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**An automated on-line turbulent flow liquid-chromatography technology coupled to a high resolution mass spectrometer LTQ-Orbitrap for suspect screening of antibiotic transformation products during microalgae wastewater treatment**

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**Highlights:**

- An on-line LC-HRMS methodology with automated suspect screening data analysis.
- Tentative identification of 40 tentative transformation products of 9 antibiotics.

- Understanding biotic and abiotic mechanisms in antibiotic removal by microalgae.
- Detection of TPs in a microalgae photobioreactor treating toilet wastewater.

## ABSTRACT

The evaluation of wastewater treatment capabilities in terms of removal of water pollutants is crucial when assessing water mitigation issues. Not only the monitoring of target pollutants becomes a critical point, but also the transformation products (TPs) generated. Since these TPs are very often unknown compounds, their study in both wastewater and natural environment is currently recognized as a tedious task and challenging research field. In this study, a novel automated suspect screening methodology was developed for a comprehensive assessment of the TPs generated from nine antibiotics during microalgae water treatment. Three macrolides (azithromycin, erythromycin, clarithromycin), three fluoroquinolones (ofloxacin, ciprofloxacin, norfloxacin) and three additional antibiotics (trimethoprim, piperidic acid, sulfapyridine) were selected as target pollutants. The analysis of samples was carried out by direct injection in an on-line turbulent flow liquid chromatography–high resolution mass spectrometry (TFC-LC-LTQ-Orbitrap-MS/MS) system, followed by automatic data processing for compound identification. The screening methodology allowed the identification of 40 tentative TPs from a list of software predicted intermediates created automatically. Once known and unknown TPs were identified, degradation pathways were suggested considering the different mechanisms involved on their formation (biotic and abiotic). Results reveal microalgae ability for macrolide biotransformation, but not for other antibiotics such as for fluoroquinolones. Finally, the intermediates detected were included into an in-house library and applied to the identification of

tentative TPs in real toilet wastewater treated in a microalgae based photobioreactor (PBR). The overall approach allowed a comprehensive overview of the performance of microalgae water treatment in a fast and reliable manner: it represents a useful tool for the rapid screening of wide range of compounds, reducing time invested in data analysis and providing reliable structural identification.

Keywords: Automated suspect screening; Transformation products; Transformation mechanisms; Microalgae; Antibiotics; Photobioreactor.

## 1. Introduction

In the last decade, the overuse and misuse of antibiotics has promoted the incidence of an ever-growing spectrum of known and unknown compounds in urban wastewater effluents [1,2]. The presence of these pollutants in wastewater effluents may lead to potential ecological effects and promote bacterial resistance even at low concentration [3,4]. In fact, antibiotic resistant bacteria have been classified by the World Health Organization (WHO) as one of the three biggest threats to public health in the 21<sup>st</sup> century [5]. Since conventional wastewater treatment plants (WWTPs) are not designed to eliminate these emerging contaminants [6,7], the study of new and alternative wastewater treatment technologies becomes crucial to attain optimal removal efficiencies and increase the knowledge about their environmental fate [8].

Bioremediation technologies have been recognized as potential and alternative systems to provide high-removal rates on treated effluents [9]. Among the existing bioremediation technologies, microalgae-based water treatment has been lately suggested as solar power-driven, ecologically friendly and sustainable reclamation strategies [10,11]. In addition, they exhibit higher tolerance to antibiotics than bacterial species, as they are not target organisms for these compounds [12]. Microalgae has been proven to be also effective for elimination of organic substances [13], which cannot only attributed to biotransformation but also to photodegradation and uptake

processes [14]. Despite numerous studies have been focused on pharmaceutical removal in microalgae water treatment [9–11,13–17], few attention has been paid to the study of transformation products generated from the target pollutants [18]. The presence of these unknown compounds can play an important role since they might be more persistent and/or toxic than the parent compound [19]. The main difficulty to overcome their identification lies in the lack of pure analytical standards and fast analytical methods to confirm their presence along water treatment [20]. Hence, new analytical approaches comprising reliable structural identification are of high interest to easily overcome this tedious task. To this regard, high resolution mass spectrometry (HRMS) with electrospray ionization (ESI) is considered the analytical technique most widely used, since it makes possible to detect hundreds of unknown compounds in a single run [21]. Suspect screening methodologies, where tentative compounds are suggested by using libraries or prediction tools [20,22–25], are the most applied analytical strategies for the tentative identification of compounds in samples. Up to now, this approach has been widely used by different authors throughout post-acquisition data processing [1,26]. However, the reported workflows comprise several steps such as chromatographic data processing, data reduction, MS library search and MS/MS spectra elucidation [27]. To greatly facilitate analyte identification, most of the studies rely on online databases. However, manual data compiling is always required and the number of compounds identified are limited to those entities already known [28]. According to this, automated TP identification by using prediction tools may represent an important advance to detect new unknown chemicals [29–31], especially when new and alternative water treatment technologies are evaluated. Additionally, such automated data treatment tools for suspect screening analysis would allow the simultaneous evaluation of several target substances in just one experiment, avoiding the performance of multiple single experiments for each compound. On the other hand, although the application of the on-line turbulent flow chromatography for the identification of TPs in real wastewater treatment matrices is not new [15,32,33], this

technology permits an on-line direct clean-up of dirty wastewater samples with less sample manipulation and better performance to detect TPs at low concentration levels [34]. Therefore, the application of this technology together with automatic software data processing may represent a useful tool for the rapid screening of wide range of suspect compounds, not only reducing the time invested in sample analysis but also in data treatment to finally attain reliable structural information.

The main objective of this study was to develop an automated analytical methodology to understand the transformation and fate of nine antibiotic compounds during microalgae water treatment, both at batch scale and in a pilot photobioreactor treating toilet wastewater. The screening methodology was based on the analysis by an on-line turbulent-flow liquid chromatography coupled with high resolution mass spectrometry (TFC-HPLC-LTQ-Orbitrap) methodology together with an advanced software data processing tool.

This study provided valuable information about transformation of selected antibiotics to better evaluate the scope of microalgae as an alternative wastewater treatment. In addition, the study of TPs allowed to understand the abiotic and biotic processes involved in pollutant removal.

## **2. Materials and methods**

### **2.1. Chemicals and reagents**

Azithromycin (AZI), erythromycin (ERY), clarithromycin (CTM), ofloxacin (OFC), ciprofloxacin (CFC), norfloxacin (NFC), sulfapyridine (SPY), trimethoprim (TMP) and pipemidic acid (PMA) were purchased at high purity grade (>95%) from Sigma-Aldrich (Steinheim, Germany). Ultra-pure water, acetonitrile and methanol LiChrosolv grade were supplied from Merck (Darmstadt, Germany). Solid phase extraction (SPE) cartridges Oasis HLB (60 mg, 3 mL) were from Waters Corporation (Milford, MA, USA).

## 2.2. Microalgal batch experiments

Microalgal batch experiments were performed within 14-day by testing three different experimental conditions for each microalga studied: i) light-biomass (selected microalgae with light irradiation) ii) light-abiotic (irradiation of light without algae) and iii) dark-abiotic (without algae and without light). For microalgal live conditions *Chlamydomonas reinhardtii* (UTEX ID 2243), *Chlorella sorokiniana* (UTEX ID 1663), *Dunaliella tertiolecta* (UTEX ID LB999) and *Pseudokirchneriella subcapitata* (UTEX ID 1648) were selected and grown in their respective synthetic culture mediums: Tris-Acetate-Phosphate (TAP) for *C. reinhardtii* and *C. sorokiniana*, Artificial Sea Water for *D. tertiolecta* and in Bold 3N for *P. subcapitata*. More detailed information about experimental set-up can be found elsewhere [35]. Light experiments were carried out under continuous fluorescent lamp irradiation ( $172 \pm 18 \mu\text{mol}/(\text{m}^2 \cdot \text{s})$  irradiance level), measured by a light meter (LI.189, LI-COR Quantum/Radiometer/Photometer, USA) at a controlled temperature ( $25 \pm 1 \text{ }^\circ\text{C}$ ) and 120 rpm (orbital shaker Kuhner, LS-X, Switzerland). All experiments were carried out in triplicate by spiking the nine antibiotics simultaneously at a final concentration of 100  $\mu\text{g}/\text{L}$  each in 250 mL of synthetic medium. 1 mL of samples were collected in amber glass vials at initial time, and after 7 and 14 days of treatment. Samples were freeze-dried and stored at  $-80 \text{ }^\circ\text{C}$  until analysis. Reconstitution was performed in 100  $\mu\text{L}$  of methanol-water (5:95) before their injection in the TFC-LC-LTQ-Orbitrap-MS/MS system.

## 2.3. Microalgal photobioreactor

A microalgal photobioreactor treating the toilet wastewater was used to evaluate the elimination and transformation of the 9 antibiotics selected. The experimental set-up of this experiment has been previously described [36] as well as its microbial characterization [37]. Briefly, urban wastewater was collected from the toilet drainage of the “Chemical, Biological and Environmental Engineering Department” (Universitat

Autònoma de Barcelona, Barcelona, Spain) and pumped to an enclosed 1200 L multitubular microalgal photobioreactor (PBR). Three samples from the inlet wastewater and three samples PBR effluent were taken in three non-consecutive days after the theoretical hydraulic steady state of twelve days was reached. To enhance concentration of TPs, 25 mL and 50 mL for influent and effluent respectively were pre-concentrate up to 1 mL by using the SPE methodology previously reported [38].

## 2.4. Analytical methodology and data processing

### 2.4.1. TFC-LC-MS/MS analysis

Samples were analyzed using an on-line turbulent flow liquid-chromatography system coupled to a high-resolution mass spectrometer (HRMS). For sample purification and separation purposes, the Aria TLX-1 chromatographic system (Thermo Fisher Scientific) was used. The system comprised a PAL auto sampler and two mixing quaternary pumps (eluting and loading pumps). 20  $\mu$ L of samples were directly injected into the chromatographic system. The clean-up step was performed in a Cyclone (50  $\times$  0.5 mm, 60  $\mu$ m particle size, 60 A pore size; Thermo Fisher Scientific, Franklin, MA) and the compounds were separated using a ZORBAX Eclipse XD-C18 (150  $\times$  4.6; 5  $\mu$ m; Agilent Technologies, Santa Clara, CA, USA). Detailed information about the solvent gradient used can be found in Table S1, and an example of Total Ion Chromatogram (TIC) in Fig. S1. The total chromatographic run time was 18 min.

The LC system was connected to a LTQ-OrbitrapVelos<sup>TM</sup> (Thermo Fisher Scientific Company; Villebon-France) equipped with a diverter valve and an Electrospray Ionization Source (ESI). The analysis was performed in positive and negative ionization modes. As no results were found for negative mode experiments, data processing was carried out in positive mode only. Chromatograms and mass spectra were acquired in *Data Dependent Acquisition* (DDA) in two parallel scan events: the first one (1) was acquired in full-scan mode within a mass-to-charge ( $m/z$ ) range of 100-800  $m/z$  at a



resolving power of 60000 FWHM (MS) followed by (2) fragmentation of the most intense ion masses detected (MS/MS) at 30000 FWHM. These MS/MS experiments were performed applying a dynamic mass exclusion mode to discriminate co-eluted compounds: ions fragmented more than 3 times during 25 seconds were further ignored for fragmentation during the following 30 seconds (corresponding to peak plus tailing). Mass spectrometry conditions were set up as follows: spray voltage, 3500V; capillary temperature, 300°C; sheath gas pressure, 40 arb; and aux gas flow rate, 20 arb; collision energy, 35 eV CID, isolation width, 2 Da. For some particular TPs, a tougher fragmentation through 55 eV HCD was used for final identification. The entire system was controlled via Aria software, version 1.6, under Xcalibur 2.1 software. For inlet and PBR effluents wastewater samples, the previous analytical methodology was adapted: the in-house library containing the information about all tentative TPs identified in the batch experiments was used as a prescreening list to be used as criterion to trigger MS fragmentation in the second scan event for MS/MS fragmentation and confirmation.

#### **2.4.2. Automated data processing**

An automated data-processing methodology by using Compound Discoverer 1.0 (Thermo Scientific) connected to Mass Frontier 7.0 software (Thermo Scientific) was applied for the identification of the TPs generated. The overall workflow describing all steps involved in data processing is presented in Fig. 1.

Prior to automatic software data processing, computational data files (chromatograms and mass spectra) were loaded into the software. Target antibiotic structures (9 antibiotics) were pinpointed as parent compounds as well as the potential chemical transformations to be applied to them by software simulation: methylation, oxidation, reduction, hydroxylation, reductive defluorination, oxidative defluorination, decarboxylation, oxidative deamination to alcohol, oxidative deamination to ketone,

desaturation, dehydration, hydration, acetylation, carboxylation, piperazinyl dealkylation, sulfation, sulfonamide alkylation and sulfur dioxide reduction. A combination of a maximum number of two dealkylation steps for a maximum of three consecutive chemical transformations were selected. Using all this prior information, a list with predicted TPs was created during the automatic data processing run.

Automatic data processing starts with MS data filtering in the  $m/z$  range between 100 Da and 800 Da, and by setting a peak intensity threshold at 10 signal-to-noise ratio. To compensate small differences in retention times, chromatographic alignment was performed by using a mass tolerance error of 5 ppm and a maximum retention time shift of 0.5 min. In parallel, the list containing the 9 parent compounds and their predicted TPs was automatically generated including the corresponding exact masses and the software transformation applied. This list was automatically compared with experimental data by using an MS mass tolerance of 5 ppm and a minimum chromatographic peak intensity of 1000 counts. Those parent and predicted compounds successfully matched in samples were included into a list of detected compounds. For confirmation purposes, MS/MS spectra were automatically elucidated by using predicted fragment structures with a mass tolerance of 5 ppm and a signal-to-noise ratio of 10. The percentage value obtained for each detected compound (FISH scoring) indicated the MS/MS reliability on automatic compound identification.

After software data processing, results were filtered by selecting those compounds with FISH values  $\geq$  than 65% [39] with at least two characteristic fragments matched with predicted fragment structures. As a final step to avoid false positives, the predicted TP structures and their elucidated MS/MS spectra were manually reviewed. Both parent and confirmed TPs were included into an in-house library and used for the detection of TPs in microalgal-based photobioreactor samples.

### **3. Results and discussion**

### 3.1. Software data processing

Four data sets (one for each microalga experimental set) were automatically processed by the software (each set lasting 13 hours on the software run). Each data set included the experimental files obtained from light-biomass experiments with a particular microalga, a light-abiotic and a dark-abiotic experiment. Automatic processing reduced the number of chromatographic peaks up to 10% without manual refining. In contrast to other methodologies described, this extent on data reduction was only achieved with a combination of automatic and manual processing [40–42]. 73 suspected compounds out 12291 predicted were tentatively detected in samples after the automatic data processing, including the 9 parent compounds and 64 TPs. After manual review, the confirmed list was reduced to the 9 parent compounds and 40 TPs: 8 TPs for AZI, 6 for ERY, 2 for CTM, 8 for OFC, 5 for CFC, 5 for NFC, 3 for PMA, 2 for TMP and 1 for SPY. Among them, 19 TPs were reported as direct matches, where TP structures were directly proposed by the software from automatic MS/MS spectra elucidation. On the other hand, 21 TPs were reported as shifted matches, where TP structures proposed required additional transformations to define the final chemical structures. An example of direct and shifted matches is presented in Fig. S2. Automated MS/MS elucidation with annotation of the corresponding tentative fragments contributed to reduce the processing time for TPs identification. This workflow allowed the elucidation of a high number of potential TPs without performing degradation experiments for each of the compounds separately, saving time and laboratory resources. Nonetheless, although this methodology provided a rapid tool for peak filtering with less handling operation, manual work was necessary in a final step to avoid false positives and evaluate findings. The final 40 TPs were registered in an in-house library, which included for each compound, its retention time, elemental composition, fragmentation ions, mass error, double bond equivalents (RDB) and the tentative chemical structure (Table S2). The presence of the 9 parent compounds and their 40 suspect TPs was monitored

along the experiments performed, both in batch and in microalgal photobioreactor treating toilet wastewater. Their relative concentrations were calculated and presented in Figs. 2-5.

### **3.2. Evaluation of antibiotic transformations during microalgae batch experiments**

Antibiotic transformation was investigated along microalgae treatment experiments.

While the results about removal of parent compounds are discussed in detail elsewhere [35], in this work the results about antibiotic transformations are presented and discussed separately in three different groups: macrolides (azithromycin, erythromycin, clarithromycin), fluoroquinolones (ofloxacin, ciprofloxacin, norfloxacin) and other additional and non-related antibiotics (trimethoprim, pipemidic acid, sulfapyridine). Suggested transformation pathways are presented in Figs. 2a-4a.

Relative percentages ( $A/A_0$ )-(%) for each TP (area of the peak detected divided by the area of the chromatographic peak of the parent compound at initial time) at 7 and 14 days of treatment were calculated and summarized in Figs. 2b-4b. The confirmation and quantification and of the individual TPs would require reference standards, though most of them are not commercially available.

#### **3.2.1. Macrolide transformation**

Sixteen major intermediates were tentatively identified coming from macrolide degradation including 8 TPs from azithromycin, 6 TPs from erythromycin and 2 TPs from clarithromycin (Table S2). A shared degradation pathway containing all the TPs detected along the batch experiments are presented in Fig. 2a. Additionally, the relative percentages of those intermediates with values higher than 3% are shown in Fig. 2b for each experimental condition after 7 and 14 days of treatment.

According to dark-abiotic experiments performed in the corresponding TAP, artificial sea water and bold 3N mediums, macrolide elimination achieved a mean percentage of

22±16% for AZI, 29±14% for ERY and 22±32% for CTM suggesting a direct contribution of abiotic factors on macrolide degradation. Among the intermediates generated, TP590 (up to 33% of the initial AZI), TP575 (up to 12% of initial ERY) and TP589 (up to 6% of initial CTM) were the most common structures identified (Fig. 2b); all formed by O-dealkylation of L-clandinosose moiety (Fig. 2b). On the other hand, TP717 (also named erythromycin D) was generated throughout hydroxylation reaching a ratio formation of about 20% of initial ERY. Their presence was suggested to come from the instability of macrolides in aqueous solution [43,44], confirmed by their presence at initial time at low concentration levels (lower than 3% of initial presence of the corresponding antibiotic; data not shown). In light-abiotic experiments, similar removal rates of parent compounds up to 23±12% for AZI, 34±14% for ERY and 21±33% for CTM were obtained compared to dark-abiotic experiments; as well as similar TP formation profiles. Even though some authors reported the major role of light in the generation of O-dealkylated compounds [45,46], our results cannot confirm a contribution of this factor to such type of transformation in macrolides.

Concerning the experiments performed with the 4 microalgae selected (light-biomass experiments), macrolides treated with *D. tertiolecta* presented similar antibiotic degradation rates (c.a. 26±6%) and TP formation profile to the previous abiotic conditions tested. Although some other TPs such as TP766 and TP733 were generated at a low concentration level (up to 12%), TP590 (AZI) was still pointed out as the major intermediate detected. This microalga was thus considered to not contribute extensively on macrolide elimination through biotransformation. Likewise, degradation and TP formation in *P. subcapitata* was much lower than with the other microalgae studied. In this case, average macrolide elimination was around 20±9% and TP formation profile was lower comparing to the abiotic condition tested. On the contrary, *C. reinhardtii* and *C. sorokiniana* showed a decrease on the presence of O-dealkylated intermediates (related to abiotic transformations), along with the generation of new TPs

such as TP766 (AZI), TP764 (AZI), TP734 (AZI), TP608 (AZI), TP733 (ERY), TP719 (ERY) and TP763 (CTM). These can be specifically attributed to microalgae biodegradation rather than to abiotic transformations. In fact, for these two microalgae, the elimination of the parent compounds achieved was much higher than with the other microalgae studied, with average removal values  $57\pm 1\%$ ,  $33\pm 1\%$  and  $16\pm 28\%$  for azithromycin, erythromycin and clarithromycin respectively.

Among the TPs detected, the opening of the macrocyclic lactone ring by hydrolysis of the lactone ester group can be appointed as the most common structural modification. In the case of erythromycin, hydrolysis was first led by the generation of the intermediate TP751 (ERY) followed by condensation to TP733 (ERY), present at high levels (up to 29%). This mechanism has been widely reported by many authors [47–50] and detected in secondary effluent during soil aquifer treatment [51] and in enzymatic degradation experiments with EreB esterase [32]. In this study, this hydrolysis mechanism was also identified as the major biodegradation pathway in azithromycin biotransformation, with the generation of TP766 (AZI) up to 34% of the initial AZI (Fig. 2). The opening of lactone ring was confirmed by the loss of an instauration grade (DBE) whereas d-desosamine and L-cladinose was maintained (Table S2). Unlike for erythromycin, no further molecular condensation was observed probably due to the presence of a methyl-substituted nitrogen in azithromycin Z-W position, preventing further dehydration step [52]. In the case of clarithromycin, few intermediates were detected, pointing it out as the most recalcitrant macrolide in this study. One of the reasons might be attributed to the presence of a methyl group in R<sub>1</sub> position increasing the steric effects in some TPs to be generated.

In general, the presence of higher amounts of TPs at 14 days than 7 days of treatment in all experimental conditions indicates that, even though macrolides were apparently eliminated to certain extent, they were not mineralized. Nonetheless, they were transformed into new entities that are not necessarily degraded fast enough and could

potentially keep some of the activity of the parent compounds. As observed, the use of microalgae for antibiotic biodegradation provided a higher number of intermediates compared to abiotic experiments. Therefore, a careful evaluation of the risk that these transformation products can pose should be performed. It would permit to determine the most beneficial conditions to eliminate macrolide antibiotics.

### 3.2.2. Fluoroquinolone transformation

Eighteen major intermediates were tentatively identified coming from fluoroquinolone degradation including 8 TPs from ofloxacin, 5 TPs from ciprofloxacin and 5 TPs from norfloxacin (Table S2). A shared degradation pathway containing all the TPs detected along batch experiments are presented in Fig. 3a. Additionally, relative percentages of those intermediates with values higher than 3% are shown in Fig. 3b for each experimental condition at 7 and 14 days of treatment.

According to dark-abiotic experiments, fluoroquinolone abiotic elimination can be considered negligible. These results are in accordance with the lack of intermediates in these dark-abiotic experiments. In contrast,  $85\pm 18\%$  for OFC,  $97\pm 2\%$  for CFC and  $95\pm 7\%$  for NFC were eliminated when light was irradiated in light-abiotic experiments. Several authors have indicated the sensibility of fluoroquinolone to photodegradation, which is pointed out as the most significant transformation mechanism in aquatic systems [53–57]. Amongst the identified TPs via phototransformation (Fig. 3a), ofloxacin demethylation to TP347 (OFC) and ofloxacin desethylation of piperazinyl ring to TP335 (OFC) were described as major intermediates detected up to 55% and 38% respectively of initial parent compounds (Fig. 3b). Ofloxacin was persistent in maintaining piperazinyl ring integrity as first-generation TPs. This might be explained by the further additional stability conferred by a methyl group in position  $R_1$  in ofloxacin structure (Fig. 3a). On the contrary, complete elimination of the piperazinyl ring in ciprofloxacin and norfloxacin to TP262 (CFC) and TP250 (NFC) respectively was

occurring more easily. From these last TPs, further reductive defluorination to TP244 (CFC) and TP232 (NFC) as third-generation TPs were identified as the major byproducts generated reaching values about 44% and 47% respectively.

Microalgal live experiments lead to lower removal rates of the parent compounds (and also a lower amount of TPs generated) than those obtained in light-abiotic experiments. In fact, fluoroquinolones were eliminated to an average value of  $36\pm 16\%$  for OFC,  $68\pm 14\%$  for CFC and  $61\pm 14\%$  for NFC in *C. reinhardtii*, *C. sorokiniana* and *P. subcapitata* experiments. This can be explained by the shielding effect posed by microalgae, partially preventing exposure of pollutants to light. Photodegradation may thus not occur to the same extent than in light-abiotic experiments. The marine microalgae *D. tertiolecta*, was the only one able to achieve fluoroquinolones removal rates as good as those obtained in light-abiotic experiments being almost eliminated at the end of the experiment. In line with it, concentration of TPs generated after 7 days of treatment was quite high, whereas their presence was almost residual at day 14. The better performance of the marine alga *D. tertiolecta* could be explained by the influence of water matrix rather than by the impact of the microalga itself. Actually, the best removal of fluoroquinolones was achieved in the light-abiotic experiments with artificial sea water whereas light-abiotic controls for the freshwater algae *C. reinhardtii*, *C. sorokiniana* and *P. subcapitata* were performed in less saline media. Matrix composition such as pH, dissolved organic content, chloride ion concentration has actually been reported to have an influence in photodegradation processes [45,54].

### 3.2.3. Transformation of other antibiotics

Six major intermediates were tentatively identified coming from other antibiotics including 3 TPs from pipemidic acid, 2 TPs from trimethoprim and 1 TP from sulfapyridine (Table S2). Degradation pathways containing all the TPs detected along batch experiments are presented in Fig. 4a. Additionally, relative percentages of those



intermediates with values higher than 3% are shown in Fig. 4b for each experimental condition at 7 and 14 days of treatment.

According to dark-abiotic experiments, the abiotic elimination of these 3 compounds can be considered negligible although some residual TPs such as TP259 (PMA), TP274 (TMP) and TP260 (TMP) were generated. These intermediates were also detected in light-abiotic experiments with the same profile, despite of the partial removal of the parent compounds ( $87\pm 2\%$  for PMA,  $14\pm 15\%$  for TMP and  $34\pm 7\%$  for SPY) at 14 days of treatment. In general, a more limited number of TPs were detected for these compounds since low mass intermediates might be overlooked by peak interferences at low  $m/z$  values. Different intermediates were further generated through phototransformation processes (observed in light-abiotic experiments) such as TP259 (PMA), TP277 (PMA), TP234 (PMA) and TP185 (SPY), though at low concentration levels.

Slightly higher removals ( $73\pm 12\%$  for PMA,  $23\pm 10\%$  for TMP and  $68\pm 20\%$  for SPY) were achieved after 14 days of treatment with microalgae in light-biomass experiments. However, levels of TPs did not increase in the same proportion except in the case of TP185 (SPY). This TP reached up to 35% of initial SPY concentration in the experiments with *C. reinhardtii* after 14 days of treatment, being SPY removed almost completely at the end of the treatment. Biotransformation would be the main mechanism involved in its generation, although it was already present in abiotic experiments and also reported along UV/H<sub>2</sub>O<sub>2</sub> experiments, though at much lower concentration. Therefore, *C. reinhardtii* was the only microalga able to biotransform sulfapyridine although no mineralization was achieved.

#### **3.2.4. Microalgae for pollutant mitigation**

Although monitoring of the antibiotics along the experiment provides information about removal ability of microalgae, a proper evaluation of microalgae performance can only

be done by measuring also the TPs generated. These TPs can retain some of the biological activity and even elicit higher toxicity than the parent compound, and therefore their generation might rather introduce additional threats to the environment. Consequently, TP elimination needs to be guaranteed in order to ensure the optimum treatment efficiency. In this study, macrolides were partially eliminated during both abiotic and microalgae live experiments. However, they were transformed into new entities that are not necessarily degraded at the end of the treatment. It is important to remark that different TPs were identified for each biotic and abiotic transformation mechanisms studied. On the other hand, fluoroquinolones were eliminated extensively by photodegradation processes compared to a partial elimination with microalgae live cultures. However, an important amount of TPs was detected in the light-abiotic experiments, which might equally pose a risk for the environment. The characterization of TPs has been pointed out as essential step to understand the effectivity of antibiotic removal treatment. Studies about their toxicity are of outmost importance to finally round up the study treatment assessment.

Concerning removal efficiency of microalgae, *P. subcapitata* was clearly not useful for antibiotic removal since no biotransformation of antibiotics was observed. On the other hand, *C. reinhardtii* and *C. sorokiniana* were especially capable for macrolide biotransformation but not for total compound mineralization. Among them, *C. reinhardtii* removed most of the recalcitrant fluoroquinolone TPs (TP244, TP232 and TP347) while they were present at higher concentrations in biotic experiments with *C. sorokiniana*. *D. tertiolecta* experiments showed that abiotic factors lead macrolide transformation with the generation of large amounts of TPs. However, mineralization of fluoroquinolones was achieved to certain extent. Thus, *C. reinhardtii* and *D. tertiolecta* were suggested to be the best microalgae to be used for pollutant removal because of their better antibiotic elimination together with the low TPs formation (in quantity and number).

However, none of them were successful to eliminate all type of antibiotics to the same extent.

### **3.3. Antibiotic transformation products in toilet wastewater treated in a microalgae photobioreactor (PBR)**

The in-house library created containing the compounds elucidated in batch experiments (Table S2) was used for comprehensive assessment of the occurrence of antibiotics and their TPs in a microalgae-based photobioreactor treating real toilet wastewater. Fig. 5 shows mean chromatographic areas of the compounds detected for the three samples taken in three non-consecutive days at inlet wastewater and in PBR effluent. Among target antibiotics selected erythromycin, ofloxacin and norfloxacin were detected at inlet WW and removed 85%, 67% and 95% respectively [36]. Concerning their transformation products, the initial presence of the metabolite TP733 (ERY) at inlet WW was attributed to human metabolization of ERY [58,59]. TP733 was partially eliminated in the photobioreactor, although it can also be generated during the treatment. In fact, PBR was dominated by microalgae from the genus *Chlorella* [37] and TP733 was the major ERY metabolite associated to *Chlorella sorokiniana* in the previous batch experiments (Fig. 2). TP717 (ERY), another erythromycin transformation product, increased its concentration after PRB treatment (aligned with ERY elimination) indicating the apparent transformation of ERY into this compound. This TP was previously described to be generated due to the instability of erythromycin in aqueous solution (Fig. 2 and Section 3.2.1). Other compounds such as TP590 (AZI) and TP763 (CTM) were detected in inlet WW despite the corresponding parent compounds were not present. While the first one was detected from abiotic factors, the latest was generated to come from biotransformation mechanism, also associated to *C. sorokiniana*. Our findings highlight the great importance of monitoring TPs since they can be present even when the parent compound is not or they can be present at higher concentrations than the corresponding parent compound, as it is the case of ERY TPs.

In the case of fluoroquinolones, the major intermediates identified were TP347 (OFC) and TP262 (NFC) and eliminated in PBR treatment up to 44% and 99% respectively. In accordance with microalgae batch experiments, these two TPs were reported as some of the most intense intermediates from ofloxacin and norfloxacin generated by phototransformation processes (Fig. 3). Additionally, they were also minimized in presence of *C. sorokiniana*. Finally, most of them were transformed to TP189, a fourth-generation TP of fluoroquinolones, suggesting a greater extent of antibiotic total elimination.

The PBR experiment showed an overall decrease of all intermediates (though not total removal) in theoretical steady state (HRT 12 days) except for TP717, which increases in concentration. Although total compound removal was not achieved, microalgae based PBR wastewater treatment was successfully applied to reduce the concentration of antibiotics and their TPs. The high concentrations of TPs in inlet wastewater demonstrates the great importance of monitoring these compounds. Thus, the development of advanced analytical methodologies based on suspect screening becomes of high interest to properly evaluate water treatment technologies and consider all potentially relevant chemicals present in water.

## Conclusions

In this study, a novel automated suspect screening methodology using an on-line TFC-LC-LTQ-Orbitrap-MS/MS was developed for the tentative identification of the major TPs of 9 selected antibiotics during microalgae treatment. The positive results indicated that the automated screening tools are a promising approach able to provide reliable information in a fast and efficient manner. By means of the tool developed, the identification of TPs was performed and the corresponding degradation pathways were built taking into account biotic and abiotic factors involved on experimental design. This permitted the evaluation of several microalgae as regards to their efficiency for

pollutant removal, allowed to distinguish between removal mechanisms involved, and also to confirm or deny pollutant mineralization.

The set of TPs identified in the batch microalgae experiments was further searched (suspect screening) in the water samples generated during the treatment of toilet wastewater in an algae photobioreactor. Many TPs were present in both raw and treated waters even when the parent compound is not detected, which highlights the relevance of monitoring both parent compounds and their TPs. Further studies are foreseen to investigate how these TPs might introduce deleterious effects in aquatic systems and how this could impact human health.

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**FIGURE CAPTIONS**

**Fig. 1.** Analytical workflow.

**Fig. 2.** (a) Tentative degradation pathways proposed for macrolides: green arrows indicate those TPs formed mainly by biotransformation; red arrows indicate those TPs formed mainly by unknown factors. Below (b) relative percentages of TPs at 7 and 15 days for the 4 algae studied in dark abiotic, light abiotic and light biomass experiments. The TPs represented were those with values >3%.

**Fig. 3.** (a) Tentative degradation pathways proposed for fluoroquinolones: yellow arrows indicate those TPs formed mainly by phototransformation. Below (b) relative percentages of TPs at 7 and 15 days for the 4 algae studied in dark abiotic, light abiotic and light biomass experiments. The TPs represented were those with values >3%.

**Fig. 4.** (a) Tentative degradation pathways proposed for other antibiotics: green arrows indicate those TPs formed mainly by biotransformation; red arrows indicate those TPs formed mainly by unknown factors. Below (b) relative percentages of TPs at 7 and 15 days for the 4 algae studied in dark abiotic, light abiotic and light biomass experiments. The TPs represented were those with values >3%.

**Fig. 5.** Area of the transformation products detected in wastewater at the inlet and outlet of photobioreactor (PBR) at 12 days HRT.



Figure 1

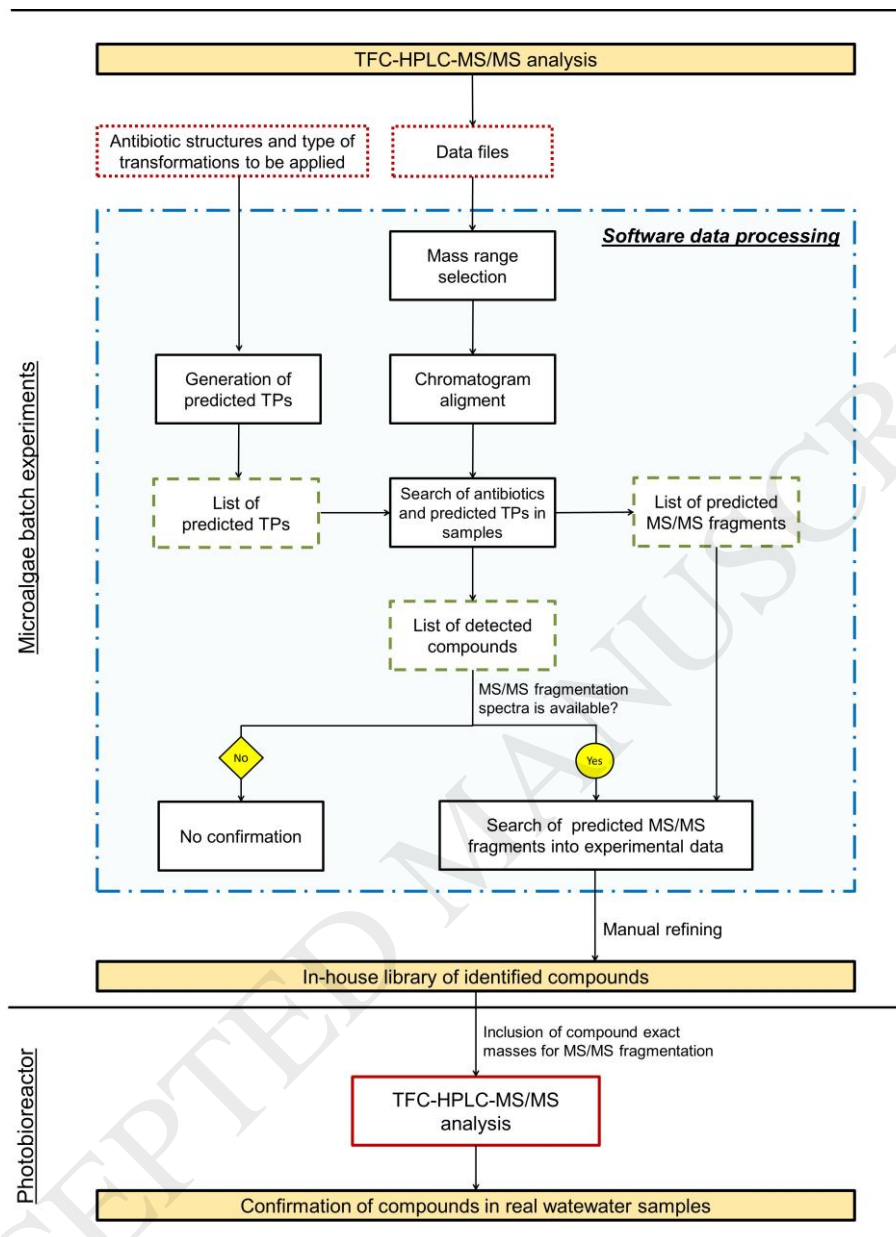


Figure 2

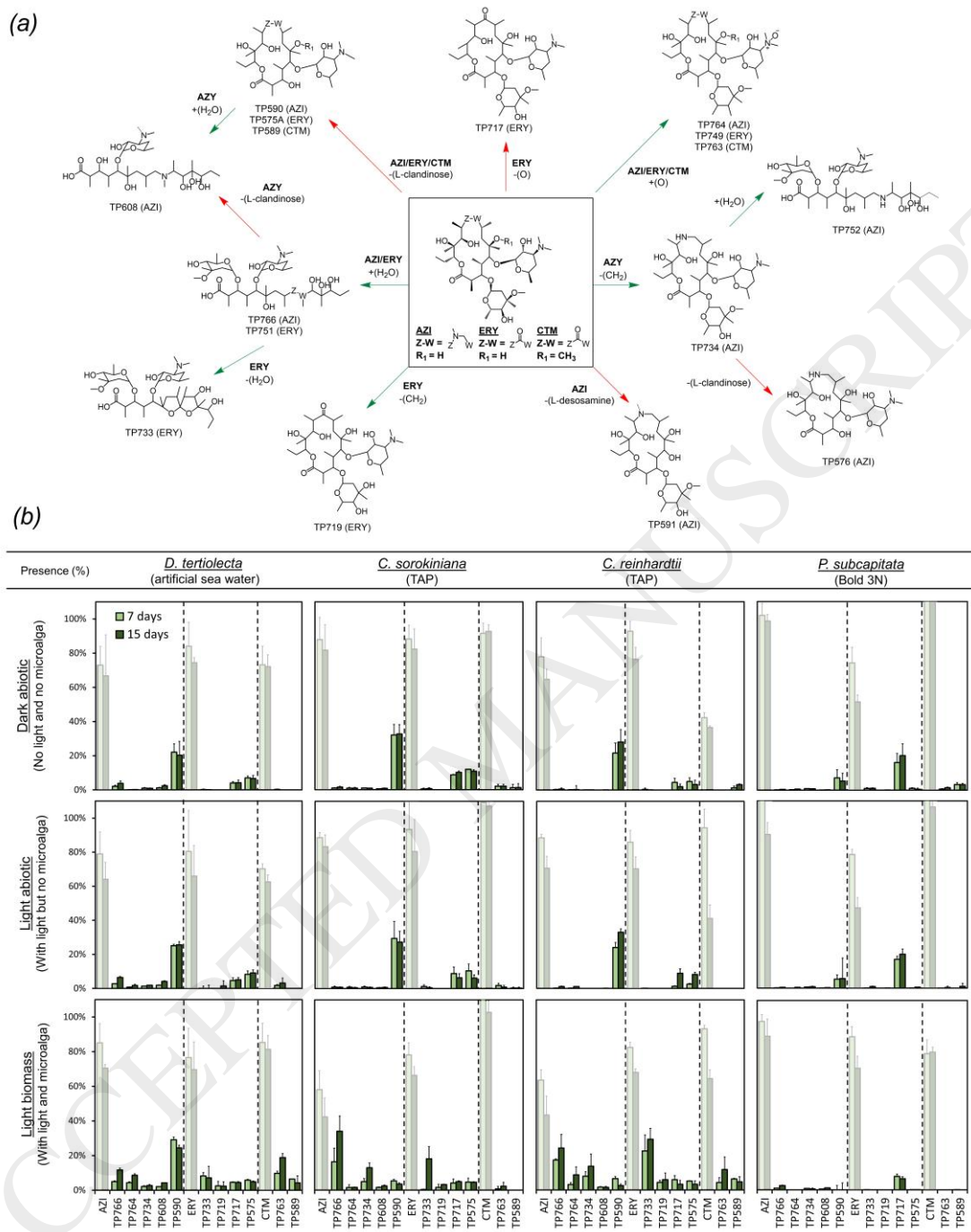


Figure 3

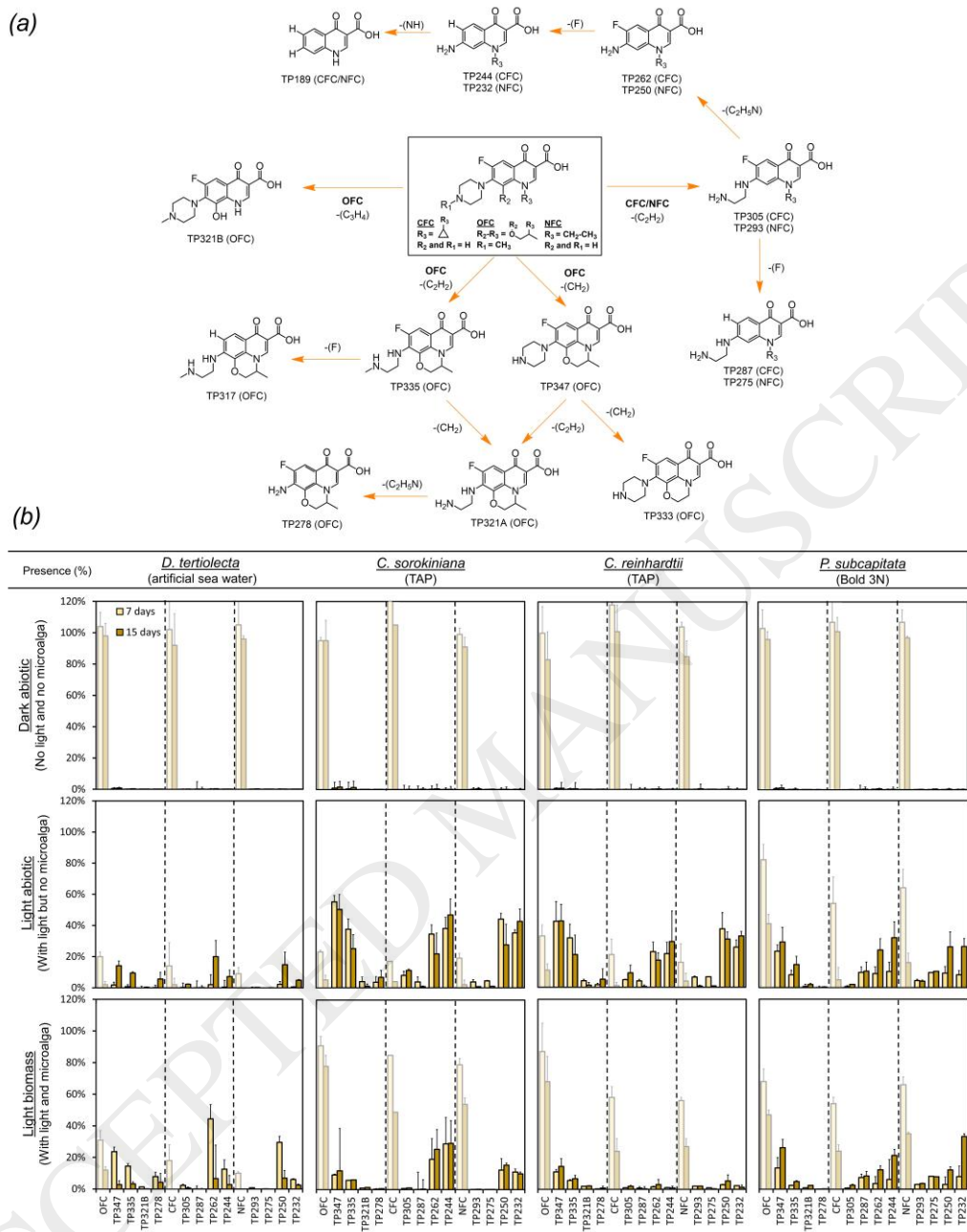
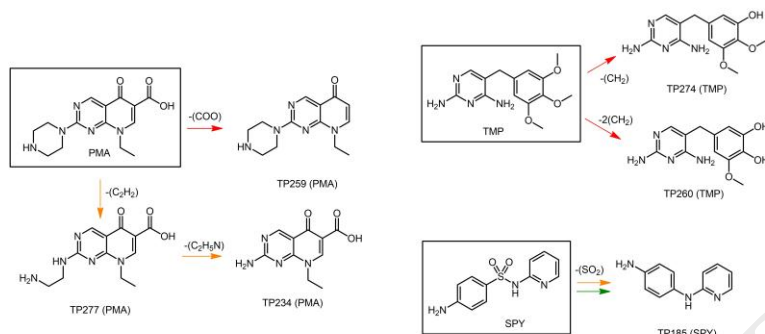


Figure 4

(a)



(b)

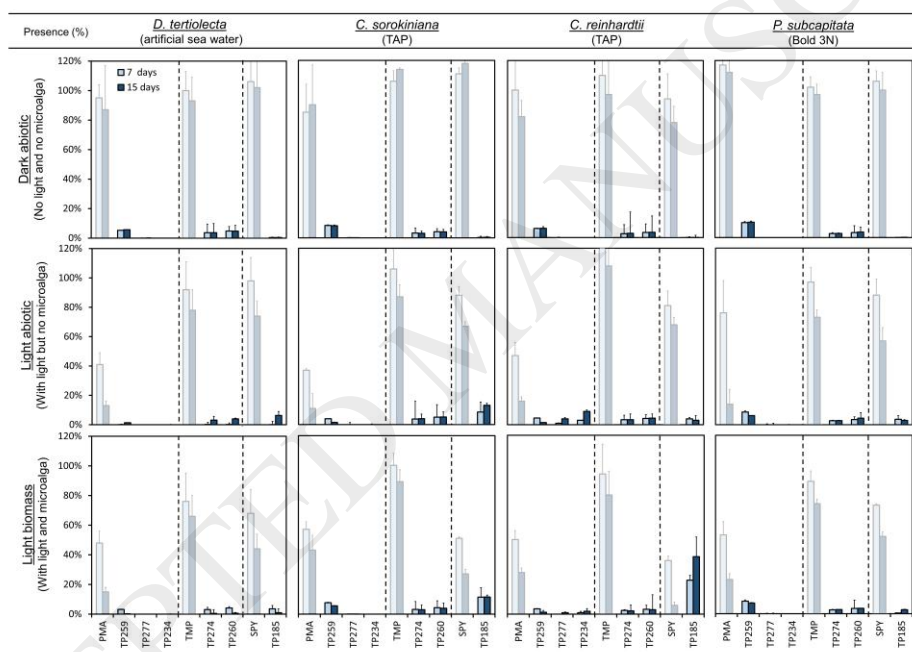


Figure 5

