 2002 ± 2170; 1223 ± 1042; 961 ± 1003; 356 ± 370; 233 ± 100; 196 ± 189; 4 ± 4 ng/L. These findings are in agreement with literature data, even though there are often contrasting results. For instance in WWTPs of Rome (Italy), Patrolecco et al. (2015) found similar levels of diclofenac (514–2230 ng/L) and carbamazepine (110–1519 ng/L). On the other hand, the same study found ketoprofen only in two out of four WWTPs, at lower amount

Table 1
Measured concentrations (ng/L) of selected pharmaceuticals in the influent and effluent of the three WWTPs during the three sampling campaigns.

WWTP	Campaign	Sampling point	Compound								
			Acetaminophen	Carbamazepine	Ketoprofen	Atenolol	Diclofenac	Amoxicillin	Doxycycline	Clarithromycin	E2
WWTP1	May 2016	In	741	205	819	561	1957	518	176	180	5
		Out	< LOD	247	182	58	2364	813	< LOD	79	5
	December 2016	In	1455	298	1006	1419	3429	5692	< LOD	309	< LOD
		Out	< LOD	147	144	83	1463	1559	< LOD	121	< LOD
	May 2017	In	5724	146	3511	631	2647	n.a.[a]	n.a.[a]	312	n.a.[a]
		Out	< LOD	196	512	56	811	n.a.[a]	n.a.[a]	< LOD	n.a.[a]
WWTP2	May 2016	Hospital	4227	73	675	864	113	470	93	546	10
		In	5264	333	512	1733	2103	662	520	155	8
		Out	< LOD	726	111	359	2199	436	< LOD	146	5
	December 2016	Hospital	28894	520	1147	1285	601	543	< LOD	2337	7
		In	8556	375	1146	3707	2641	241	86	261	< LOD
		Out	251	847	213	558	4882	7692	< LOD	565	< LOD
	May 2017	Hospital	4007	240	230	302	522	n.a.[a]	n.a.[a]	800	n.a.[a]
		In	5443	311	654	1352	1349	n.a.[a]	n.a.[a]	1303	n.a.[a]
		Out	< LOD	576	43	468	1377	n.a.[a]	n.a.[a]	503	n.a.[a]
WWTP3	May 2016	In	1181	143	351	540	1651	1345	309	53	8
		Out	< LOD	129	72	61	1564	1000	< LOD	173	5
	December 2016	In	2459	87	247	539	1766	3551	58	220	< LOD
		Out	444	150	31	299	3697	4618	< LOD	412	< LOD
	May 2017	In	4407	198	400	528	1038	n.a.[a]	n.a.[a]	407	n.a.[a]
		Out	< LOD	166	59	225	1022	n.a.[a]	n.a.[a]	455	n.a.[a]

[a] Not available.

(63–198 ng/L). In Portuguese wastewaters, similar levels of acetaminophen (3536 ng/L) were found but lower levels of diclofenac (27 ng/L) (Pereira et al., 2015). A Spanish study found higher concentration of acetaminophen (12955 ng/L), comparable concentration of ketoprofen (506 ng/L), clarithromycin (100 ng/L) and atenolol (2224 ng/L) but lower concentration of diclofenac (288 ng/L) and carbamazepine (27 ng/L) (Collado et al., 2014). Although data were comparable for acetaminophen (1422 ng/L) and atenolol (1720 ng/L) in Greek wastewaters, lower concentration of diclofenac (413 ng/l), carbamazepine (34 ng/) and ketoprofen (75 ng/L) were measured (Papageorgiou et al., 2016).

In the present study, as expected, hospital wastewater was very polluted, mostly by acetaminophen, clarithromycin and E2. Consequently, WWTP2 showed the highest influent concentrations of these three compounds but also of carbamazepine, atenolol and doxycycline. WWTP1 was mainly contaminated by ketoprofen, diclofenac and amoxicillin. The presence of hospital confirmed to be a major stressor for the quality of waters. Indeed, the only plant that does not deal with hospital wastewater (WWTP3) showed the lowest values of drugs in the influent wastewater.

The one-year sampling campaign often highlighted the correlation between measured concentration of pollutants and the season. For instance, amoxicillin, atenolol and diclofenac were more concentrated in winter than in summer (400%, 100% and 45% higher, respectively). Data indicated an opposite behaviour for ketoprofen, doxycycline and E2, detected at higher concentration in summer. As regards acetaminophen, carbamazepine and clarithromycin were instead unaffected by the time, indicating eventually a more constant use during time. These results are probably due to the different consumption of each drug during the year, depending on their therapeutic usage (Kosma et al., 2014). As expected data were not always concordant. Among seasons, different behaviours occur, affecting the actual final concentration. For instance, the concentration in wastewater is expected to be lower in the wet season, due to dilution effect (Barbosa et al., 2018). Besides other factors as adapted wastewater bacteria, high contaminant concentration, COD values, water temperature, the occurrence of stagnant surface-water zones, the presence of pollutants in the sediment or at the bed surface have proved to affect the final concentration of micropollutants (Guillet et al., 2019).

3.2. Removal of pharmaceuticals in WWTPs

According to the scientific literature, the fate of pharmaceuticals differs among WWTPs. According to Clara et al. (2005), a nearly complete removal of pharmaceuticals is associated to removal efficiencies higher than 90%, while comparable concentration of effluent and influent indicate a negligible removal. Within this range, Oppenheimer et al. (2007) classify the RE of micropollutants in three categories: excellent removal (RE > 80%), moderate (50% < RE < 80%) and poor removal (RE < 50%).

Our results confirmed the heterogeneous behaviour of pharmaceuticals: REs of selected compounds are different for each compound, each plant and each sampling time.

To better understand the fate of pharmaceuticals in a WWTP, it is important to highlight that removal pathways of organic compounds are a function of separate processes, as biotransformation/biodegradation, adsorption on excess sludge removed from the biological process and stripping (Urase and Kikuta, 2005). This latter fraction is usually neglected due to the low values of the Henry coefficients (KH) of the compounds investigated (Radjenovic et al., 2007). The other two processes cannot be here distinguished and the term “removal” is therefore used here as the results of the two phenomena. According to our results, the removal of acetaminophen and ketoprofen was complete/excellent regardless the operational parameters in the three WWTPs. In fact, for acetaminophen, RE was more than 99% in each sampling campaign and in each plant, with the exception of the December sampling in WWTP3 when the RE was 82%. Ketoprofen showed RE between 78% and 93%. Contrariwise, atenolol RE was plant dependent, ranging from medium to excellent: 90–94% for WWTP1, 65–85% for WWTP2 and 45–89% for WWTP3. This behaviour could suggest that the removal of atenolol is influenced from the operational parameters of the WWTPs, as already reported by other authors (Radjenovic et al., 2007).

Carbamazepine, diclofenac and amoxicillin were very recalcitrant to biological oxidation, presenting often a negligible removal. The average RE was instead negative, being the effluent concentration higher than in the influent. This behaviour, already observed by other authors (Collado et al., 2014), can have multiple explanations: i) the conversion of conjugated metabolites to their parent compound through enzymatic

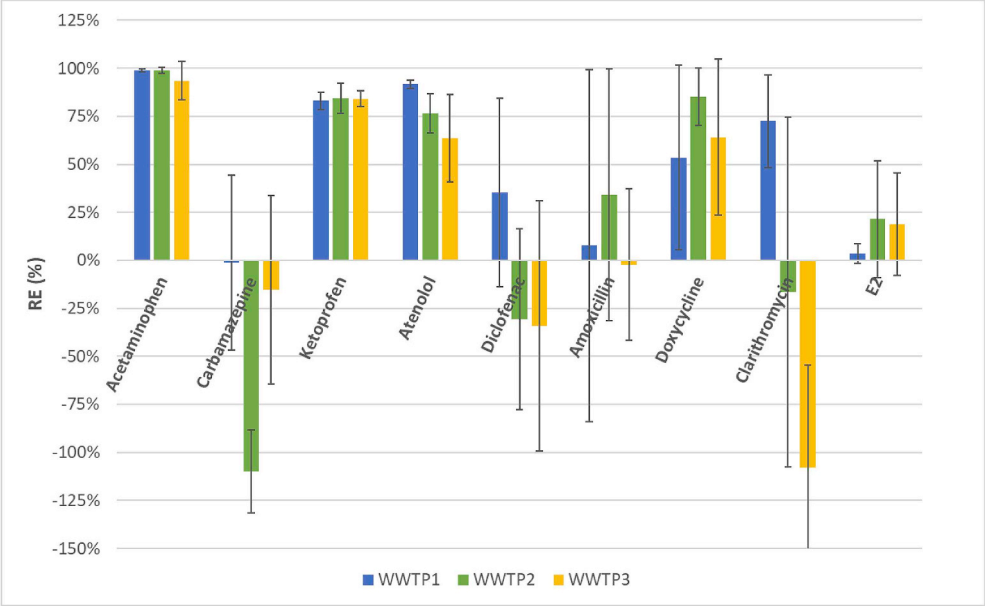

Fig. 2. Mean Removal Efficiency (RE) (%) of the three WWTPs for each studied compound.

processes in the WWTPs; ii) release of pharmaceuticals from sludge; iii) sampling variations due to long hydraulic retention times (Kosma et al., 2014) and iv) the difference in analytical capabilities for the different matrices (Oppenheimer et al., 2007). These results are in agreement with previous observations which reports carbamazepine and diclofenac among the most recalcitrant compounds (Bessa et al., 2017), while acetaminophen is reported to be a good substrate for activated sludge (Aymerich et al., 2016; Pereira et al., 2015). Finally, doxycycline, clarithromycin, and E2 showed a very variable behaviour: RE varied from 20% to 96% for doxycycline, from negative RE to 99.9% for clarithromycin and from less than 1%–43% for E2.

Regarding the differences among each plant, it can be seen that WWTP1 achieved better results for acetaminophen, ketoprofen, atenolol, diclofenac and clarithromycin (99%, 83%, 92%, 35% and 72%, respectively), while WWTP2 was more effective for amoxicillin, doxycycline and E2 (34%, 85% and 21%, respectively) (Fig. 2). This difference among the WWTPs can be partially explained by considering the different solid retention time (SRTs) of the three WWTPs: WWTP1 has an average SRT in the range of 30–40 d, WWTP2 of 20 d and WWTP3 of 15 d. As already reported by other authors (Clara et al., 2005), the operation of WWTPs with higher SRTs may increase the removal potential regarding selected micropollutants. It must be taken into account that the higher is the SRT the higher is the organic substrate removal and that in conventional WWTPs high SRTs are associated to high hydraulic retention times; in conventional WWTPs high SRTs are associated to high hydraulic retention times. Moreover, WWTP3 is the plant with, on average, lowest concentrations of pharmaceuticals, which, in general, lead to lower RE.

Comparing warm and cold seasons (Fig. 3), it is interesting to notice that average removal (considering all plants and all compounds) was higher in May (39%) than in December (19%). The main reason can be associated to the WWTPs performances in time: lower temperatures of winter season may slow down biodegradation inside the sewage system resulting in increasing concentrations in the inflow of the WWTP (Barbosa et al., 2018; Li et al., 2018). Probably, due to higher temperatures registered in May, the biodegradation rates by activated sludge was enhanced, as already observed by other authors (Česen et al., 2018; Collado et al., 2014; Papageorgiou et al., 2016). Moreover, sorption, which also play an important role in micropollutants removal, depends on temperature (Urase and Kikuta, 2005). For many compounds, sorption yields increase with decreasing temperatures while biodegradation is less effective when the temperature decreases (Deblonde et al., 2011). This phenomenon is particularly true for diclofenac, doxycycline and E2, but not for the other compounds whose REs were similar in both periods.

3.3. Ecotoxicological assessment

Six standard bioassays were used in the ecotoxicity evaluation of WWTPs influents and effluents. As regard the alga *R. subcapitata* two endpoints were evaluated, assessing the effects of the waters on the algal development and the photosynthesis yields. The organisms were chosen because representative of different trophic levels and representative of the possible effects of wastewaters on aquatic (direct) and terrestrial (indirect) ecosystem. As agricultural practices are responsible of around 70% of water used in the world (OECD, 2012), phytotoxicity studies are important to evaluate long-transfer toxicity issues. Unfortunately, these organisms are rarely taken into consideration, as demonstrated by Destrieux et al. (2017): inside a database with around 125 literature references, duckweed plant and crustacean species, followed by *R. subcapitata* and *V. fischeri*, are the most commonly used species for ecotoxicity assessment of pharmaceutical substances. In the present study, the information of alga, bacteria and crostaceous were coupled with three plants, investigating the early development phases as the germination and the root elongation.

Table 2 reports results obtained performing different bioassays. As expected, these data confirmed that a single bioassay cannot be consider exhaustive to assess the environmental toxicity of a sample. Since different organisms showed different sensitivity, only a battery of tests can produce reliable ecological predictions. As can be seen from Table 2, all the influents resulted toxic for at least one model organism. The most sensitive organisms were *V. fisheri* and *R. subcapitata*, whereas *D. magna* almost never reacted to the wastewaters: 90% of samples induced a significantly algal or bacterial inhibition, but only one sample cause the same effect on *D. magna*. These data are in accordance to several studies that indicate the alga and the bacterium as among the most sensitive and reliable organisms for wastewater quality evaluation (Abbas et al., 2018; Gorenoglu et al., 2018; Ortiz de García et al., 2014; Spina et al., 2018). As regards *D. magna,* contrasting results can be found in literature. Despite capable of responding to heavy metals (Cui et al., 2018), only a slight toxicity was associated to secondary effluents (Maselli et al., 2015; Rizzo et al., 2009). For instance, even though *D.*

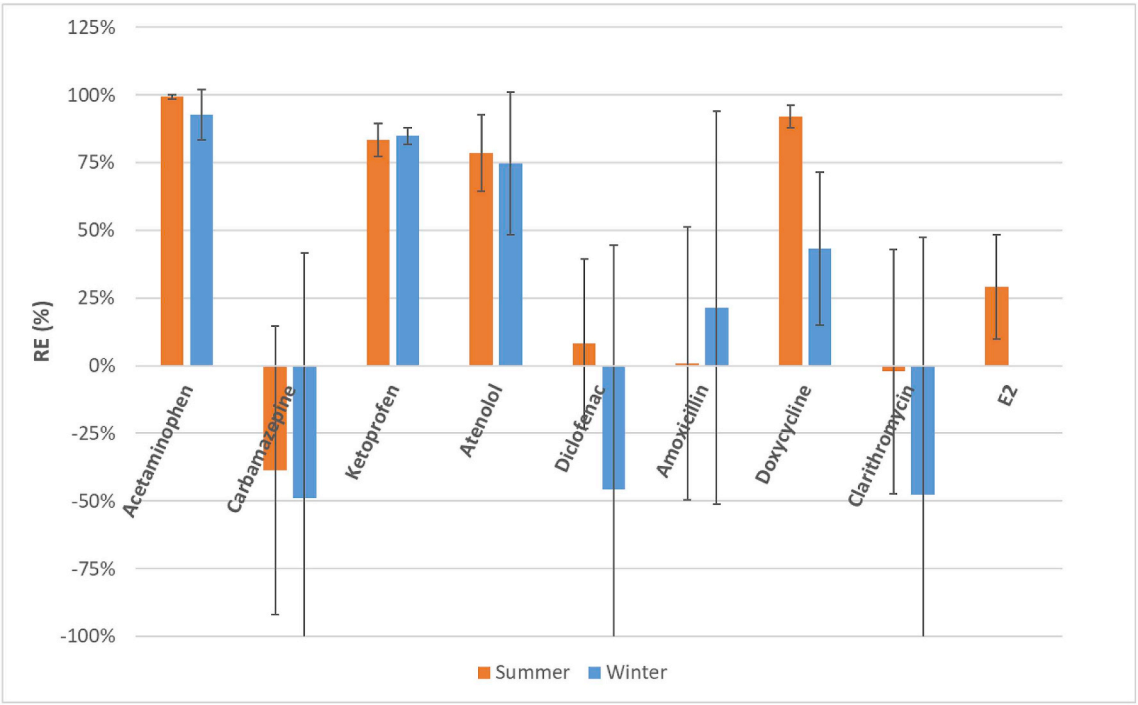

Fig. 3. Mean Removal Efficiency (RE) (%) obtained from all WWTPs for summer and winter campaign, for each studied compound.

magna was severely affected by single pharmaceutical (amoxicilline, carbamazepine, diclofenac) solutions and their mixture, it was not sensitive to a real wastewater (Rizzo et al., 2009).

Concerning phytotoxicity, an inhibition phenomenon was observed in 37% of samples. This should not mislead the attention, because 33% of samples significantly stimulated the plants development. The maximal GI% was observed for WWTP1 effluent collected in May 2016, when *S. commune* development increased up to 71% in comparison with the control. Even though this is not a common finding, few reports have already reported stimulatory effects of wastewaters on ecotoxicological bioassays, and explained by hormetic behaviour or nutritional interaction (Delistraty and Yokel, 1999; Freitas et al., 2017; Rosal et al., 2010). As highlighted by Freitas et al. (2017), the capability of waters contaminated by micropollutants of stimulating specific ecotoxicological endpoints should not be considered as a positive response. By definition, ecotoxicity includes any developmental or behavioural modification from the natural status. Even the presence of molecules that act as nutrients or growth inducers has to be carefully considered because ultimately capable of causing alterations to the receiving ecosystem (Freitas et al., 2017).

Seasonal variability was not so evident; nor the model organism or WWTPs were influenced by the season. For instance, in December,

Table 2
Inhibition percentage (I%) of the bioassays' endpoints, i.e. bioluminescence inhibition of *V. fischeri,* development inhibition of *D. magna*, growth and photosynthesis inhibition of *R. sabcapitata,* inhibition of the germination index (analysing both germination and root elongation) of *S. commune, L. sativus* and *C. sativum*. High I% values indicate a high toxicity; negative results should be interpreted as stimulatory effects, as toxic as well.

WWTP	Campaign	Sampling point	Ecotoxicological bioassays						
			V. fischeri	*D. magna*	*R. subcapitata growth*	*R. subcapitata photosynthesis*	*S. commune*	*L. sativus*	*C. sativum*
WWTP1	May 2016	In	2.3	0.0	63.3	39.8	−23.9	−16.3	27.2
		Out	−34.4	0.0	19.9	9.8	−17.2	7.6	40.7
	December 2016	In	85.7	0.0	58.4	13.1	39.2	12.4	−27.3
		Out	−24.2	0.0	12.4	0.1	20.5	16.9	17.0
	May 2017	In	−1.3	0.0	41.2	22.0	45.4	45.3	16.3
		Out	−31.6	0.0	12.8	10.0	18.4	14.6	28.4
WWTP2	May 2016	Hospital	63.9	0.0	59.7	6.4	−33.8	−7.4	−25.4
		In	94.2	0.0	72.7	7.3	−71.2	−2.6	7.6
		Out	−5.6	0.0	14.6	0.0	8.8	−1.0	14.4
	December 2016	Hospital	−12.1	0.0	20.9	2.1	2.9	23.2	−30.6
		In	67.0	0.0	53.4	13.5	−13.1	−11.3	−30.2
		Out	−40.8	0.0	23.9	−2.3	−5.1	23.5	−14.8
	May 2017	Hospital	68.3	100.0	98.2	34.1	38.6	26.2	25.2
		In	81.3	100.0	95.5	41.0	45.0	24.8	16.8
		Out	10.7	0.0	10.1	15.0	−0.3	13.8	22.5
WWTP3	May 2016	In	89.4	0.0	74.0	28.5	4.1	4.5	7.3
		Out	28.8	0.0	23.2	0.6	17.9	−21.0	−3.7
	December 2016	In	69.0	0.0	58.0	18.7	−33.4	−5.3	−27.5
		Out	−16.9	0.0	14.2	−2.1	26.7	15.0	−28.6
	May 2017	In	95.4	0.0	48.6	30.0	44.1	2.0	8.1
		Out	−31.9	0.0	16.7	1.1	39.3	6.7	32.5

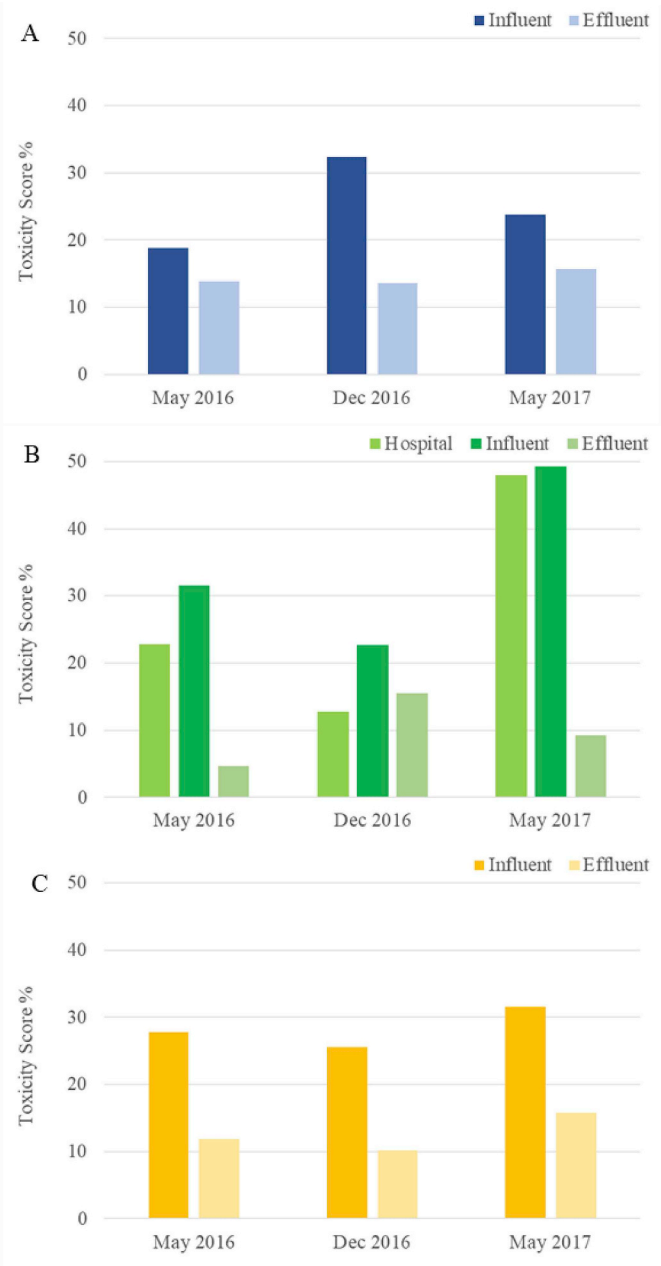

Fig. 4. Toxicity scores (TS) of the hospital wastewater (white), influents (dark grey) and effluents (light grey) of WWTP1 (A), WWTP2 (B) and WWTP3 (C) collected during the three seasons. The TS was calculated balancing the severity of the effect, the percentage of response, the statistic relevance of the response and the number of endpoints.

WWTP1 influent was more toxic for *V. fischeri*, but this was not true for *S. commune* or *C. sativum*. Hospital wastewater is the major exception, showing lower toxicity for December samples (null effect for three bioassays, moderate inhibition/stimulation for 4 endpoints) and higher toxicity for May 2017 samples (I% 25–100% for all bioassays). As already reported in literature (Rivas Ibáñez et al., 2017; Soupilas et al., 2008), these data denoted that ecotoxicity poorly reflected influents chemical composition reported in Table 1. Bioassays instead reacted to the whole micropollutants scenario (known and un-known) and to possible synergic effects.

These findings confirmed how important ecotoxicological bioassays are to describe the water quality, providing more information than direct assessment of chemicals concentration or prediction analysis could do (Affek et al., 2018; Ortiz de García et al., 2014).

WWTPs treatments significantly changed the tests responses but data were scattered and often contrasting among bioassays. As regards *R. subcapitata,* a clear detoxification was observed for both the growth (55–89% toxicity reduction) and the photosynthesis (55–100%). According to plants, in only 37% of cases, WWTPs reduced the toxicity while 22% of samples showed no difference compared to the influent. In many cases (41%), effluents were more toxic (more inhibited or more stimulated) than influents. A possible explanation can be given by the presence of transformation products that cannot be univocally considered as less toxic than parent ones (Zuriaga et al., 2019; Khaleel et al., 2019). A similar phenomenon was observed in the *V. fischeri* test. For most samples (7 out of 9), the bacterium was stimulated by effluents. This is in deep contrast with influents, which caused instead a strong inhibition of the bacteria. All the WWTPs, regardless the treatments and the seasons, seemed indeed to modify the sample composition, producing effluents with remarkable stimulatory effects. Hormesis can be partially explained the bacteria behaviour, as already demonstrated for single or mixed solutions of heavy metals (Zou et al., 2017) and pharmaceutical and personal care products (PPCPs) (Ortiz de García et al., 2016). Indeed, at low concentrations (comparable with environmental ones) 20 PPCPs, including amoxicillin, clarithromycin and diclofenac, triggered a hormetic effect on *V. fischeri* (Ortiz de García et al., 2016). In the present study, it should be important to determine which chemicals or mixtures are causing this dose response curve to promote appropriate enforcing and preventive actions.

Bioassays were not always coherent among each other and some organisms (e.g. *D. magna*) resulted not suitable effect-based monitoring. The development of coherent and reliable batteries of ecotoxicological tests have been deeply analysed in literature, combining direct and indirect approaches as well as *in vivo* and *in vitro* bioassays (Grummt et al., 2013; Huguier et al., 2015). Many efforts have been directed to demonstrate that *in vitro* tests are a powerful and useful tool to evaluate water safety, as bioassays can be usefully applied to the ecoxicological assessment of drinking waters (Grummt et al., 2013). In the present study, the panel of the tested bioassays surely needs to be integrated with other model organisms and endpoints to investigate their sensitivity to municipal and industrial wastewaters, as well as their capability to implement a more precise ecotoxicological assessment. Despite this, the proposed battery may provide realistic information about the possible impact of the effluents on the receiving aquatic ecosystem. A strategy made by one single test would be instead influenced by its sensitivity to the specific samples under investigation: choosing a unique test seems to be a no-future perspective. The same approach resulted a winning strategy also in many other reports, where ecotoxicological bioassays were efficiently applied to municipal wastewaters (Rivas Ibáñez et al., 2017), urban and hospital effluents (Wigh et al., 2016), leachates (Aydin et al., 2015), textile and tannery wastewaters (Tigini et al., 2011), etc.

Effect-based methods are nowadays recognized to be an effective tool for risk assessment of both freshwaters (Jia et al., 2019) and wastewaters (Díaz-Garduno et al., 2016). In Europe, projects as SOLUTION and BioTox, and the NORMAN network have helped to increase the available information (Grummt et al., 2013; Brack et al., 2019). Despite several recommendations given (Brack et al., 2017), to date, a precise bioassays portfolio has not yet legally accepted worldwide for pharmaceuticals and micropollutants in wastewaters. Since researchers often use different bioassays, a precise comparison among wastewaters is difficult to draw because of the non-harmonization of the applied environmental assessments. Worldwide, several classification systems have been proposed but they are mostly based on single parameter (e.g. LC50, NOEC, TU) evaluation (Četkauskaite et al., 2016). More complex data elaboration have been pursued, as the application of Hasse diagram technique to define those tests that can better describe the underground water quality (Kudłak et al., 2014). There is the need to merge a multi-data panel of ecotoxicological bioassays in a single output. The extrapolation of all the results in a toxicological index that

balances the endpoint severity and the coherence of the battery may give a powerful tool for data comparison at technical and scientific level. Fig. 4 shows the TS data. Influents showed to be an actual risk for the ecosystem, with a toxicity ranging from moderate to very high. The worst scenario was associated to the WWTP2 sample collected in May 2017: both hospital and influent samples were very highly toxic with a TS value closed to 50%. WWTPs were effective in TS decrease (from 26.8 to 85.2%) but it did not always mean a change in the toxicity assessment: with just two exceptions, moderate toxicity was associated to the effluents. The best performances were associated to WWTP2 in May 2016 and 2017: the high or very high toxicity of the influent was reduced almost to absent toxicity in the effluents. Governance and protection agencies may exploit a toxicity scale strategy to compare heterogeneous and even contrasting results that contribute to the ecotoxicological assessment of the battery.

4. Conclusions

Results of the present study confirmed pharmaceutical compounds as widespread pollutants in wastewater. The nine pharmaceuticals of interest were detected in the influents at least in one sampling campaign. In agreement with literature data, pharmaceuticals concentration in the effluents was not negligible, indicating that conventional WWTPs are not completely efficient in drugs removal. Since WWTPs with higher solid retention time obtained higher removal of micropollutants, it could be assumed that high retention time is indicated to enhance drugs removal. Due to the different sensitivity of the organisms, a single bioassay cannot be considered exhaustive to assess the environmental toxicity but only a battery of tests can produce reliable ecological predictions. Thanks to the development of toxicity scale, the impact on the ecosystem was assessed: WWTPs caused a significant reduction of the risk, but the effluents cannot be considered harmless (moderate toxicity). Despite WWTPs often reduced the toxicity, even though in many cases effluents were more toxic than influents. This observation, together with chemical data, clearly indicate WWTPs as an important source of pharmaceuticals in the Arno river with an important environmental toxicity; therefore, the reduction of pharmaceutical load originated from point source such us WWTPs would ask in the future the adoption of refinery steps in WWTPs able to increase RE of drugs.

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