https://doi.org/10.1016/j.scitotenv.2017.12.145

Municipal wastewater spiramycin removal by conventional treatments and heterogeneous photocatalysis

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https://doi.org/10.1016/j.scitotenv.2017.12.145

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https://doi.org/10.1016/j.scitotenv.2017.12.145

Abstract

This study assessed the effects and removal options of the macrolide spiramycin, currently used for both in human and veterinary medicine- with a special focus on advanced oxidation processes based on heterogeneous TiO_2–assisted photocatalysis. Spiramycin real concentrations were investigated on a seasonal basis in a municipal wastewater treatment plant (up to 35 μg L^{-1}), while its removal kinetics were studied considering both aqueous solutions and real wastewater samples, including by-products toxicity assessment. High variability of spiramycin removal by activated sludge treatments (from 9% (wintertime) to >99.9% (summertime)) was observed on a seasonal basis. Preliminary results showed that a total spiramycin removal (>99.9%) is achieved with 0.1 g L^{-1} of TiO_2 in aqueous solution after 80 min. Integrated toxicity showed residual slight acute effects in the photocatalytic treated solutions, independently from the amount of TiO_2 used, and could be linked to the presence of intermediate compounds. Photolysis of wastewater samples collected after activated sludge treatment during summer season (SPY 5 μg/L) allowed a full SPY removal after 80 min. When photocatalysis with 0.1 g L^{-1} of TiO_2 was carried out in wastewater samples collected in winter season (SPY 30 μg L^{-1}) after AS treatment, SPY removal was up to 91% after 80 min.

Keywords

Advanced oxidation processes; mass spectrometry; antibiotics; TiO_2; toxicity

Highlights

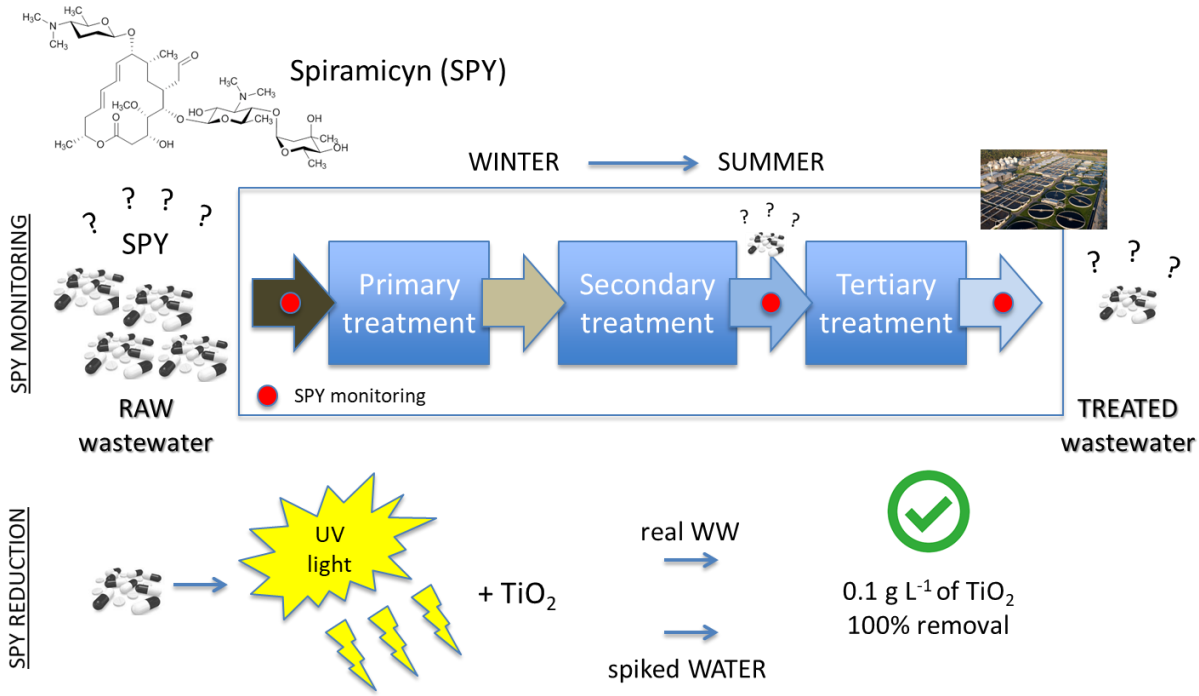
- SPY in WWTP before and after wastewater treatment was up to 35 μg L^{-1}
- SPY reduction was more effective in summer than winter by AS treatment
- Photocatalysis (winter samples) (0.1 g TiO_2 L^{-1}, 80 min) reduced SPY up to 91%

https://doi.org/10.1016/j.scitotenv.2017.12.145

- After treatment, ecotoxicity was 7-18% due to residual oxidation by-products

https://doi.org/10.1016/j.scitotenv.2017.12.145

Graphical Abstract



https://doi.org/10.1016/j.scitotenv.2017.12.145

1. Introduction

Antibiotics released into the environment through the wastewater cycle are considered contaminants of emerging concern belonging to the class of micro- and nano-pollutants similarly to engineered nanomaterials (Minetto et al., 2016), textile dyes and other textile by-products and personal care residues (Lofrano et al., 2016a; Libralato et al., 2011).

In the European Union between 2010 and 2014, the overall human antibiotic consumption showed a significant increasing trend with a large inter-country dissimilarity (i.e. from 1.1 packages/1000 inhabitants per day in Sweden up to 3.8 packages/1000 inhabitants per day in Italy) (ECDC, 2014). Sarmah et al. (2016) stated that veterinary antibiotics might play a leading role in wastewater contamination largely contributing to the final load of drugs discharged into the environment on a specific geographical basis (e.g. presence of intensive livestock breeding). According to Wang and Tang (2010), the total worldwide amount of used antibiotics (medical and veterinary) reached up to 2 10^5 ton/y. The consumption of antimicrobials by livestock is expected to increase from 63151 ± 1560 ton in 2010 to 105596 ± 3605 ton in 2030 (Van Boeckel et al., 2015). As antibiotics are poorly adsorbed in animal guts, a great part of them is excreted into faeces and urine, and, frequently, in a form that is not metabolized. When zootechnical wastewater is discharged into sewage (i.e. with or without *in situ* pre-treatment) and subsequent into municipal wastewater treatment plants (WWTPs), a significant increase in the antibiotic load is expected at the influent (Sarmah et al., 2016). Indeed, several studies showed that conventional WWTPs could not completely remove antibiotics, thus, they can finally enter the aquatic and terrestrial environment via conventional effluent and sewage sludge disposal (Watkinson et al., 2007; Batt et al., 2007; Zuccato et al., 2010; Gracia-Lor et al., 2012; Michael et al., 2013; Birošová et al., 2014). According to Zuccato et al. (2010), wastewater samples from northern Italy WWTPs (Milan, Como and Varese) presented an amount of antibiotics ranging within 115–237 g per 1000

https://doi.org/10.1016/j.scitotenv.2017.12.145

inhabitants per year in both influent and effluent and, thus, being potentially released into the receiving water bodies. Macrolides, particularly clarithromycin, spiramycin, and quinolones are the most abundant antibiotics in untreated wastewater. After penicillin and quinolones, ECDC (2014) estimated that macrolides are the third class of antibiotics consumed in Italy.

Antibiotics both taken singly and as mixtures showed to influence both the structure and function of algal communities (Wilson et al., 2003). They showed to influence the development, transfer, or spread of antibiotics resistant bacteria and/or antibiotics resistant genes in a long-term perspective (Ferro et al., 2015). They can impair human embryonic cells and affect zebrafish liver cells proliferation (Pomati et al., 2006, 2007), but data are still scarce for (environmental) risk assessment.

The environmental concern associated to the release of antibiotics in the aquatic ecosystems is expected to increase over time especially when considering the reuse of conventionally treated wastewater (e.g. industry, hospital and household) increasing the risk of drinking water contamination and, thus, non-voluntary human exposure (Kim and Aga, 2007; Benotti et al., 2008). Consequently, the European Commission updated the Watch List of substances for Union-wide monitoring (Commission Implementing Decision 2015/495) including erythromycin, clarithromycin, and azithromycin.

To face up the antibiotic removal, the performance of traditional activated sludge (AS) WWTP should be improved including further treatments like advanced oxidation processes (AOPs) such as ozonation, Fenton, photo-Fenton oxidation, and heterogeneous photocatalysis that are gaining growing interest as complementary treatments (De Luca et al., 2013; Carotenuto et al., 2014; Lofrano et al., 2016b; Lofrano et al., 2017; Rasheed et al., 2017a; Rasheed et al., 2017b). Amongst AOPs, TiO_2-assisted photocatalysis is being considered as an effective and sustainable technology for the degradation and detoxification of complex organic chemicals (Vaiano et al., 2015). The photocatalytic process consists in utilizing the

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ultra-violet (UV) irradiation (λ <380 nm) to photoexcite a semiconductor catalyst in presence of oxygen. Within this scenario, oxidizing species (i.e. bound hydroxyl radical (•OH) or free holes) attack oxidable substances producing a progressive breaking down of macro-molecules yielding to CO_2, H_2O and diluted inorganic acids. The most commonly used catalyst is the semiconductor TiO_2, mainly because it is an abundant, non-expensive, and relatively low toxic product (Malato et al., 2002).

Nevertheless the huge amount of papers about AOPs, gaps into the knowledge about the proper management of antibiotics in wastewater treatment is still present mainly due to the absence of toxicity identification evaluation of treated effluents that only rarely consider a complete battery of toxicity tests and final toxicity data integration. Scarce information exists about spiramycin (SPY) behaviour in wastewater. SPY is a macrolide antibiotic widely used to treat human (e.g. oropharynx, respiratory system, and genito-urinary tract) and veterinary infections (e.g. cryptosporidiosis and toxoplasmosis). This research investigated on a seasonal basis SPY presence in wastewater samples collected before and after treatment in a municipal AS WWTP (Campania Region, Italy) to monitor the state-of-the-art and assess the potentiality for its reduction/removal at real scale via AOPs. The photocatalytic degradation of SPY was evaluated by using a range of TiO_2 concentrations both in biologically treated wastewater samples and aqueous solutions. Photo-degradation by-products were investigated, and toxicity of samples using a full battery of bioassays was provided to complete the characterization of the performance of the treatment process.

2 Materials and methods

2.1 Wastewater treatment plant and sampling

Samples were collected from a municipal WWTP, located in Campania region (Italy) receiving wastewater collected from urban households, agro-industries, zootechnical

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activities, hospices and other facilities. The WWTP had an average capacity of 300 000 p.e., and a flow rate ranging between 30 000 m^3 d^{-1} (winter) up to 60 000 m^3 d^{-1} (summer) due to seasonal activities (e.g. cannery industries). The treatment process includes: i) Mechanical pre-treatment (screening and pumping stations, grit and oil removal); ii) Rainwater section (primary sedimentation and aerated storage); iii) Secondary treatment (nitrification-denitrification and final settling); and iv) Tertiary treatment (gravity filtration on sand), and disinfection with peracetic acid.

Two weeks seasonal sampling campaigns were carried out at the WWTP in winter and summer 2015 with three sampling points: i) influent; ii) wastewater after the biological treatment; iii) effluent. Samples were collected three times per day and mixed to obtain a composite sample. During sampling, no rainfall events were registered and daily WWTPs hydraulic loading rates were nearly constant. Samples were kept at 4 $^{\circ}C$ in the darkness during the way back to the laboratory. Wastewater were characterised for COD, TSS, NH_3, NO_3, NO_2, according to APHA (2012).

2.2 Materials and analytical procedures

SPY (Sigma-Aldrich) presents high solubility in water and is freely soluble in ethanol 96% ($C_{43}H_{74}N_2O_{14}$; 443.053 g mol^{-1}; solid appearance: white or yellow-white powder). We selected the P25 TiO_2 (80% anatase and 20% rutile) for heterogeneous photocatalysis (Evonik, Essen, Germany). Sigma Aldrich (Sant Louis, MO, USA) supplied high performance liquid chromatography (HPLC) grade water, methanol, acetonitrile and formic acid. The ultraviolet–visible (UV-Vis) spectra were recorded using a spectrophotometer (Varian, Cary 50). The degradation of SPY dispersed in ultra-pure water was followed by HPLC-UV (Finnigan Surveyor LC Pump Plus, USA) equipped with a reversed phase C18 analytical column (Phenomenex Luna, 3 μm, 2.1 mm × 150 mm) with UV-Vis spectrophotometer

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(Finnigan Surveyor UV-VIS Plus Detector, USA). The compound was eluted using as mobile phase a mixture of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile-methanol (1:1 v:v) (eluent B) at a flow rate of 0.2 mL min^{-1}. The initial concentration was 10% B followed by a linear gradient from 10 to 70% B over the course of 3 min and 80% over the next 2 min. Finally, eluent B was lowered to 10 % in 1 min. Before the next injection, the system could equilibrate for 9 min. The injection volume was 20 μL and the wavelength set for the quantification was 230 nm according to the maximum light absorption of SPY (inlet Fig. 1). Under these conditions, the retention time of SPY was about 9.7 min (Fig.1). The limit of quantification (LOQ) was 0.1 μg mL^{-1}. Data were collected by ChromQuest version 3.1.6 software (Thermo Electron 2003). The quantification of SPY in wastewater was performed using an LC-MS equipped with a 1525 binary pump (Waters, Milford, MA, USA) and a Phenomenex Luna-C18 column (3 μm, 2.1 mm × 150 mm). The eluents and the condition were the same used for the HPLC analysis Before the next injection, the system could equilibrate for 4 min. The injection volume was 10 μL. Under these conditions, the retention time of SPY was 5.8 min (Fig. 1S). The limit of quantification (LOQ) was equal to 0.1 ng mL^{-1}.

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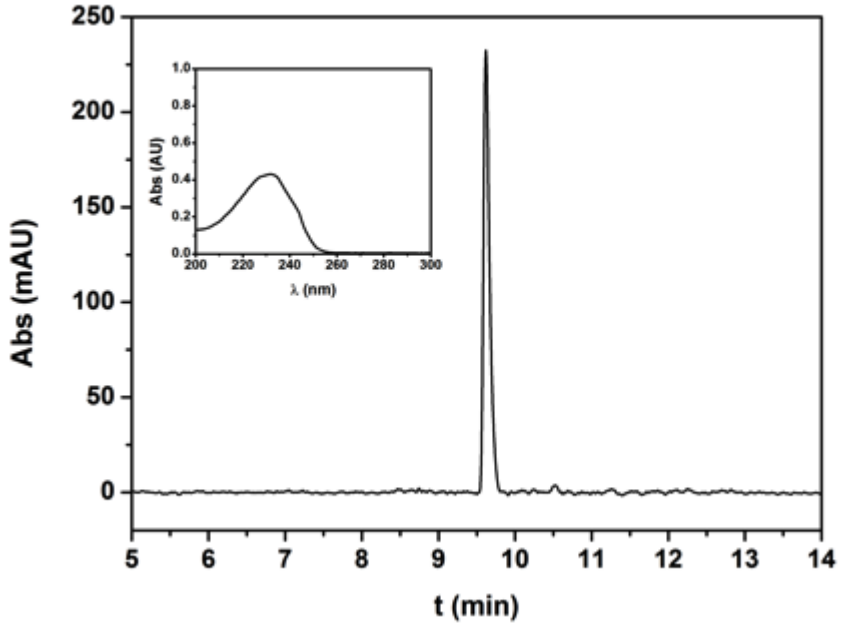

Figure 1 HPLC chromatogram of SPY; (inlet) UV spectra of SPY.

A Quattro micro API (Micromass, Manchester, UK) triple quadrupole tandem mass spectrometer operating in the multiple reaction monitoring (MRM) positive ion mode was used for SPY detection in wastewater. Data acquisition was accomplished by MassLynx version 4.1 software (Micromass, Manchester, UK). The following conditions were found to provide the optimum signal: ion source temperature, 100 °C; desolvation temperature, 250 °C; cone gas, 30 L h^{-1}; desolvation gas, 500 L h^{-1}; cone voltage, 20 V; collision energy, 10 eV; and capillary voltage, 3.0 kV. One MRM transitions were analysed: 422.3–176.0 m z^{-1}. Quantification was accomplished using an external standard method. Instrument calibration included the analysis of standards at 10, 100, 500, 1000 nmol L^{-1}. A blank sample was analyzed between each sample to verify that the measured SPY concentrations were not false positives.

For the by-products identification mass spectra were acquired with a Bruker solarix XR Fourier transform mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped

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with a 7T refrigerated actively shielded superconducting magnet (Bruker Biospin, Wissembourg, France). The samples were ionized in positive ion mode using ESI (Bruker Daltonik GmbH, Bremen, Germany).

Sample solutions were continuously supplied using a syringe pump at a flow rate of 120 μL h^{-1}. The detection mass range was set to 100-1000 m z^{-1}. The mass spectra were calibrated externally with a solution of sodium trifluoroacetate in water in positive ion mode using a linear calibration.

2.3 Experimental plan

A first set of investigation was aimed at evaluating the effects of TiO_2 in dark conditions to set the background level of SPY removal and potential adsorption. Two concentrations of SPY (10 and 70 mg L^{-1}) were selected to evaluate the influence of the initial antibiotic concentration, in addition the solution at 70 mg L^{-1} SPY was used to facilitate the identification of the potential oxidation by-products. Photolysis experiments were carried out at 20 °C in a 250 mL magnetic stirred cylindrical Pyrex vessel filled with 200 mL of ultra-pure water solution (10-70 mg L^{-1} of SPY). In photocatalysis experiments, various TiO_2 concentrations (0.1, 0.5, 1, 2 and 4 g L^{-1}) were added to the same solutions (10-70 mg L^{-1} of SPY) at natural pH = 5.5.

The reaction vessel was placed in a chamber and illuminated for 5, 10, 20, 40, and 80 min with a xenon arc lamp (450 W, Lot Oriel Group, Italy) equipped with special glass filtering the transmission of wavelengths below 320 nm to use the radiation able to activate the catalyst. The irradiation was determined by the potassium ferrioxalate actinometry (Hatchard and Parker, 1956) was 4.5 x 10^{-7} Einstein s^{-1}. After the photocatalysis process, samples were slowly filtered through 0.45 μm pore size mixed esters membrane (Millipore, Billerica, MA, USA) to remove the catalyst. Dark experiments were carried out with the lamp switched off.

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Finally, dark photolysis and photocatalysis experiments were carried out on the samples from the effluent of the biological treatment during summer and winter seasons, once the optimum TiO_2 concentration was determined.

2.4 Ecotoxicity and data analysis

Toxicity was investigated in accordance to Lofrano et al. (2016) via a battery of acute (A) and chronic (C) toxicity tests including biological models belonging to various trophic levels like *Vibrio fischeri* (A), *Raphidocelis subcapitata* (C), and *Daphnia magna* (A). Toxicity tests were carried out on untreated 10 mg L^{-1} SPY solution (pure substance) and after the photocatalytic treatment with various TiO_2 concentrations (0.1, 0.5, 1, 2 and 4 g L^{-1}) for 80 min. Toxicity tests with *V. fischeri* (NRRL-B-11177) were carried out according to ISO (2008). The luminescence was measured with a Microtox® analyzer (Model 500, AZUR Environmental) after 5 and 15 min at 15 °C. Tests were carried out in triplicate. Data were analysed with Microtox Omni® software and the result expressed as percentage of bioluminescence inhibition (%). Microalgae growth inhibition test with *R. subcapitata* was carried out according to ISO (2012). Cultures were kept in Erlenmeyer flasks. The initial inoculum contained 10^4 cells mL^{-1}. The specific growth inhibition rate was calculated considering 6 replicates exposed at 20 ± 1 °C for 72 h under continuous illumination (6000 lx). Effect data were expressed as percentage of growth inhibition. Toxicity tests with *D. magna* were carried out according to ISO (2013) and Maselli (2017). Newborn daphnids (< 24 h old) were exposed in four replicates for 24 and 48 h at 20 ± 1 °C under continuous illumination (1000 lx). Before testing, they were fed with *R. subcapitata* (300,000 cells mL^{-1}) *ad libitum*. Toxicity was expressed as the percentage of dead organism and corrected for the effects in negative controls (0 g/L TiO_2) according to Abbott's formula. All toxicity tests included the assessment of negative and positive controls in accordance with the specific

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reference method. Toxicity was expressed as percentage of effect or as effective concentration causing the 5 (EC5), 20 (EC20) and 50% (EC50) effect to the exposed population. After the verification of homoschedasticity (F test, $p < 0.05$) and normality (Shapiro-Wilk test, $p < 0.05$) of toxicity data, the significance of differences between average values of different experimental treatments and controls was assessed by the analysis of variance (ANOVA, $p < 0.05$). When ANOVA revealed significant differences among treatments, *post-hoc* tests were carried out with Tukey's test ($p < 0.05$). Statistical analyses were performed using Microsoft® Excel 2013/XLSTAT©-Pro (Version 7.2, 2003, Addinsoft, Inc., Brooklyn, NY, USA).

Toxicity data have been integrated according to Persoone et al. (2003) approach for natural water. According to Libralato et al. (2010) and Lofrano et al. (2016), the hazard classification system based on percentage of effect (PE) includes a Class I for PE < 20% (score 0), Class II for 20% ≤ PE < 50% (score 1), Class III for 50% ≤ PE < 100% (score 2), Class IV when PE = 100% in at least one test (score 3) and a Class V when PE = 100% in all bioassays (score 4). Finally, the integrated class weight score was determined by averaging the values corresponding to each microbiotest class normalised to the most sensitive organism (highest score).

3 Results and discussion

3.2 Heterogeneous photocatalysis of SPY in aqueous solutions

3.2.1 Effect of SPY concentration

Screening experiments carried out in the dark at 10 mg L^{-1} and 70 mg L^{-1} of SPY in distilled water considering a concentration equal to 0.1 g L^{-1} of TiO_2 proved that adsorption was negligible in the antibiotic removal (data not shown). As reported in Figure 2, a slight decrease in SPY concentration could be observed during photolysis experiments. After 80 min of irradiation, antibiotic removal was set at 11% for 70 mg L^{-1} of SPY. As for most of the organic compounds, SPY photolysis resulted strongly influenced by both the wavelength and

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intensity of UV source (Lofrano et al., 2016b). Chekir et al. (2013) reported a SPY (10 mg L^{-1}) removal < 7% after 6 h of irradiation with 2 Phillips lamps (PL-L 24 W/10/4P; λ_{max} = 365 nm).

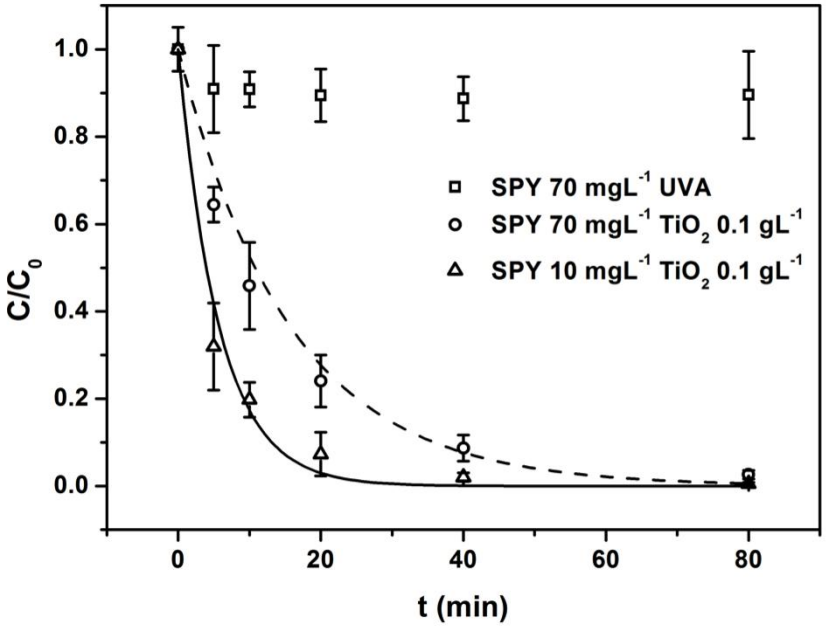

Figure 2 Photocatalytic kinetic curves of SPY (10 and 70 mg L^{-1}) after 5, 20, 40, 80 min at 0.1 g L^{-1} of TiO_2 at pH 5.5; error bars represent standard error (n = 3).

According to Calza et al. (2010), the complete disappearance of the drug (15 mg L^{-1}) occurred through a pseudo-first-order decay (1,500-W Xenon lamp equipped with a 340-nm cut-off filter simulating AM1 solar light). In sterilised water, $t_{1/2}$ was 48 h, further reduced to 25 h when SPY was spiked in river water.

In photocatalysis experiments, the action of 0.1 g L^{-1} of TiO_2 combined with UV radiation after 80 min increased the reduction up to 99% and 97% for 10 mg L^{-1} and 70 mg L^{-1} of SPY, respectively. Antibiotic reduction was faster at 10 mg L^{-1} of SPY. The degradation rate was inversely dependent to the initial SPY concentration following the pseudo-first-order kinetic model: i) $k = 0.17 \pm 0.02$ min^{-1}, $t_{1/2} = 4.0 \pm 0.4$ min at 10 mg L^{-1} of SPY; ii) $k = 0.064 \pm 0.003$ min^{-1}, $t_{1/2} = 10.8 \pm 0.5$ min at 70 mg L^{-1} of SPY. Similarly, Chekir et al. (2013) reported

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289 reduction rates equal to 95.6%, 89.9%, and 78.4% for 10, 20, 40 mg L^{-1} of SPY, respectively,
290 under simulated sunlight after 360 min with 0.25 g L^{-1} of TiO_2.
291
292 **3.2.2 Effect of catalysts in SPY aqueous solution**
293 In order to evaluate the catalyst effect, several loads (0.1, 0.5, 1, 2, 4 g L^{-1} of TiO_2) were
294 tested in photocatalysis of 10 mg L^{-1} of SPY. After 80 min of photo-oxidation, significant
295 SPY removal (> 99.9%) was achieved with 0.1 g L^{-1} of TiO_2 (Figure 3). During treatment,
296 SPY reduction increased with TiO_2 concentration up to a degradation of >99.9% using 2 g L^{-1}
297 of TiO_2 after 10 min of contact time. A complete SPY removal was achieved as well after 40
298 min of photo-degradation with 1 g L^{-1} of TiO_2. Beyond this value, the degradation remained
299 approximately constant and the rate constant evolution was low. Above 2 g L^{-1} of TiO_2,
300 particles caused a shadowing effect reducing the penetration ability of the radiation, thus
301 reducing the formation of hydroxyl radicals being responsible of SPY oxidation. Chekir et al.
302 (2013) achieved a 96% SPY removal (10 mg L^{-1}) after 360 min of photo-oxidation using 0.25
303 g L^{-1} of TiO_2.
304 The optimum TiO_2 concentration must be determined time-by-time to avoid the use of an
305 excess of the reactive agent ensuring that the absorption of radiation photons is maximized for
306 an efficient degradation (Lofrano et al., 2016b).
307 The photocatalytic reduction of SPY (10 mg L^{-1}) followed a pseudo first order (PFO) kinetic
308 equation, corresponding to photocatalytic degradation rate constants reported in Table 2 with
309 the half-life ($t_{1/2}$) values.
310

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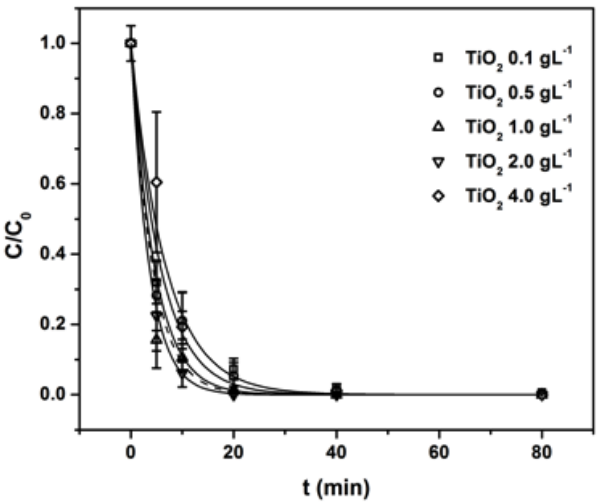

311

312 **Figure 3** Photocatalytic kinetic curves of SPY (10 mg L^{-1}) after 5, 10, 20, 40, 80 min at 0.1,

313 0.5, 1.0, 2.0, 4.0 g L^{-1} of TiO_2 at pH 5.5; error bars represent standard error (n = 3).

314

315 **Table 2** Half-life ($t_{1/2}$) of SPY (10 mg L^{-1}) and the pseudo first order constant (*k*) .

TiO_2	**$t_{1/2}$**	***k***
g L^{-1}	min	min^{-1}
0.1	4.0 ± 0.4	0.180 ± 0.020
0.5	4.7 ± 0.2	0.148 ± 0.006
1.0	3.1 ± 0.3	0.220 ± 0.020
2.0	2.5 ± 0.1	0.282 ± 0.006
4.0	2.9 ± 0.3	0.240 ± 0.030

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3.2.3 Degradation products

Degradation products may promote microbial resistance, above all if the active part of the molecule remains unmodified, and/or generate more toxic effects than their parent compounds. Consequently, monitoring not only drug degradation but also its metabolites is of increasing relevance in evaluating their environmental impact. Calza et al. (2010) reported that most transformation products formed after photocatalytic treatment of 15 mg L^{-1} SPY with 0.1 g L^{-1} of TiO_2 reached their maximum amounts up to 15 min of irradiation. Some of them (m/z 526, 859,699) were removed after 60 min, whereas the smaller molecules (m/z 144, 160,176, 336) took more time to be degraded disappearing only after 120 min. A similar behaviour was observed in the present study, monitoring the transformation products as a function of the irradiation time.

Figure 4 reports the ESI-MS spectra on untreated SPY (70 mgL^{-1}) and photocatalysis-treated solutions with 0.1 $gTiO_2$ L^{-1} over the time. The ESI-MS spectra showed a progressive degradation of SPY and the formation of several intermediates and/or by-products after 10 min of photocatalysis the m/z 143, 175, 335, 525, 698, 701, 814, 828, 859, 891, were detected and after 20 min the m/z 159 appeared. The molecules associated to the peaks identified in Figure 4 are shown in Figure 5. All of them were still present after 80 min of photocatalysis.

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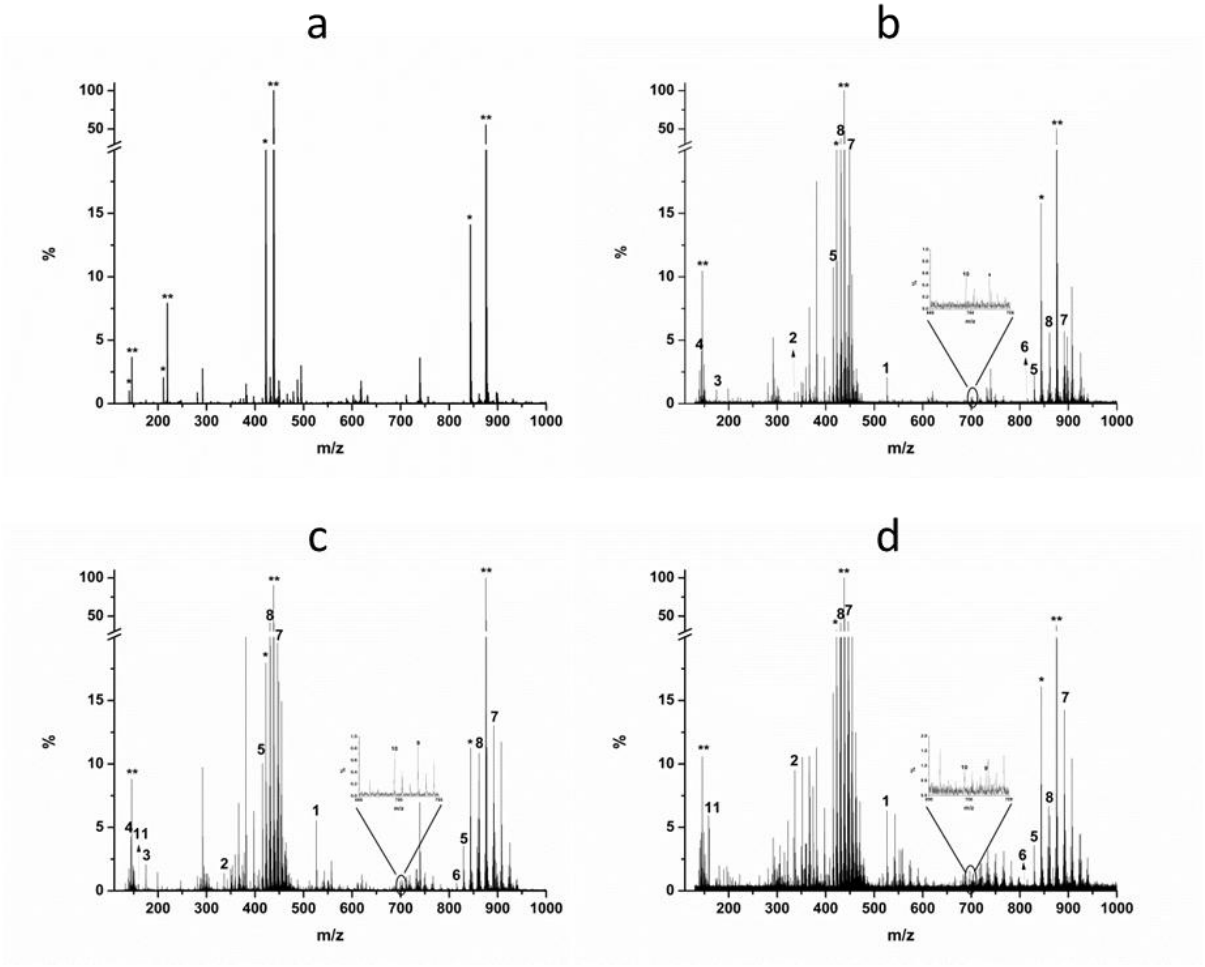

Figure 4 ESI-MS spectra in positive ion mode on untreated SPY (70 mgL^{-1}) (a), SPY photocatalysis (70 mgL^{-1}) at 0.1 gL^{-1} of TiO_2 after 10 min (b),20 min (c) and 80 min (d); *SPY and ** [SPY + CH_3COOH] formed during the injection phase in ESI-MS.

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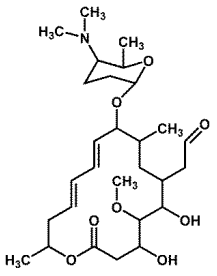
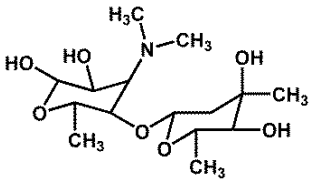
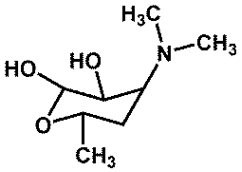
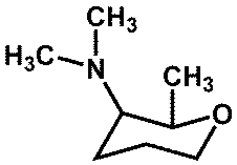
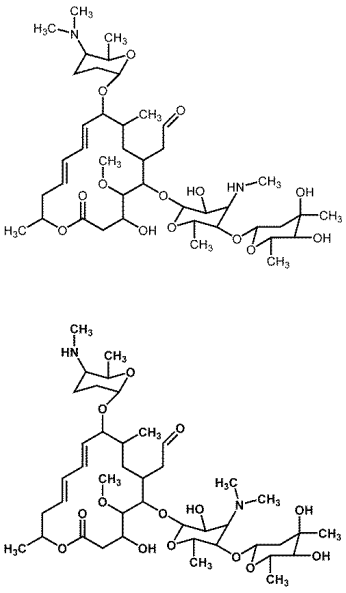
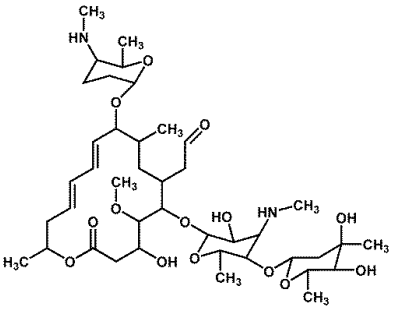
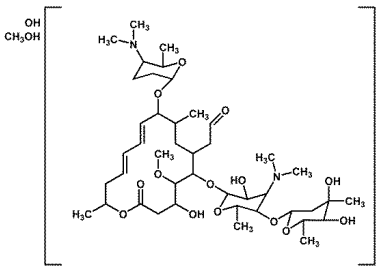
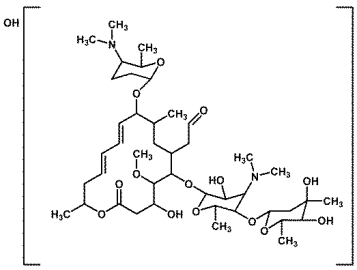
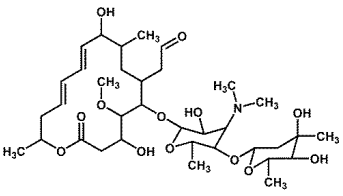
 **1 (M.W. 525)**	 **2 (M.W. 335)**	 **3 (M.W. 175)**
 **4 (M.W. 143)**	 **5 (M.W. 828)**	 **6 (M.W. 814)**
 **7 (M.W. 891)**	 **8 (M.W. 859)**	 **9 (M.W. 701)**

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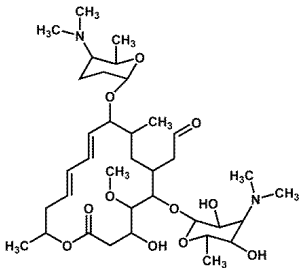
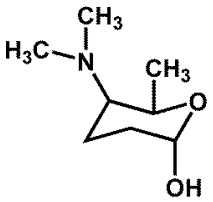
 **10 (M.W. 698)**	 **11 (M.W. 159)**	

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349 **Figure 5** Molecules associated to the peaks identified in Figure 4.

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3.1 Occurrence and removal of SPY in WWTP

Real SPY concentrations detected in wastewater samples are reported in Table 1. Wintertime showed a significantly higher presence of SPY compared to summertime. In winter, the average SPY concentration was 32 μg L^{-1} in both influent and effluent specimen thus being significantly higher than the average concentrations of SPY measured in effluents from nine Italian WWTPs (75 ng L^{-1}) (Zuccato et al., 2005). Birošová et al. (2016) observed a similar trend highlighting higher levels of antibiotics in raw wastewater samples from two Bratislava WWTPs (Slovakia) in February than in August. The clarithromycin concentration ranged from 0.7 μg L^{-1} in August up to 2.520 μg L^{-1} in February. McArdell et al. (2003) reported that winter antibiotic concentrations might be two times higher than in summertime.

Table 1 Occurrence of SPY in WWTP (Campania, Italy) before (influent), after activated sludge (AS) biological treatment and at the final discharge point (effluent); SPY reduction rates (%) were reported within the same season (RR_W = Reduction Rate influent-effluent) and between winter- and summertime (RR_B).

	Influent	**After AS treatment**	**Effluent**	**RR_W**
	μg L^{-1}			**%**
Winter (February)	35 ± 7	34 ± 7	32 ± 7	9
Summer (July)	5 ± 2	< 0.1	< 0.1	>99.9%
RR_B (%)	86%	>99.9%	>99.9%	-

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Municipal WWTPs are the major pathway for the disposal of antibiotics, and the concentrations measured in influent can reflect the levels of antibiotics in its collection area (Al-Rifai et al., 2011; Jelić et al., 2009, Li et al., 2013). Li et al. (2011) observed as the sums of the average daily mass flow of antibiotics from influent of two WWTPs in Hong Kong changed significantly according to the living standard of the serving regions even in the same city. Thus, the higher value detected in winter samples could be partially attributed to the higher frequency of antibiotic prescription in Campania Region and/or its misuse (i.e. the higher SPY consumption during winter caused by the higher amount of potential infections and the lower ability of WWTPs in wintertime to effectively reduce/remove such a highly concentrated and recalcitrant compound due to low environmental temperatures).

In the surroundings of the WWTP, there are no pharmaceutical industries or hospitals and, thus, the average concentrations could reflect mainly the domestic antibiotic consumption in the area. Nevertheless, the WWTP receives wastewater from nearby livestock husbandries, thus the veterinary-related load could be relevant and, currently, still unknown. In fact, in the early 1960s, SPY was the first macrolide intended for animal use (EMA, 2016). In EU, SPY use as growth promoter in feed was withdrawn in 1998 (Council Regulation (2821/98/EC), but it is still viable as therapeutic drug for cattle, poultry and pigs (Anadón et al., 2012). Companies in EU are not required to provide background information about the amount of marketed veterinary drugs, thus the volume of used antibiotics is often very difficult to identify (EMA, 2016). Although data on antibiotics used on a species-by-species basis are rarely available, it is known that animal doses are significantly higher than humans (i.e. per unit of body weight) (EMA, 2016) explaining, at least in part, the amount of SPY detected in WWTP influent. The significant variation of antibiotics in influent during summer sampling could be due to many reasons, including antibiotics consumption pattern, seasonal and daily fluctuation of hydraulic loads.

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According to Table 1, SPY reduction rates (RR_W) also varied seasonally form 9% in winter to >99.9% in summer. Zhou et al. (2013) reported that removal percentage for the macrolides in AS plants can vary from 8.79–89.4% which agrees well with our data. Any or very slight removal rates could be observed during winter season after both AS and at the final discharge. Accordingly, insufficient degradation of some macrolides, by AS processes was also reported by Birošová et al. (2016). In the summer period, SPY was completely removed already after the secondary treatment (RR_B). In Bejing (China) WWTP, Li et al. (2013) stated that macrolides, including SPY, were persistent during conventional AS treatment (365 ng L^{-1} in influents, 353 ng L^{-1} in secondary effluents). Only after ultrafiltration coupled to ozonation, all target antibiotics were effectively reduced (from 85% up to >99.9%) decreasing their environmental risk.

3.3 Photolysis and heterogeneous photocatalysis of SPY in real wastewater

The photolysis of wastewater samples collected after the biological treatment during the summer period (SPY 5 µg/L) allowed to achieve >99.9% removal of SPY after 80 min (Figure 6). The degradation followed a PFO kinetic corresponding to a rate constant of 0.094 ± 0.003 min^{-1} and a half-life ($t_{1/2}$) of 7.4 ± 0.2 min. Although the solar radiation is expected to be less efficient than UV one, these results could contribute to explain the high removal rate of SPY in WWTP during summer time.

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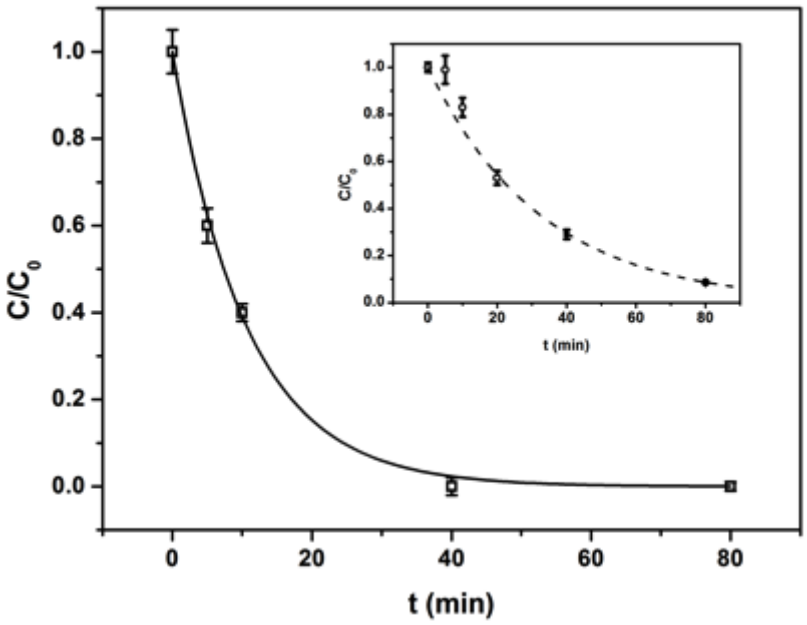

Figure 6 Photolysis of wastewater samples collected after the biological treatment during summer season (SPY 5 µg/L); (inlet) Photocatalysis with 0.1 g/L of TiO_2 of wastewater samples collected in winter season after biological treatment (SPY 30 µg/L); error bars represent standard error (n = 3).

When photocatalysis was carried out on the same wastewater samples with 0.1 g L^{-1} of TiO_2, a complete removal of SPY was attained only after 5 min. Thus, no kinetic study could be carried out. This result was attributed to the very low concentration of scavenger compounds in wastewater including organic substance (Table 1S), which made the hydroxyl radicals available for the oxidation of target pollutant. When photocatalysis was carried out in wastewater samples collected in winter season after the biological treatment (SPY 30 µg L^{-1}), a reduction rate of 91% was achieved after 80 min of treatment (Figure 6 inlet).

3.2.4 Ecotoxicity

Results from quality assurance and quality control procedures were in line with the relative toxicity test protocols for both negative and positive controls. The effects of SPY to *V.*

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fischeri, *R. subcapitata* and *D. magna* as EC5, EC20 and EC50 were reported in Table 3. Species presented a great range of sensitivities: *V. fischeri* < *D. magna* < *R. subcapitata*. Microalgae were the most sensitive biological model. SPY EC50 for *V. fischeri* was determined for the first time presenting no relevant effects, even though it has been already used for SPY contaminated wastewater sample assessment (Calza et al., 2010). SPY EC50 for *R. subcapitata* was like Halling-Sørensen (2000) (1.3-4.0 mg/L) and Minguez et al. (2016) (4.12-6.01 mg/L); SPY EC50 for *D. magna* was in accordance with Minguez et al. (2016) (> 100 mg/L). Effective concentrations for all species were several times greater than the real detected SPY concentration in untreated wastewater (5-35 μg L^{-1}, Table 1).

The application of risk quotients considering the predicted environmental concentration (PEC) and predicted no effect concentration (PNEC) showed that SPY did not present any risk of direct toxicity, but the potentiality to induce antibiotic resistance cannot be considered in such traditional risk evaluation and are still under investigated.

About SPY spiked samples, toxicity data were displayed considering single species effects in Figure 7; results were integrated according to Persoone et al. (2003) in Table 4. Outcomes from *V. fischeri* (15 min) (Figure 4 A) showed that toxicity was not significantly influenced ($p < 0.05$) by the amount of TiO_2 (0.1-4 g L^{-1}) used during the treatment process with an average residual toxicity of 7-18%.

Data from *R. subcapitata* (15 min) (Figure 4 B) showed that toxicity was not significantly influenced ($p < 0.05$) by the amount of TiO_2 (0.1-4 g L^{-1}) used during the process. Average residual toxicity was always > 40% and up to 52%; no EC50 values could be estimated. Daphnids evidenced a significant increasing toxicity trend in treated SPY solutions from 0.1 to 4 g L^{-1} TiO_2, with an average residual effect always ≥ 13% (Figure 4 C).

The toxicity of SPY spiked samples after treatment could be associated to SPY by-products that were not completely removed as previously described. Residual toxicity was not

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satisfactorily reduced below 10% effect that is the commonly accepted "no effect" threshold in many toxicity tests considering negative controls (Lofrano et al., 2016b). Further optimization of the treatment process will be necessary to meet the goal of toxicity reduction up to < 10% effect.

About toxicity data ranking and integration (Persoone et al., 2003) (Table 4), effects were singly scored from *no acute hazard* (i.e. mainly due to *V. fischeri*), and *slight acute hazard* (i.e. mainly due to *D. magna*) to *acute hazard* (i.e. *R. subcapitata*). Integrated results supported the general issue related to the presence of residual slight acute hazard in treated samples without any distinction about the amount of TiO_2 used within the treatment process.

Table 3 Toxicity effects of SPY on *V. fischeri* 15 min, *R. subcapitata* and *D. magna* 48 h as effective concentrations (EC) able to promote 5 (EC5), 20 (EC20) and 50% (EC50) effect.

	EC5	**EC20**	**EC50**
Species		mg L^{-1}	
*V. fischeri**	1348**	2944**	8263**
R. subcapitata	0.02 (0.09-0.03)	0.25 (0.16-0.36)	4.0 (3.2-5.0)
D. magna^{+}	70 (37-99)	281 (257-304)	503 (478-530)

*15 min; ** forecast values; $^{+}$48 h

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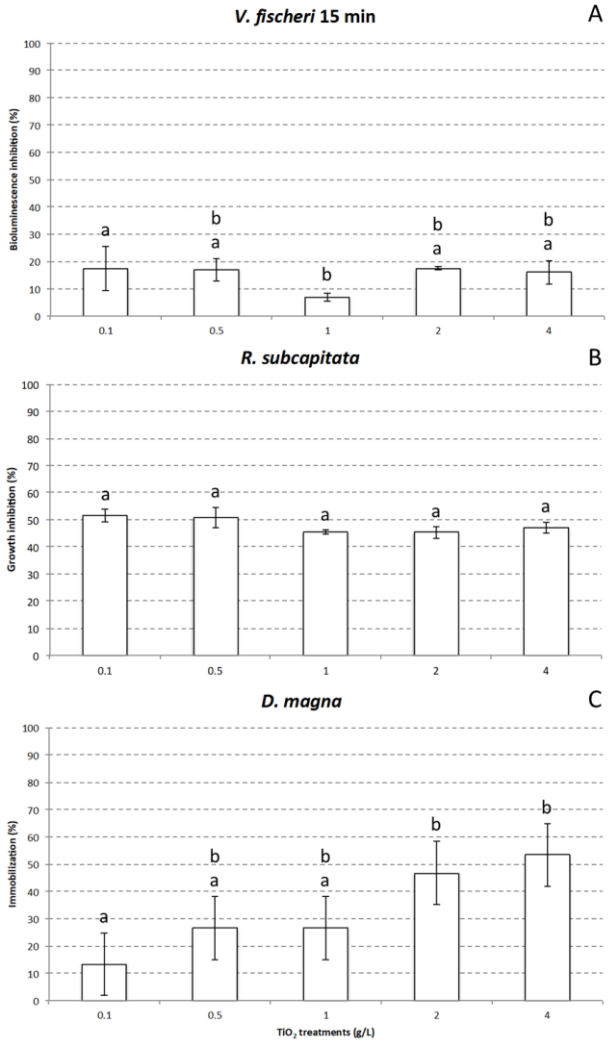

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473 **Figure 7** Toxicity results of treated SPY solution (10 mg L^{-1}) after 0.1, 0.5, 1, 2 and 4 g L^{-1} of
474 TiO_2 including *V. fischeri* bioluminescence inhibition after 15 min (A) contact time, *R.*
475 *subcapitata* growth inhibition (B), and *D. magna* mortality after 48 h (C) contact time; data
476 with different letters (a–b) are significantly different (Tukey's, $p < 0.05$); error bars represent
477 standard error (n = 3).

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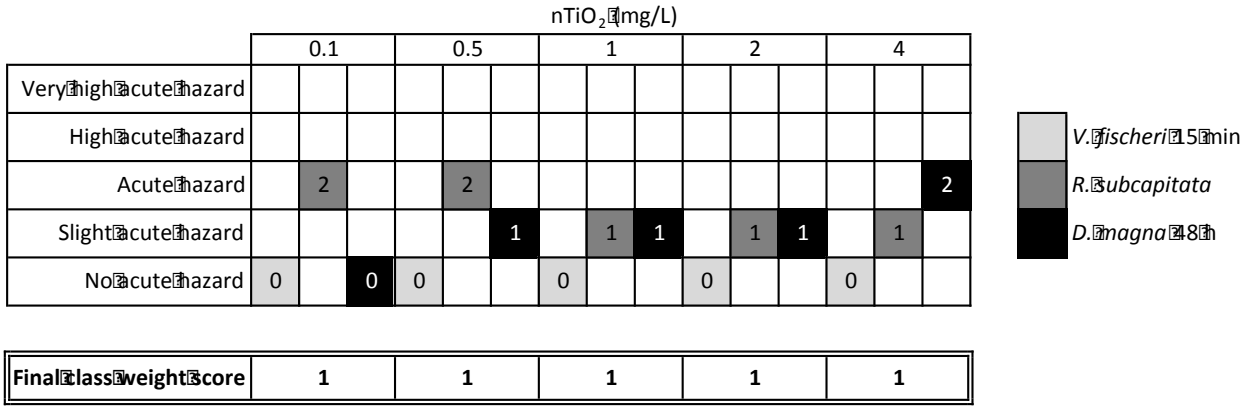

Final class weight score	1	1	1	1	1

Table 4 Integrated assessment of toxicity data from treated (0.1, 0.5, 1, 2 and 4 min g L^{-1} of TiO_2) SPY solutions including *V. fischeri* 15 min, *R. subcapitata* and *D. magna* 48 h according to Persoone et al. (2003) toxicity class weight score for the classification of natural waters.

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4. Conclusions

- The antibiotic concentration in municipal wastewater can significantly vary on a seasonal basis and between different WWTPs; therefore, a preliminary monitoring is necessary for a proper management strategy on a case-by-case basis;
- The photocatalytic process showed to be very efficient in SPY removal (> 90%) also at relatively high concentrations as those detected in the monitored WWTP compared to conventional AS treatments;
- The optimal range of TiO_2 concentration must be determined time-by-time to avoid the use of an excess of the reactive agent ensuring that the absorption of radiation photons is maximized for an efficient degradation;
- Acute effects were still present in treated effluents up to more than 50%. Further optimization of the process is necessary to meet the goal of reducing toxicity < 10% threshold effect.

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Captions figures

Figure 1 HPLC chromatogram of SPY - inlet: UV spectra of SPY.

Figure 2 Photocatalytic kinetic curves of SPY (10 and 70 mg L-1) after 5, 20, 40, 80 min at 0.1 g L-1 of TiO2 at pH 5.5; error bars represent standard error (n = 3).

Figure 3 Photocatalytic kinetic curves of SPY (10 mg L^{-1}) after 5, 10, 20, 40, 80 min at 0.1, 0.5, 1.0, 2.0, 4.0 g L^{-1} of TiO_2 at pH 5.5; error bars represent standard error (n = 3).

Figure 4 ESI-MS spectra in positive ion mode on untreated SPY (70 mgL^{-1}) (a), SPY photocatalysis (70 mgL^{-1}) at 0.1 gL^{-1} of TiO_2 after 10 min (b),20 min (c) and 80 min (d); *SPY and ** [SPY + CH_3COOH] formed during the injection phase in ESI-MS.

Figure 5 Molecules associated to the peaks identified in Figure 4.

Figure 6 Photolysis of wastewater samples collected after the biological treatment during summer season (SPY 5 µg/L); inlet Photocatalysis with 0.1 g/L of TiO_2 of wastewater samples collected in winter season after biological treatment (SPY 30 µg/L); error bars represent standard error (n = 3).

Figure 7 Toxicity results of treated SPY solution (10 mg L^{-1}) after 0.1, 0.5, 1, 2 and 4 g L^{-1} of TiO_2 including *V. fischeri* bioluminescence inhibition after 15 min (A) contact time, *R. subcapitata* growth inhibition (B), and *D. magna* mortality after 48 h (C) contact time; data with different letters (a–b) are significantly different (Tukey's, $p < 0.05$).

https://doi.org/10.1016/j.scitotenv.2017.12.145

Captions of tables

Table 1 Occurrence of SPY in WWTP (Campania, Italy) before (influent), after activated sludge (AS) biological treatment and at the final discharge point (effluent); SPY reduction rates (%) were reported within the same season (RR_W = Reduction Rate influent-effluent) and between winter- and summertime (RR_B).

Table 2 Half-life ($t_{1/2}$) of SPY (10 mg L^{-1}) and the pseudo first order constant (*k*).

Table 3 Toxicity effects of SPY on *V. fischeri* 15 min, *R. subcapitata* and *D. magna* 48 h as effective concentrations (EC) able to promote 5 (EC5), 20 (EC20) and 50% (EC50) effect.

Table 4 Integrated assessment of toxicity data from treated (0.1, 0.5, 1, 2 and 4 min g L^{-1} of TiO_2) SPY solutions including *V. fischeri* 15 min, *R. subcapitata* and *D. magna* 48 h according to Persoone et al. (2003) toxicity class weight score for the classification of natural waters.

Supplementary material for on-line publication only

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https://doi.org/10.1016/j.scitotenv.2017.12.145