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Abstract

The aqueous environmental fate of two antibiotics, lincomycin and clarithromycin, and an antiepileptic drug, carbamazepine, was investigated by monitoring drugs decomposition and identifying intermediates in Po river water (North Italy). Initially, control experiments in the dark and under illumination were performed on river water spiked with drugs to simulate all possible transformation processes occurring in the aquatic system. Under illumination, these pharmaceuticals were degraded and transformed into numerous organic intermediate compounds. Several species were formed and characterised by analysing MS and MS^n spectra and by comparison with parent molecule fragmentation pathways. River water was sampled at three sampling points in an urban area. The selected pharmaceuticals were detected in all samples. Eight transformation products identified in the laboratory simulation were found in natural river water from carbamazepine degradation, three from clarithromycin and two from lincomycin. Their transformation occurring in aquatic system mainly involved mono- and poly-hydroxylation followed by oxidation of the hydroxyl groups.

Keywords: Carbamazepine Clarithromycin Lincomycin Orbitrap Intermediates

Introduction

For decades, the scientific community attention was devoted to the study of conventional pollutants or macropollutants, which include hundreds of chemical compounds traditionally under regulation in the field of environmental monitoring. These contaminants comprise chlorinated compounds, dioxins and pesticides, whose high environmental persistence is linked to their lipophilicity and

stability. The development of new and more sensitive methods of detection, identification and quantification of trace elements and the evaluation of toxicological effects, has moved the attention toward the so-called emerging pollutants, defined as substances not previously known or of newly identification (Richardson and Ternes *2011*; Daughton *2001*). Pharmaceuticals are included in this broad family of substances, as they are ubiquitous environmental pollutants that contaminate the environment through a number of point sources (Khetan and Collins *2007*; Daughton and Ternes *1999*). They are at present a major source of bioactive molecules in the water. Their low volatility forces their spreading by transport in water and their polar nature prevents the removal from aqueous system by facilitating the dispersion through the food chain (Bottoni and Fidente *2005*). Early research related to the presence of such substances in the environment were conducted in the USA in 1976 and in England in 1985, but more detailed and systematic studies only began since the early 1990s; recently, monitoring studies on rivers and wastewater treatment plants was published (Zuccato et al. *2006*). The growing awareness of this new form of environmental contamination led the scientific community to address an increased interest in pharmaceutical products. Many studies showed the presence of these compounds in surface water, groundwater and drinking water (Heberer et al. *2002*; Kummerer *2004*). Improper disposal of unused or expired medicines, industrial and hospital waste, facilities for aquaculture, the run-off from farms and livestock contribute significantly to the detection of such compounds in natural waters and other environmental media. However, the main source for pollution, responsible for 70–80 % of the contamination, is excretion by pharmacologically treated patients (Zuccato et al. *2000*).

Some of these compounds show long environmental persistence, being only partially or slowly biodegradable. Other compounds have a rather less persistence but, due to their continuous release into the environment or the formation of transformation products arising from their degradation, may impact on the aquatic ecosystem and human health.

This study followed from a project devoted to the identification of PhACs in Po river water (Northern Italy). Three pharmaceuticals, carbamazepine, lincomycin and clarithromycin, included in the priority list of pharmaceutical products, were detected. Their occurrence has been frequently highlighted in surface waters, so that continuous monitoring and toxicological studies are required (Castiglioni et al. *2005*); however, the information available about the fate of these three drugs in aquatic system appear rather low (Zuccato et al. *2010*; Miao et al. *2003*). Systematic data are scarce and insufficient for an environmental risk assessment. An effort was recently bring in by a study focused on antibiotics monitoring; it showed that urban STPs are the main source of their inflow in the environment and only some classes of antibiotics, i.e. macrolides and quinolones contributed significantly to the environment contamination (Zuccato et al. *2010*).

With this in mind, this study was aimed to enlighten the fate of the selected drugs in Po river water, focusing on the drugs degradation and identification of their transformation products and trying to recognise the main transformation routes followed by these drugs when discharged in the environment. The goal was attained in two steps. Firstly, laboratory experiments were performed by using river water spiked with drug(s) under dark or simulated solar light. Previous studies were concerned with the mechanism of TiO_2-mediated photolysis of lincomycin (Calza et al. *2012a*), clarithromycin and carbamazepine (Calza et al. *2012b*), where unknown degradation products were identified and characterised by multiple stage mass spectrometry; the photocatalytic process can be used to artificially produce degradation compounds similar to those formed in oxidation/reduction of metabolic and environmental pathways (Calza et al. *2004*, *2010*, *2011*).
Secondly, all the possible main and secondary transformation products (TPs) were searched for in several samples collected from the Po river.

Materials and reagents

Carbamazepine (CAS 298-46-4, 99 % purity), lincomycin (CAS 7179-49-9, 99 % purity) and clarithromycin (CAS 81103-11-9, ≥95 % purity) were provided from Sigma-Aldrich.
Chemicals used for preparation of the chromatographic eluents used in different analysis were: formic acid (99 %) from Merck and acetonitrile from Scharlau (AC0331 Supergradient HPLC grade).

Procedures for irradiation

Po river waters sampled on Turin on 1 February 2010, enriched with selected drugs (15 mg L^{-1}) just after sampling and then irradiated. Irradiations were carried out in Pyrex glass cells and performed using a Philips TLK/05 lamp (40 W/m^2) with the maximum emission at 360 nm. The temperature reached during the irradiation was 38 ± 2 °C.

Sample preparation

Po river water samples were collected in a sampling campaign performed in Turin at three sampling points, all located close to the city centre and far from wastewater input, from 1 February 2010 to 8 March 2010. Samples were collected at 1 m depth, 2 m far from the river border using brown glass bottles; samples are then kept in the dark and promptly analysed. Field blanks were also collected close to the Po river source to ensure samples not contaminated with selected drugs. River water

main quality parameters were as follows: pH = 6.6; dissolved oxygen, 4.2 mg/L; chemical oxygen demand, 4 mg/L, and electric conductivity, 360 μS/cm.

River Po water samples were concentrated by solid-phase extraction (SPE) using 500 mg/3 mL Strata X (Phenomenex) cartridges. Water samples (200 mL) were spiked with 200 μL isoxsuprine (1 mg/L) used as recovery standard. Elution was performed with 2 mL CH_3OH and 2 ml of 2 % ammonia in CH_3OH. Eluted solutions were dried under nitrogen flux and reconstituted with 200 μL 0.05 % formic acid, then directly analysed by HPLC/MS.

Quantitative data were obtained through an external calibration after normalisation on isoxsuprine signal. Limit of detection after concentration on SPE cartridges was 0.5 ng/L. The same extraction procedure was applied to a standard mixture analysis of an ultrapure water sample spiked with drugs and subjected to illumination. The extraction recovery was evaluated to be >90 % percentage for all TPs, performing the analysis of photo-degradation mixture before and after SPE.

Analytical techniques: HPLC-MS

Chromatographic separations, monitored using an MS analyser, were run on a Phenomenex LUNA C18 column, 150 × 2.0 mm, using an Ultimate 3000 HPLC instrument (Dionex). Injection volume was 20 μL and flow rate was 200 μL/min. Gradient mobile-phase composition was adopted: 5/100 in 0/21 min of acetonitrile/0.05 % formic acid.

An LTQ Orbitrap mass spectrometer (ThermoFisher) equipped with an ESI ion source interface was used. The LC column effluent was delivered to the ion source, using nitrogen as sheath and auxiliary gas. The source voltage was set at 4.5 kV. The heated capillary temperature was maintained at 265 °C. Prior to analysis, the acquisition method used was optimised by tuning sections for the parent compound (capillary, magnetic lenses and collimating octapoles voltages) to achieve maximum sensitivity. The tuning parameters adopted for the ESI source (ion positive mode) were: capillary voltage, 11.00 V and tube lens, 70 V; for ions optics—multipole 00 offset, −1.25 V; lens 0 voltage, −4.00 V; multipole 0 offset, −4.50 V; lens 1 voltage, −10.00 V; gate lens voltage, −74.00 V; multipole 1 offset, −11.00 V; and front lens voltage, −4.75 V. Mass accuracy of the recorded ions (vs. calculated) was ±5 millimass unit (without internal calibration).

Results and discussion

Drugs degradation in laboratory simulation

Po river water was spiked with selected pharmaceuticals and subjected to different treatments; drugs disappearance profiles are collected in Fig. 1. Previous measurements on sterilised water showed that photolysis and thermal decomposition did not contribute to drugs decomposition in the considered times (Calza et al. *2012a, b*). In river water, in the dark a slight degradation occurred with less than 10 % abated after 16 days, implying an almost negligible contribution of biotic processes to the drugs degradation.

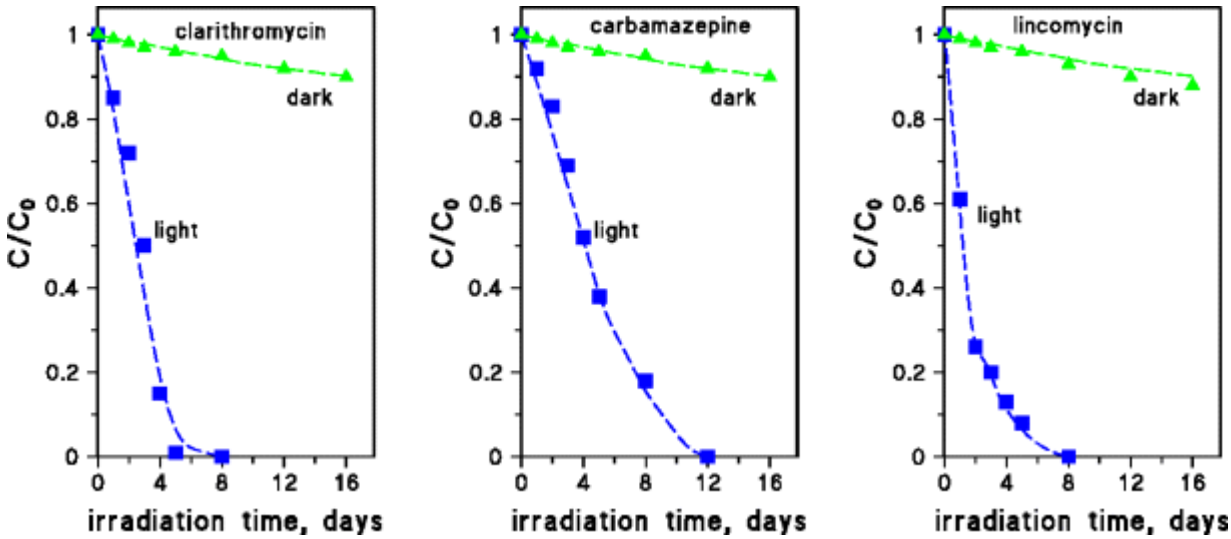


Fig. 1 Degradation of clarithromycin, carbamazepine and lincomycin in Po river water in dark condition or under light exposure

When river water samples were subjected to irradiation, the degradation occurred and the concentration of lincomycin, clarithromycin and carbamazepine was halved after, respectively, 2, 3 and 4 days of light exposure. All drugs were completely degraded within 12 days of irradiation, demonstrating that indirect photolysis processes empower their abatement in the aquatic compartment.

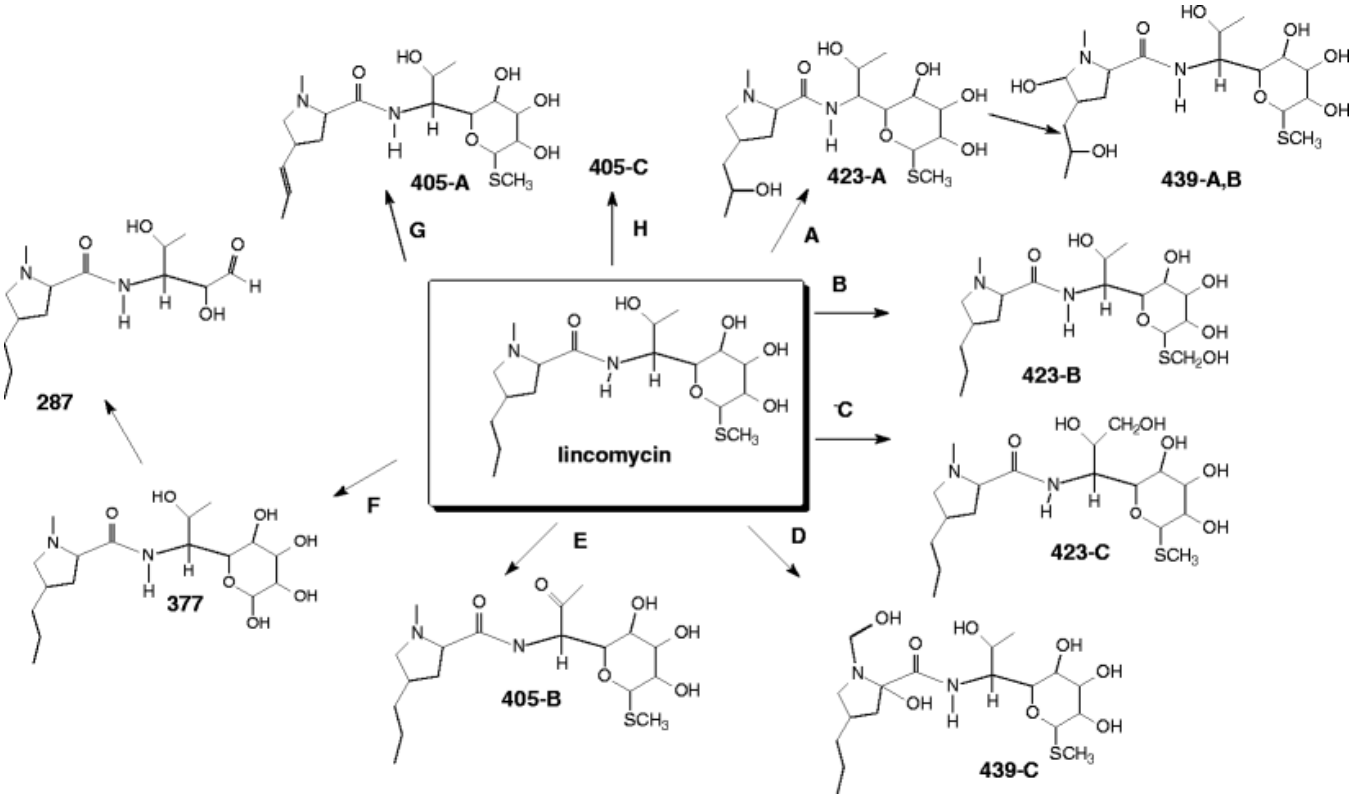
Drugs transformation in laboratory simulation

Lincomycin

A previous study on lincomycin in sterilised water had shown that, through homogeneous photolysis, two TPs were formed, namely 377 (formed through detachment of the thiomethyl group and hydroxylation) and 423-A (monohydroxylated derivatives) (Calza et al. *2012a*).

These TPs were also detected in dark experiments conducted in river water, implying that their formation could involve both biotic and photochemical processes; additionally, another monohydroxylated derivatives (namely 423-C) and oxidised lincomycin (405-B) are formed (see

Scheme 1). These TPs matched with compounds identified through a photocatalytic process and previously characterised by HPLC/HR-MS ^{n} (Calza et al. *2012a*); these are collected in Table S1 as Electronic supplementary material (ESM). TPs temporal profiles of formation/disappearance proved as these processes did not occur simultaneously, but the oxidation reaction appeared to be a kinetically favourite (data shown in Fig. S1 in the ESM).



Scheme 1 Proposed transformation pathways followed by lincomycin in river water under laboratory simulated conditions

Upon light exposure, lincomycin transformation in river water easier occurred and proceeded through the formation of the TPs plotted in Fig. 2 and Scheme 1. Most of them coincide with those formed through the TiO_2 photo-induced process (see Table S1). Among the 21 TPs previously characterised (Calza et al. *2012a*), 11 of them were also found in spiked river water samples, whose formation involved mono- and dihydroxylation (423 and 439), thiomethyl group detachment (377), ring cleavage (287) and oxidation to a keto group (405). It has to be underlined that while the above mentioned TPs (377, 423-A, 423-C and 405-B) could also be formed through biotic or direct photolysis processes, the other compounds were detected in irradiated Po river samples only, so that their formation could be attributed to indirect photolysis processes mediated by natural species, e.g. dissolved organic matter, nitrite and nitrate ions, H_2O_2 and iron species (Boule et al. *2005*). A new isomeric form for TPs 405, labelled 405-C, was identified in river water samples only and started an additional transformation route. Unfortunately, its MS ^{n} spectra did not show the formation of

structural-diagnostic ions helpful for the characterisation of this new TP and, for such, a structure is not shown in Scheme 1.

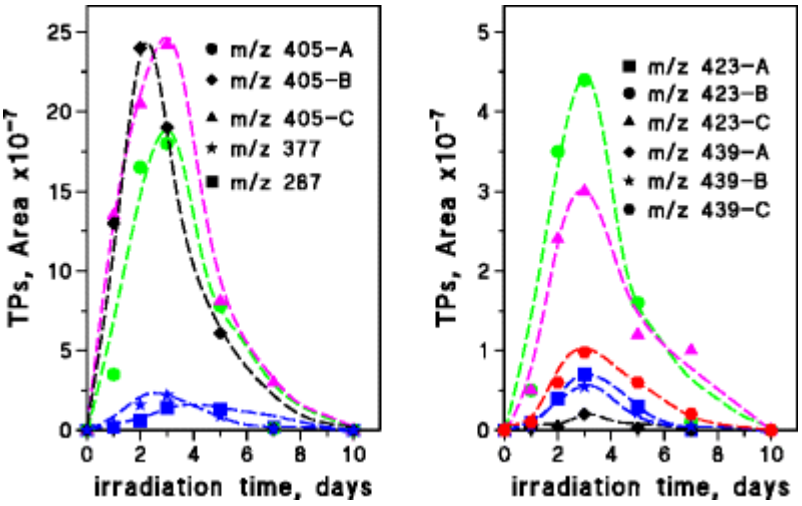


Fig. 2 Lincomycin transformation products produced through homogeneous photolysis in Po river water

Based on their temporal profiles, all these TPs could be formed following eight initial parallel transformation pathways. As a consequence of discussion above, pathways B, D and G started by indirect photolysis processes only and should be utilised as "environmental markers" for the photoinduced processes occurring in the aquatic system. Four routes (namely A, C, E and F) could involve also microbial degradation, while two pathways (namely A and F) could be also promoted by direct photolysis process, as discussed above. It has to be noted that the maximum concentration of TPs 287 and 439 was reached at an irradiation time larger than that of the other degradation compounds, suggesting the occurrence of consecutive pathways depicted in Scheme 1.

Clarithromycin

Experiments previously conducted on clarithromycin in sterilised water revealed the formation of numerous species (Calza et al. *2012b*). In the samples subjected to homogeneous photolysis, the formation of 14 TPs occurred and comprises mono- and di-hydroxyl clarithromycin derivatives or oxidation products. Under heterogeneous photocatalysis, 21 degradation products were identified and characterised by MS^n spectra analysis (all collected in Table S2).

Tests conducted in River water in the dark showed the formation of three monohydroxy derivatives (namely 764-B, 764-C and 764-D), whose formation was necessarily promoted by micro-organisms (see Figure S2 for temporal profiles).

Upon light exposure, river water samples had highlighted the formation of monohydroxylated derivatives (764) too; these TPs could be formed through combined biotic and abiotic processes.

Furthermore, fifteen intermediates were only formed upon irradiation. All the identified species are well matched with the TPs formed through a photocatalytic process (Table S2).

Species with greater abundance appear to be TPs involving monohydroxylation/dehydrogenation (762), di-hydroxylation (780), detachment of cladinose (622) and monohydroxylation (764), as assessed in Fig. 3. TPs 796 (trihydroxylation), 794 (trihydroxylation/oxidation) and 746 (oxidation) were also detected in lower amount. Among these, TPs 762, 746, 764 and 780 formation could be ascribed to direct photolysis or biotic processes, while TPs 794, 622 and 796 formation has to be mainly attributed to an indirect photolysis process.

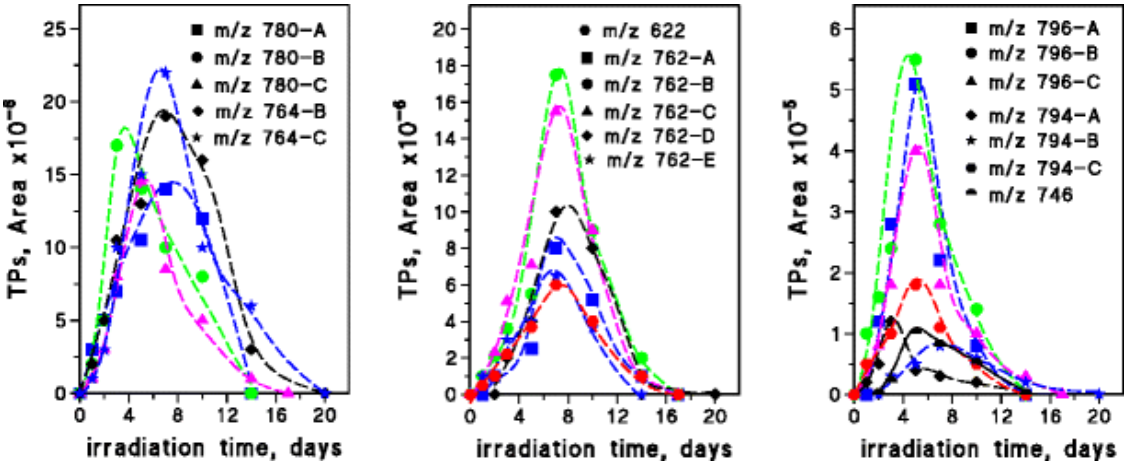


Fig. 3 Clarithromycin transformation products produced through homogeneous photolysis in Po river water

Carbamazepine

Any TPs were detected in tests conducted in the dark, so permitting to exclude biotic contribution to the carbamazepine degradation in the considered time. Conversely, UV–vis irradiation for times up to 16 days revealed the formation of most of the intermediates already highlighted by the photocatalytic process, with the only exception of TP 267 (see Table S3) (Calza et al. *2012b*). All TPs formed in river water are plotted in Fig. 4, where their temporal evolution profiles are reported. Carbamazepine transformation in spiked river water proceeded through hydroxylation with the formation of TPs 253, 269 and 285 (mono-, di- and trihydroxy carbamazepine), hydroxylation and ring contraction (253-A, 269-H and 285-B), hydration of the C_{10}–C_{11} double bond (TPs 271 and 287) and intramolecular cyclization (TP 251). Some carbamazepine monohydroxyl derivatives, recognised as 10,11-dihydro-10,11-epoxycarbamazepine, 2-hydroxycarbamazepine (namely 253-B), 3-hydroxy-carbamazepine (namely 253-C) and 10,11-dihydro-10,11-di-hydroxycarbamazepine (namely 271) were known to be also human metabolites (Maggs et al. *1996*; Breton et al. *2005*) and

have been already detected in aqueous matrices arising from wastewater treatment plants (Miao et al. *2005, 2003*). Conversely, formation of other TPs could be attributed to photoinduced pathways.

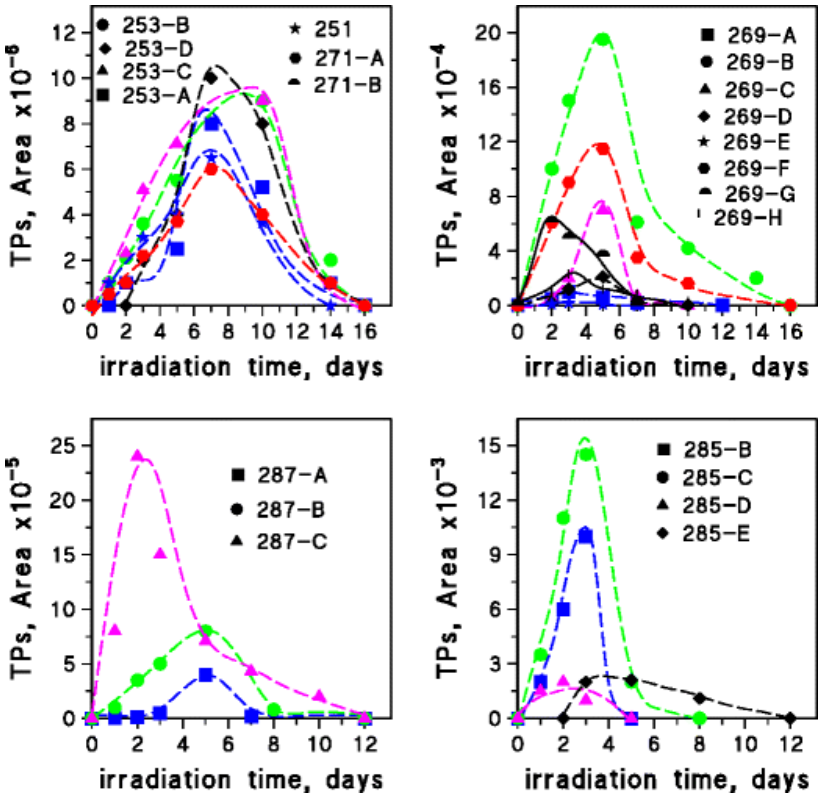


Fig. 4 Carbamazepine transformation products produced through homogeneous photolysis in Po river water

In-field analysis

Selected drugs and their transformation products were searched out in all Po river samples, collected as described in "Sample preparation". Carbamazepine, lincomycin and clarithromycin were detected in all samples, together with several TPs, all plotted in Fig. 5.

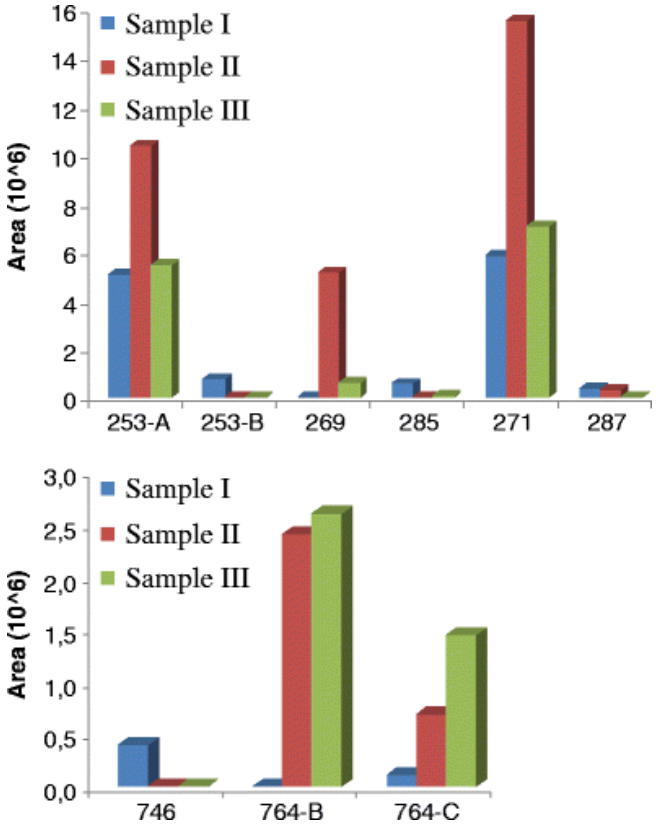


Fig. 5 Drugs transformation products detected in Po river samples; top carbamazepine and bottom clarithromycin.

Sum of TPs main areas have been utilised to estimate the amount of metabolised drugs. The relative ratios between sum of the TPs area and area of parent compound was calculated

Among the detected pharmaceuticals, carbamazepine was the most abundant, with an average concentration of 74.4 ng/L. This high value is not surprising since the occurrence of carbamazepine in river water and effluents from different sewage treatment plants in Italy has been often highlighted (Castiglioni et al. *2005*; Andreozzi et al. *2003*) at concentrations quite high (several hundreds of nanograms per litre). Its high concentration should be partially attributed to its stability and inefficiency in removal treatments (Zuccato et al. *2005*; Castiglioni et al. *2005*; Andreozzi et al. *2003*), combined with an increased drug consumption in recent years.

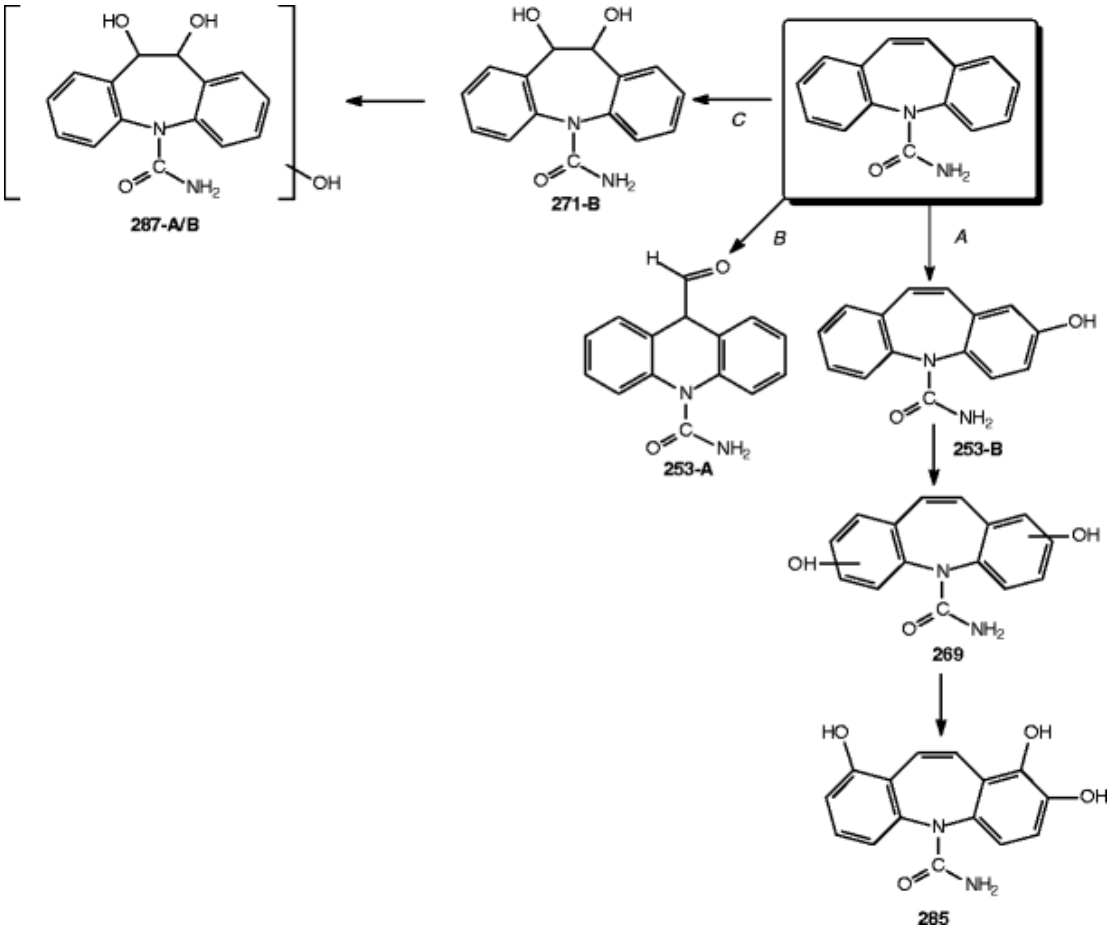
Lincomycin was detected at an average concentration of 20.0 ng/L; this quantity is within the range of available values. The occurrence of lincomycin in Po river has been often highlighted and is very variable; since 1997 its concentration was estimated to be 4.6 ng/L (Castiglioni et al. *2004*), while subsequent studies reported concentrations up to 32 ng/L (Zuccato et al. *2005*); recent data revealed that the drug is present at rather lower concentrations, ranging from 3 to 8 ng/L (Zuccato et al. *2010*). As lincomycin is a drug intended to both human and veterinary use, these high levels could be attributed to the large number of animal farms in the vicinity of river Po (Calamari et al. *2003*).

Finally, note that the monitoring of lincomycin in water exiting the wastewater treatment plants had shown an incomplete drug removal (Zuccato et al. *2010*). This, together with the excretions of treated patients, was the major sources for drug release in the aquatic compartment.

Clarithromycin concentration in the three analysed samples settled at an average value of 4.6 ng/L. It should be noted that the estimated amounts were within the range of concentration monitored into previous River Po samples, where the presence of clarithromycin was highlighted at concentrations between 2 and 20 ng/L (Zuccato et al. *2010*; Calamari et al. *2003*). Clarithromycin was one of the more abundant antibacterial found at the entry of sewage treatment plants (concentrations of several hundreds of nanograms per litre) (Zuccato et al. *2010*), and the processes of mechanical and chemical treatment were not sufficient to ensure its complete removal.

Along with the selected drugs, several TPs already identified in laboratory experiments were also detected in natural Po river water, whose formation mostly occurred through a combination of biotic and abiotic pathways. Their relative amount is plotted in Fig. 5.

The search for TPs of carbamazepine in river water samples has led to the identification of numerous compounds. All the recognised TPs may be linked through the transformation pathways summarised in Scheme 2: a qualitative assessment can highlight that the main transformation products were those arising from hydration on the C_{10}–C_{11} double bond and monohydroxylation. Due to the discussion above, these TP_S could be formed following three initial pathways, involving hydroxylation (pathway A), hydroxylation and ring contraction (pathway B) and hydration on the double bond (pathway C). The compounds 253-B and 271 should be formed from human metabolism or abiotic processes, as they were recognised as the most important metabolites of carbamazepine (Petrovic and Barcelò *2007*; Miao et al. *2003*). Conversely, the formation of TPs 253-A, 269 and 285 has to be attributed to abiotic pathways.

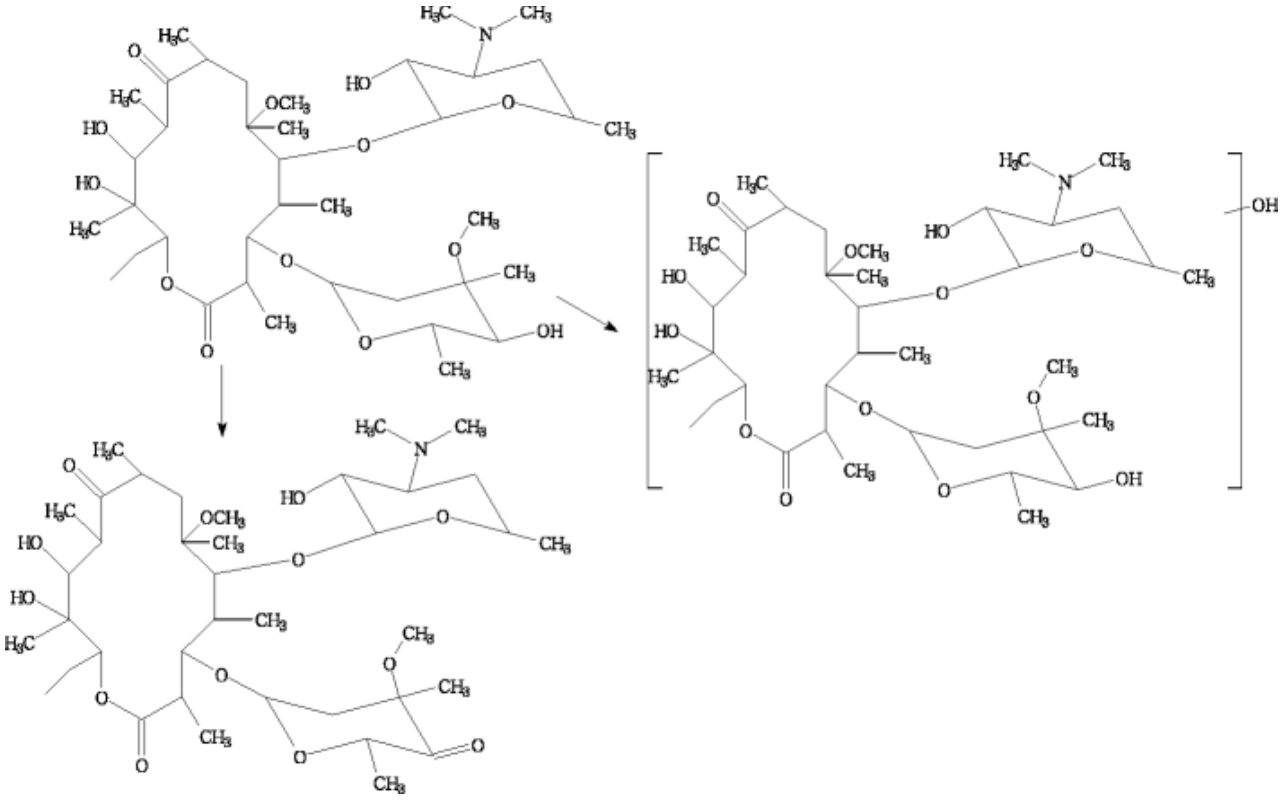


Scheme 2 Transformation products formed by carbamazepine in river water into in field samples

Based on the peak areas, the sum of all TPs found roughly approximately 50 % of the drug amount, so underlining that almost 50 % of the drug was degraded. The temporal profiles depicted in Fig. 4 show for all the detected TPs a similar kinetic of formation/disapperance, so that, by analysing their relative ratios, it is possible to understand which are the predominant transformation pathways followed in river water. Compounds 253, 269 and 285 are closely related through a hydroxylation mechanism that involves a sequence of hydroxyl entrance; the lower amount detected is due to the consecutive pathways depicted in Scheme 2. Compounds 271 and 287 could be formed through human metabolism or photolysis as well. Analysis of the relative TPs ratios obtained in laboratory and in field samples show that ratio 253/271 passed from 1.33 (laboratory data) to 0.7 for field samples. This could be the expression of a remarkable effect of human metabolism in the drug transformation, particularly for pathway C. Nevertheless, even if compound 253-B, formed through pathway A, could be formed as human metabolite, di- and tri-hydroxycarbamazepine formation should be attributed to a photochemical process, in agreement with the carbamazepine-modelled fate (De Laurentiis et al. *2012*). It permits to consider these compounds as markers of a photo-

initiated carbamazepine transformation in the aquatic environment. Pathway B should be attributed to carbamazepine phototransformation, too.

TPs arising from clarithromycin degradation were found in all samples and Scheme 3 collects all the identified species. It shows the formation of mono-hydroxylated (764-B and 764-C) and oxidised (746) species only, confirming that the degradation of clarithromycin proceeds mainly by oxidation and hydroxylation; the former can also be a sign of a preference for the aquatic compartment. From a qualitative assessment based on the TPs peak areas, the sum of all TPs found roughly approximate 60 % of the drug amount, so underlining that also in this case the drug is largely transformed.



Scheme 3 Proposed transformation pathways followed by clarithromycin in river water into in field samples

Conversely, the search for TPs of lincomycin led to the sole identification of two isomeric forms of monohydroxylated, namely 423-B and 423-C in all samples.

Conclusions

Clarithromycin, lincomycin and carbamazepine were detected in all river samples, together with a number of degradation compounds previously characterised through a photocatalytic process coupled with high-performance liquid chromatography (HPLC)/high-resolution mass spectrometry

(HRMS) analysis, allowing the monitoring of the drug presence and its transformation products in environmental analysis. It may be noted that carbamazepine appears at higher concentrations than the other two drugs: this is consistent with the degradation tests conducted in the laboratory that have shown that the former has longer persistence in the aquatic compartment.

HRMS, in this case combined with high performance liquid chromatography is a technique that plays a key role in the investigation of environmental processes and in the study of the fate of pollutants.

This approach has permitted not only to assess the selected drugs presence in natural waters but also to identify which of the transformation routes recognised in simulation experiments, also occurred in the aquatic environment. Several TPs were identified. Some of them seems to be formed through a biotic or abiotic process. Specifically for the case of carbamazepine, it was possible to find some key TPs that could be considered as markers for its photochemical environmental transformation in the aquatic environment.

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