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Degradation of the tricyclic antipsychotic drug chlorpromazine under environmental conditions, identification of its main aquatic biotic and abiotic transformation products by LC–MS^n and their effects on environmental bacteria

Christoph Trautwein[a,b,c,1], Klaus Kümmerer[c,*]

[a] Department of Environmental Health Sciences, University Medical Centre Freiburg, Breisacher Str. 115B, D-79106 Freiburg, Germany
[b] Institute for Biology II, Microbiology, University Freiburg, Schänzlestrasse 1, D-79104 Freiburg, Germany
[c] Institute for Environmental Chemistry, Leuphana University Lüneburg, Scharnhorststrasse 1, D-21335 Lüneburg, Germany

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ABSTRACT

The search for environmental transformation products of organic pollutants (like drugs) is a difficult task and usually only few compounds are detected. This might be due to effective degradation but could also be a result of analytical deficits dealing with complex matrices. Especially transformation products of very low concentrations in sludge were difficult to identify so far. Additionally, the use of standard separation techniques might lead to the loss of isomeric compounds, which possess identical spectroscopic and spectrometric properties. To date no complete study investigating the environmental fate of any tricyclic antipsychotic drug has been reported. Therefore, this study investigated the popular neuroleptic drug chlorpromazine and its potential transformation by all main environmental pathways: aerobic and anaerobic biodegradation as well as abiotic photolytic degradation by sunlight. Analysis of test samples by high performance liquid chromatography coupled to multiple stage mass-spectrometry (HPLC–MS^n) allowed the detection of numerous compounds. Further, the use of a special software allowed distinguishing between transformation products of small intensities and background "noise" caused by sludge or matrix. Three aerobic tests of different bacterial density (the *Closed Bottle* test, OECD 301D; the *Manometric Respiratory* test, OECD 301F; the modified *Zahn–Wellens* test, 302B; one anaerobic test (a modified anaerobic degradation test according to ISO 11734) as well as a photodegradation test were performed in the present study. According to the individual test guidelines, chlorpromazine had to be classified as not biodegradable in all of the biodegradation tests. However, a special chromatographic column and gradient along with mass spectrometric fragmentation experiments of higher order uncovered the presence of a total of 61 abiotic and biotic transformation products which where formed during the course of the tests. The structures of three aerobic and one anaerobic biotransformation products were elucidated by HPLC-UV-Flourescence–MS^n. Photodegradation showed almost complete elimination of chlorpromazine after 4 h of irradiation with a xenon arc lamp. 57 photoproducts were found and for 28 of them LC–MS^n fragmentation experiments ($n = 4$) were performed. The molecular structures of the three main photolysis products were elucidated. The identified transformation products are expected to be found in the aquatic environment, yet nothing is known about their ecotoxicological properties. As some of the performed tests showed toxic effects of chlorpromazine or its transformation products on bacteria, further risk assessment upon this drug and its fate is strongly recommended.

1. Introduction

The risk and fate of pharmaceuticals in the environment is a topic that nowadays is well-established considering its awareness in science and society. However, there still does not exist enough knowledge and no consensus on how to overcome this challenge. Reduction of drug disposal and the design of biodegradable drugs [1] are under investigation. The same is true for new technical processes in wastewater treatment and freshwater preparation [2]. Many substances are detected in surface water bodies, groundwater, and soil [3]. As a result, questions arise about their impact upon flora and fauna, including mankind [4]. Still, for many of those compounds, little is known about their fate during sewage treatment. This includes the formation of potential transformation products in the environment caused by bacterial biotransformation or abiotic transformation, like

* Corresponding author. Tel.: +49 4131 677 2893; fax: +49 4131 677 2894.
E-mail addresses: christoph.trautwein@uniklinik-freiburg.de (C. Trautwein), klaus.kuemmerer@uni.leuphana.de (K. Kümmerer).
[1] Tel.: +49 761 270 82470; fax: +49 761 270 82130.

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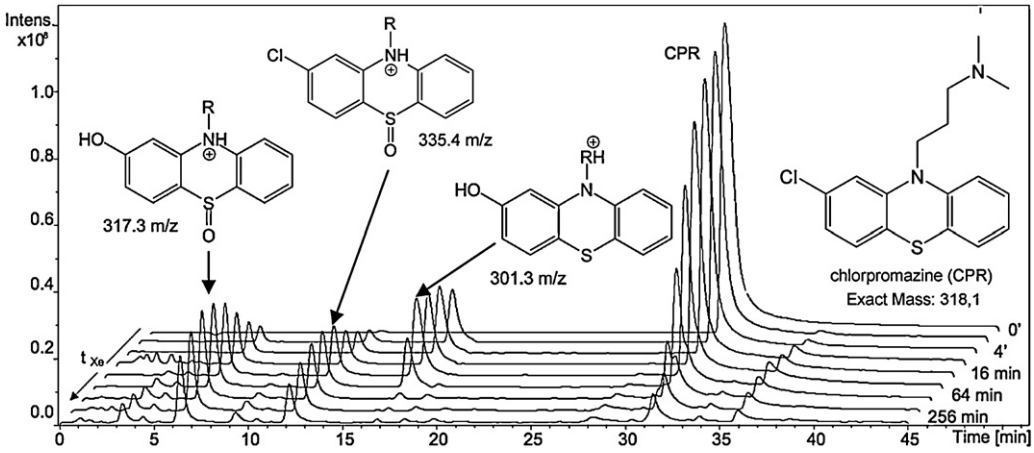

Fig. 1. Total ion currents (TICs) of samples taken during the course of the photodegradation test and structures of chlorpromazine and its three main photolysis products; t_{Xe} = irradiation time with xenon lamp.

irradiation and advanced effluent treatment. New treatment processes, such as ozonation or ultraviolet treatment, can be deficient and incomplete, creating new organic pollutants. These transformation products can be toxic to aquatic organisms and because of their physical–chemical properties they can persist in the aquatic water cycle [5].

Pharmaceuticals were first detected in the aquatic environment in the late 1970s [6]. Since then, a variety of PPCPs (pharmaceuticals and personal care products) have been found in several environmental compartments [7]. The increasing sensitivity of modern instrumental chemical analysis led to the detection of more and more of these compounds [8] and still does. Yet little is known about their environmental fate [9]. However, the identification of transformation products of PPCPs and other chemicals, which are formed in the environment or within technical treatment by biotic or abiotic degradation, is a relatively new field of research. It was shown that environmental bacteria cannot only form the same degradation products as the human metabolism [10], but also generate entirely new dead-end transformation products, which so far have not been reported before [11]. Natural sunlight is another environmental factor that can transform certain pharmaceuticals to unknown degradation products [12,13]. Therefore, risk assessment of these compounds is a big task, since it is known that these compounds can be even more toxic or persistent than their parent drugs [14].

Chlorpromazine (CPR) (Fig. 1), a phenothiazine compound with a pk_a of 9.3 and log p_{ow} of 5.35 [15], was the first modern antipsychotic drug and released in 1952. Besides its main indication for the treatment of schizophrenia and mental disorders, several other possible applications for the future have been found over the last years. CPR showed strong neuroprotection at the cellular level and might be a new drug against Creutzfeld–Jacob's and Parkinson's disease [16,17]. Further, it was shown that CPR has potential as supporting agent in cancer treatment [18,19]. Especially its antibiotic properties make it a promising candidate for treatment of multi-resistant microbes [20,21]. CPR is also discussed as reserve-drug against malaria and tuberculosis in developing countries [22]. Whereas some of its antibiotic properties are conducted directly by CPR itself through strong inhibition of P-glycoprotein-mediated transport [23], other studies show that activation of CPR by light exposure has special bactericide effects [20]. It was also this mechanism that made CPR famous for side effects such as photoallergy and phototoxicity during its early years since introduction. Patients treated with CPR who stayed in the dark did not show any symptoms, while the same patients under day light exposure showed strong skin reactions. CPR was shown to accumulate in the eye lens and cornea, where formation of photoproducts can lead to severe tissue damage and eventually blindness [24].

In the human body, CPR is extensively metabolized to phase I and phase II metabolites, some of them being responsible for the pharmacological activity. So far, 77 human metabolites have been separated by thin-layer chromatography [25], whereas up to 168 human metabolites are theoretically possible [26]. However, it was shown that mutations in the human cytochrome P450 2D6 lead to the excretion of unmetabolized CPR [27]. The main metabolic pathways are oxygenation reactions at the carbon, sulfur, and nitrogen atoms and oxidative side chain degradation following glucuronic acid and sulfate conjugation [28,29]. No degradation of the heterocyclic phenothiazine core was found so far [30]. 7-Hydroxy-CPR, 3-hydroxy-CPR, CPR-N-oxide, and desmethyl-CPR may contribute to the pharmacological effects of CPR [31]. About half of the metabolites are found in urine, whereas the remainders are found in feces. In urine, unmetabolized CPR is excreted at less than 1% and conjugated or unconjugated metabolites range from 20 to 70%. 5–6% of a dosage is excreted via biliary elimination in the feces [32]. For long-term therapy, some metabolites could still be detected 18 months after the last administration. As it has been shown that, in general, conjugates can be environmentally transformed back to their phase I metabolites [9], this might also be the case for CPR conjugates.

So far, no data about bacterial aerobic biodegradation has been reported in literature, but some information is available about fungal metabolism. The filamentous fungus *Cunninghamella elegans* produced five major metabolites of CPR, which had all been found in mammal metabolism before [33]. However, nothing is known about anaerobic biodegradation of CPR yet.

Considering abiotic degradation of CPR by natural sunlight, several studies investigated the photosensitive properties of this drug: under aerobic conditions, twelve photoproducts have so far been separated chromatographically and it was shown that formation of those products went along with brown and later violet colorations [34]. Strong differences in photochemistry have been found between presence and absence of oxygen in the test solutions, with formation of polymers under anaerobic conditions [35]. Irradiation with short UVB (270 nm) leads to photoionization, whereas irradiation with long UVA (320–400 nm) produces free radical intermediates [36]. Yet most of the photoproducts of CPR have not been characterized. It was shown that CPR can photochemically modify DNA, RNA, cell membranes, and soluble proteins [37]. Free radicals were shown to add easily to guanosinemonophosphate [38] and photomutagenesis was tested positive in the Ames test at a CPR concentration of 10 μg/mL [39]. Photoallergy is thought to be caused by the covalent modification of

proteins or other molecules to produce an antigen [40]. Photooxidation and formation of free radicals were shown to be responsible for phototoxic effects and photosensitization [41]. By contrast, it was shown that photo-dimers and -polymers of CPR cause haemolysis of human blood cells without the presence of light [42]. However, for most phototoxic reactions, it remains unclear whether they are catalyzed by sensitized CPR itself, or by a photoproduct [40]. It was also demonstrated that UVB-induced histamine release of mast cells was increased by CPR at concentrations higher than 1 μM [43], explaining allergic skin reactions.

Toxicity against environmental aquatic organisms has also been reported: After 148 h, CPR showed an EC_{50} of 0.92 mg/L for the duckweed plant *Lemna minor* [44]. In another study, CPR as well as its photoproducts was very toxic to the protozoan *Spirostomum ambiguum* with LC_{50} values around 0.5 mg/L after 24 h [45].

After almost 60 years of usage and more than 15 years of research on fate and effects of pharmaceuticals present in the environment, only rare data on the environmental fate and effects of CPR is available. To overcome this unsatisfying situation, this study investigated the biodegradation in batch tests of the OECD and ISO series and used a photodegradation test system to investigate the formation of abiotic transformation products. LC–MS^n allowed for the identification of some of the transformation products formed in these tests.

2. Materials and methods

2.1. Abiotic photolysis

The abiotic photodegradation test (PHOTO test) under natural light conditions was assessed using a UV/VIS xenon lamp (TXE 150, UV-Consulting Peschl, Mainz) that emits spectra similar to natural sunlight (300–800 nm), therefore simulating the photochemical reaction of CPR in surface waters. A stock solution of 50 mg/L of CPR was prepared with deionized water in a 1 L vessel and stored in the dark until use. Temperature conditions were set to 20 °C and monitored during the test. 750 mL of the test solution were transferred into the reactor and the test started by switching on the lamp. In 2^n min (n = 0–8) time intervals, samples were taken for DOC, pH and LC–MS analysis until 256 min. DOC and pH were measured directly after samples were taken whereas samples for LC–MS analysis were stored at −20 °C until LC–MS^n analysis was performed.

2.2. Biodegradation

2.2.1. Closed Bottle test (OECD 301 D)

The *Closed Bottle* test (CBT) is recommended as a first, simple test for the assessment of the biodegradability of organic compounds in the environment. Substances that pass the test are classified as *readily biodegradable*. They are also assumed to be readily biodegradable in a sewage treatment plant (STP) and therefore are therefore expected not to reach or accumulate in the aquatic environment [46]. The CBT was performed according to the OECD test guideline [47] with different test bottles (Table 1) of low nutrient content, low bacterial density (10^1–10^4 colony forming units CFU/mL) and at room temperature (20 ± 1 °C) in the dark as described elsewhere in detail [11]. The concentration of CPR in the test bottles was adjusted to 2.6 mg/L, corresponding to a $ThOD_{NH_3}$ (theoretical oxygen demand without considering a possible nitrification) of 5 mg/L. All test vessels were inoculated with an aliquot of the effluent from the municipal STP in Kenzingen (Germany; 13,000 population equivalents). Two drops of inoculum (about 60 μL) were added to 1 L of medium, which resulted in approximately 500 CFU/mL.

The progress of aerobic biodegradation was monitored by measuring the oxygen concentration in the test vessels with an optode oxygen sensor system (Fibox 3 PreSens, Regensburg, Germany), which is based on the physical principle of dynamic luminescence [48,49]. Measurements of oxygen and temperature were performed daily during the test period in order to get kinetic information about the degradation process. Samples at the beginning and end of the test were taken and stored at −20 °C until subsequent LC–MS^n analysis. According to the test guidelines, a test compound is classified as "readily biodegradable" if biodegradability, expressed as a percentage of oxygen consumed in the test vessel (ThOD), exceeds 60 within a period of ten days after the oxygen consumption reached 10% ThOD.

2.2.2. Manometric Respiratory test (OECD 301 F)

The *Manometric Respiratory* test (MRT) was performed in accordance with the test guidelines [50] in the dark at room temperature (20 ± 1 °C) under gentle stirring as described elsewhere in detail [11]. Different test bottles were used for assessing biodegradation, toxicity, and abiotic degradation (Table 1). The MRT is performed with higher inoculum density than the CBT. The OxiTop® OC110-system (WTW GmbH, Weilheim, Germany) was used as measuring system.

The test bottles contained 15.5 mg/L CPR, corresponding to a $ThOD_{NH_3}$ of 30 mg/L. All test bottles were inoculated with an aliquot from the effluent of the STP Kenzingen (population equivalent 13,000). To 1 L of medium, 80 mL of inoculum were added, resulting in a high bacterial diversity of approximately 10^4–10^5 CFU/mL in each test bottle. The validity criterion is a removal of 60% ThOD within a 10 days window for the quality control. Aerobic biodegradation was monitored by measuring the microbiological oxygen consumption by CO_2 production. Samples at test begin and end were taken and stored at −20 °C until subsequent LC–MS^n analysis.

2.2.3. Zahn–Wellens test (OECD 302 B)

The *Zahn–Wellens* test (ZWT) can assess whether an organic compound or mixture of compounds is to any extent aerobically biodegradable (*inherent biodegradability*) under optimized conditions (like in a STP). The nutrient content and bacterial density and diversity are very high because activated sludge is used as inoculum. The ZWT was performed according to the test guidelines as described elsewhere [11,51]. Different test vessels were used (Table 1).

The sludge used in the ZWT was obtained from the municipal STP of Forchheim (AZV Breisgauer Bucht, Germany; 600,000 population equivalents). Validity criteria are 70% DOC removal in the "quality control" vessel within 14 days and biodegradation in the "test" vessels has to take place gradually over days or weeks. CPR concentration in the test vessels was adjusted to 87.1 mg/L, corresponding to a DOC of 50 mg/L. The dry matter content of sludge in all of the test vessels was 1 g/L, and pH was adjusted to between 6.5 and 7.5 with 1 molar NaOH or H_2SO_4 solutions using a pH meter (Beckmann Coulter 10 pH meter, Fullerton, CA, USA). The test was performed under gentle stirring and aeration at 21–25 °C at diffuse room light.

Elimination by sorption was accounted for DOC values measured after 3 h. Each of the daily samples was analyzed in a triplicate. Parts of the centrifuged and filtrated samples were stored deep-frozen (−20 °C) for subsequent LC–MS^n analysis.

2.2.4. Anaerobic degradation test (ISO 11734: 1995)

Anaerobic degradation (ANAD) was assessed according to ISO 11734, which was modified in some technical terms in order to continuously measure the biogas production in the test bottles [52]. This test allows the evaluation of the ultimate anaerobic biodegradability of organic compounds in digested sludge. Normal dwell time

Table 1
Composition of biodegradation test series in *the Closed Bottle* test (1–4), *Manometric Respiratory* test (1–5), *Zahn–Wellens* test (1–3, 6) and anaerobic degradation test (1–4).

Test series	1 Blank	2 Quality control	3 Test compound	4 Toxicity control	5 Sterile control	6 Negative control
Mineral medium	+	+	+	+	+	+
Inoculum	+	+	+	+		
Chlorpromazine (CPR)			+	+	+	+
Reference substance		+		+		
Sodium azide					+	
Copper sulfate						+

of anaerobic sludge in the digestion towers varies between 25 and 30 days. In this test, an extended incubation time of 56 days without stirring was utilized, considering the fact that the test system does not work with pure but diluted sludge and relatively high test compound concentrations are used. Different test bottles were used (Table 1).

For preparation of the anaerobic test media and washing of the sludge, three vessels (10 L each) of deionized water were prepared and then degassed with pure nitrogen for several hours prior to use in order to expel dissolved oxygen. Test media containing resazurine as oxygen indicator was created according to the test guideline [52]. All further steps dealing with the anaerobic sludge were performed under an anaerobic N_2/H_2 atmosphere which was guaranteed using a COY® type B anaerobic chamber system (COY Laboratory Products, Grass Lake, MI, USA).

Final test volume of each bottle was 800 mL. Three blank bottles were prepared with 720 mL of medium and 80 mL inoculum. Reference substances according to the guideline, namely sodium benzoate, phenol, and polyethylenglycole 400 (PEG 400) were used. Reference controls were run in parallel with a concentration of 50 mg/L DOC of each compound, using 710 mL of medium, 80 mL of inoculum, and 10 mL of reference compound. CPR was run in triplicate with 710 mL of medium, 80 of inoculum, and 10 of standard solution, resulting in a final concentration of 96/L in each bottle (corresponding to 50/L DOC). The toxicity control bottle of CPR contained only 700 medium, but besides 10 of CPR standard solution an additional 10 of PEG 400 solution.

Fresh anaerobic sludge (dry matter content ≈30 g/L) was taken from the municipal STP of Forchheim (AZV Breisgauer Bucht, Germany; 600,000 population equivalents) and transported in firmly closed 2 PET bottles (in total 8) inside an insulating polystyrene box in order to maintain it close to 39 °C as the digestion towers of the STP are run with temperatures of approximately 39 °C. The same temperature was set for the incubator. Prior to use, the sludge was washed two or three times with anaerobic media in order to reduce the background of inorganic carbon. Final inorganic carbon was 7.1/L (should be <10 mg/L according to the test guideline). DOC was measured with a TOC-Analyser (TOC 5000/ASI 5000 version 3.10, Shimadzu, Duisburg).

As the dry matter content in each test bottle should range between 1 and 3/L, the sludge was diluted with anoxic media prior to use. Final dry matter content in each test bottle was approximately 1/L. After sludge addition, pH was measured and if necessary adjusted to a value of 7.2, using low concentrated NaOH and HCl solutions.

In contrast to stepwise pressure measurements described in the ISO norm via an injection needle which is connected to a barometer, the OxiTop® OC110-system with measuring heads was used for pressure measurements. Pressure values were automatically recorded every 4 on the chip in the measuring heads and afterwards transferred to a computer for further processing.

After 56 days of incubation, 4 of 5 molar HCl were injected into the bottles in order to expel dissolved CO_2 into the gas phase. The following injection of 3 of 6 molar KOH into one of the quivera leads to the absorption of all CO_2 of the gas phase. The remaining pressure corresponded to the methane gas produced during the course of the test. The amount of produced CO_2 and CH_4 as well as the biodegradation degrees were calculated according to the test guideline. The test was valid if the reference substance reached a degree of >60% degradation in the plateau phase and if the resazurine indicator in the test bottles did not turn pink. Samples at test begin and test end were stored at −20 °C for subsequent LC–MS^n analysis.

2.3. Primary elimination and identification of transformation products by HPLC–MS^n

An HPLC system (HPLC 1100 series, Agilent Technologies, Waldbronn, Germany) consisting of a G1312A binary pump, a G1329A thermostatic autosampler, a G1316A column oven (set to 40 °C), and a G1322A degasser was used for chromatographic analysis. Separation was performed on a monolithic silica gel column with a characteristic bimodal pore structure (*Chromolith Performance* RP-18 endcapped 100–3, 2 μm, Merck Chemicals, Darmstadt) coupled to a precolumn (*Chromolith Guard Cartridge* RP-18e 5–3, 2 μm, Merck Chemicals, Darmstadt). For elution, 10 ammonium acetate in deionized water (solution A) and 100 methanol LC–MS grade (solution B) were used by applying the following linear gradient: 0 min 20% B, 25 min 40% B, 30 min 52% B, 43 min 65% B, 44 min 95% B, 45 min 95% B, 45.01 min 20% B. The sample injection volume was 50 μL and the flow rate was 1.0 mL/min. Post time was set to 2 min; resulting in a total run time of 47.01 min. CPR standards (1, 2.5, 5, 10, 25 and 50 mg/L) were used to establish a calibration curve. Regression coefficients were $r^2 = 1000$ in the CBT/MRT, $r^2 = 0.993$ in the ZWT, $r^2 = 0.979$ in the ANAD test, and $r^2 = 0.980$ in the PHOTO test. Each sample was measured twice and every eight samples, a water blank, followed by a CPR standard and another water blank were introduced for quality control reasons.

For UV detection, a UV/VIS detector (Agilent G1314A) was used and absorbance maxima at 210, 250, 260, 270 and 300 nm were measured. Fluorescence detection was assessed with an Agilent G1321A fluorescence detector (excitation 270 nm, emission 450 nm).

Mass spectra were obtained using a Bruker Daltonic Esquire 6000 plus ion trap mass spectrometer equipped with a Bruker data analysis system (Bruker Daltonik GmbH, Bremen, Germany). The mass spectrometer was connected to the Agilent 1100 Series HPLC system. For ionization, an atmospheric pressure interface (API) with electrospray ionization (ESI) was used with generation of positive (+) charged molecular ions. Before analysing the samples, the mass spectrometer was tuned for CPR, optimizing the ionization source parameters, lens voltages, and trap conditions in the *SmartTune* mode of the Esquire software while infusing a standard solution of CPR (10 mg/L) via a syringe pump (Coler-Parmer 74900 series) at a flow rate of 225 μL/h.

The operating conditions of the ion generation were: −500 V end plate and −3100 V capillary voltage relative to the needle, 50.00 psi (343 kPa) nebulizer pressure, 12.0 L/min nitrogen dry gas flow at a dry temperature of 365 °C, and capillary exit voltage 114.9 V.

Operating conditions of the ion transport and focusing were: 40 V skimmer (cone voltage), Octopole RF (radio frequency)

Amplitude 150.0 Vpp and −60 V at lens two. The ion trap parameters were set with a target mass of 319 m/z and maximum accumulation time of 200 ms at a scan range from m/z 20 to 1000. For sample measurements without fragmentation experiments, averages were set to six spectra with an accumulation time of 15 ms. For further investigation of transformation products, the samples taken at the end of each degradation test were analyzed by the Auto MS^n mode, where transformation products with highest peak intensity were isolated and fragmented up to MS^4 in order to gain more structural information. Here, averages were set to one spectrum with an accumulation time of 75 ms and a precursor selection for further fragmentation of the three most abundant ions was chosen. Absolute threshold for MS^2 was set to 5000 ion counts, for n > 2–20. Unfortunately, high frequent isolation and fragmentation procedures of the Esquire 6000 plus apparatus bring along some mass shifts during measurements. Therefore, the m/z values of some isolated ions differ from the targeted precursor up to ±0.5 m/z. The same is to be directed toward product ions. However, as these inaccuracies are the same for all target compounds, these data could still be used for structural elucidation.

Total ion currents (TICs) of each time point were first subtracted by Bruker's *Metabolite Detect* (MD) software (*metabolitetools 2.0 SR1*, Bruker Daltonik GmbH, Bremen, Germany) with the blank of each test series and measurement point. Afterwards, the LC–MS data was subtracted with the ion background at the beginning of the chromatographic runtime. This procedure was done to assure that all remaining peaks only belonged to CPR or its transformation products and no other compounds derived from the inoculum or even system contamination. Peak detection was performed using the *Dissect* algorithm in Bruker's *Data Analysis* software (*dataanalsis 4.0 SP2*, Bruker Daltonik GmbH, Bremen, Germany). This algorithm finds compounds on a LC–MS chromatogram even if the peaks overlap completely. It is based on the principle that all ions having a maximum intensity at the same time belong to the same compound. Internal S/N threshold was set lowest (0.01) and maximum number of overlapping compounds to 3. Cut-off intensity for mass spectrum calculation was set to 1%.

For the most intense peaks, fragmentation experiments were performed up to MS^4. Therefore, the precursor ion was fragmented first. The three most abundant product ions were then selected and fragmented in the same scheme again and so on up to MS^4 if peak intensity was high enough. However, in practice, sometimes up to four product ions were generated, which is caused by the fact that during the elution window of a peak, chemical ionization features of the compound change, which results in formation of new fragments with other m/z values. In order to interpret the acquired MS^n data correctly, similar fragmentation studies upon CPR and its metabolites were evaluated and compared [53–55].

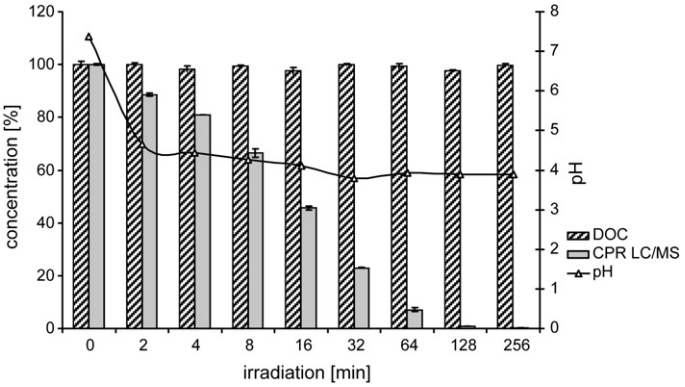

Fig. 2. Photodegradation of chlorpromazine (CPR) and results of DOC (dissolved organic carbon), LC–MS and pH measurements as a function of irradiation time.

2.4. Chemicals

All the chemicals used for the preparation of nutrients, chromatographic solvents and DOC analysis were of at least >98.5% purity. Methanol used for chromatographic elution was of LC–MS grade (*Chromasolv*® 99.9%, Sigma–Aldrich, Fluka Analytical, Darmstadt). CPR and promazine (>98% purity) were purchased from Sigma–Aldrich (Munich, Germany).

3. Results

3.1. Photolytic degradation of chlorpromazine

LC–MS analysis of photolysis samples showed first order kinetics (Fig. SI-1) and a different TIC curve at each time step of the measurement series (Fig. 1). A strong decrease of the peak at the retention time of CPR (319.2 m/z at t_r = 31 min) could be observed and new peaks (mainly at earlier retention times) emerged along the irradiation with the xenon lamp (Fig. 1). However, DOC concentrations remained constant at high level, indicating no mineralization. At the end of the test, a DOC elimination of 0.1% was found, but only residuals of CPR were detected according to LC–MS analysis, accounting for a primary elimination of 99.7% (Table 2) and transformation of CPR but no mineralization during the course of the test (Fig. 2). The pH values turned from neutral (pH = 7.4 at 0 min) to acidic (pH = 3.9 at 256 min) (Fig. 2). This was shown to be due to photolytic dechlorination and 2-hydroxylation of CPR with resulting proton release into the test solution.

The color of the test suspension turned from clear at the beginning of the photolysis experiment to pink and ruby colored at its end. In order to also identify transformation products of small

Table 2
Test concentrations and results of chlorpromazine (CPR) degradation/elimination in the photolysis (PHOTO) test, *Closed Bottle* test (CBT), *Manometric Respiratory* test (MRT), *Zahn–Wellens* test (ZWT) and anaerobic degradation (ANAD) test; DOC, dissolved organic carbon.

Test series	Test compound		Toxicity control		Sterile/negative control	
CPR concentration	Biological degradation/DOC elimination	LC–MS elimination	Biological degradation	LC–MS elimination	Biological degradation/DOC elimination	LC–MS elimination
PHOTO test [50.0 mg/L]	0.1%	99.7%	–	–	–	–
CBT 1 [2.6 mg/L]	1.3%	2.2%	52.6%	4.5%	–	–
CBT 2 [2.6 mg/L]	2.4%	2.5%	49.2%	2.6%	–	–
MRT 1 [15.5 mg/L]	−2.9%	7.4%	33.7%	5.5%	1.0%	0.6%
MRT 2 [15.5 mg/L]	−25.2%	5.9%	–	–	–	–
ZWT 1 [87.1 mg/L]	−1.3%	70.6%	–	–	−42.1%	61.0%
ZWT 2 [87.1 mg/L]	−8.5%	73.5%	–	–	–	–
ANAD test 1 [96.0 mg/L]	−83.7%	6.3%	−81.0%	28.3%	–	–
ANAD test 2 [96.0 mg/L]	−83.3%	12.3%	–	–	–	–
ANAD test 3 [96.0 mg/L]	−80.3%	21.9%	–	–	–	–

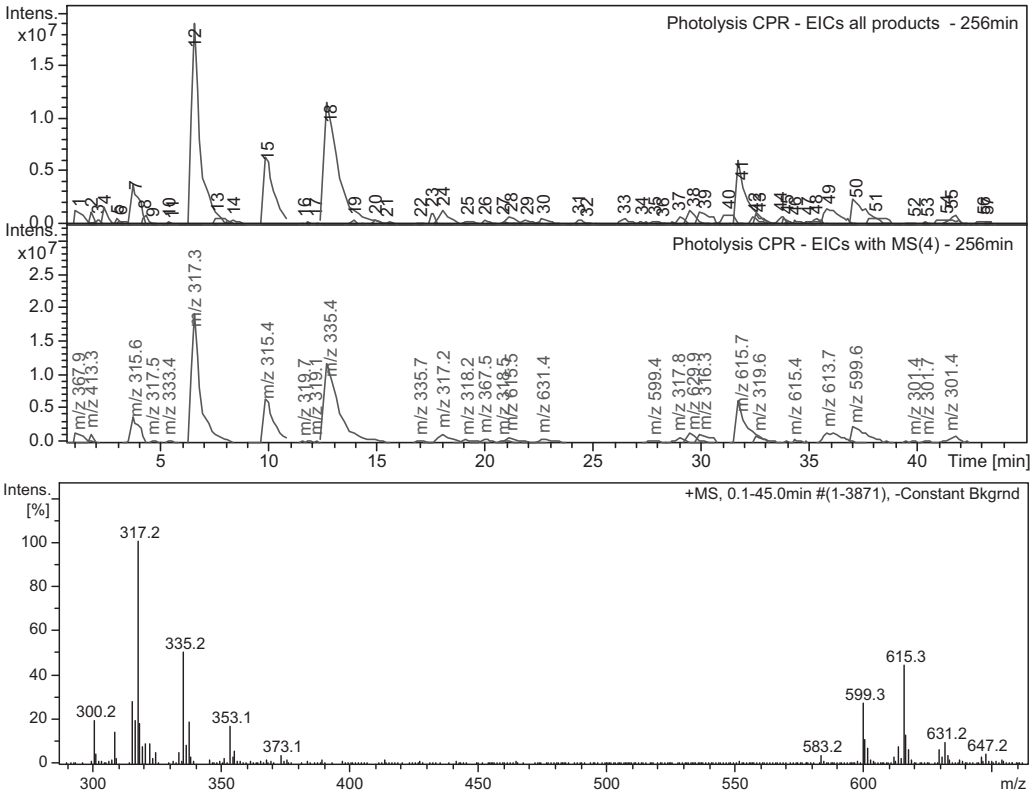

Fig. 3. Peak detection with the *Dissect* algorithm (first line) and extracted ion currents (EICs) with MS^4 experiments in the photolysis test of chlorpromazine (CPR) taken at test end after 256 min irradiation (second line); averaged mass spectrum over the total chromatographic run time of 45 min (third line).

intensities, CPR data files of each time point were subtracted with the MD software by the water blank data file which was run at the beginning of the test (see Section 2.3). Thereby, peaks were eliminated which did not belong to the analyte or its transformation products. The *Dissect* algorithm of the Bruker *Data Analysis* software revealed 58 peaks at test end, of which 57 should belong to the photodynamic degradation of CPR (Fig. 3 – first line). One peak belonged to residual concentrations of CPR. For the 29 most intense peaks (Fig. 3 – second line) with high S/N ratios, MS^{1-4} spectra were acquired, using the Auto MS^n mode of the mass spectrometer software. Depending on the peak intensity and fragmentation characteristics of each transformation product, up to MS^4 spectra were generated (Tables SI-1 and SI-2) in order to gain structural information on the photoproducts. An averaged mass spectrum over the whole chromatographic window showed a familiar pattern of the most intense m/z values: peaks around the precursor of CPR (319.2 m/z) indicate multiple oxygenation during the photolysis experiment as well as formation of dimers at higher m/z values which also have oxygen-related mass differences of 16 m/z between each other (Fig. 3 – third line). Mass spectrometric data of the three main transformation products were used for structural elucidation.

The most intense peak, $m/z = 317.3$, was found at $t_r = 6.6$ min with an S/N > 1000 (Table 3 and Fig. 3). For structural elucidation, this peak was isolated and fragmented (see Section 2.3). The precursor ion at 317.3 m/z gave several product ions (Table 4), which were fragmented again so that a complex fragmentation pattern of mass spectra emerged (Fig. 4). Those data were used to clarify the structure of the photolysis product. A MS^2 fragment with 269 m/z indicates extrusion of a SO moiety (−48 m/z) and therefore oxygenation at the sulfur atom. Furthermore, MS^{2-4} fragments with 139 m/z indicate hydroxylation at one of the benzene rings. Fig. 5 gives a proposal for the structure of $m/z = 317.3$ according to the acquired MS^{1-4} data. The 2nd most intense peak, $m/z = 335.5$ eluted at $t_r = 12.9$ min with an S/N > 500 (Table 3 and Fig. 3). Isolation and fragmentation again gave characteristic product ions up to MS^4 (Table 4), which were used in order to elucidate the molecular structure of this compound (Fig. SI-2). Again, fragments with a loss of −48 m/z to their precursor ion (MS^2 287 m/z and MS^3 242/211 m/z) indicate extrusion of a SO moiety and therefore a sulfur oxidized precursor ion. Fig. SI-3 shows the possible fragmentation pattern and confirmation of this transformation product as chlorpromazine sulfoxide at $m/z = 335.5$ according to the acquired MS^{1-4} data. A 3rd photolysis product at $t_r = 16.8$ min and $m/z = 301.4$ m/z was the most intense peak from 2 min until 32 min of irradiation, but was almost undetectable at 64 min (Fig. 1). This degradation product was confirmed by MS^{1-4} data (Fig. SI-4) as 2-hydroxypromazine, as a result of dechlorination followed by hydroxylation (Fig. SI-5). Molecular structures of the remaining 25 photoproducts were elucidated using the MS^{1-4} data of Tables SI-1 and SI-2.

3.2. Biodegradation of chlorpromazine

3.2.1. Aerobic degradation: Closed Bottle test (OECD 301 D)

Validity criteria of the OECD test guideline (>60% ThOD of the quality control substrate (sodium acetate) have to be degraded) were met [47]: sodium acetate reached a biodegradation of 79 ± 3.1% ThOD after ten days (84 ± 3.5% at test end) (Fig. SI-6) and oxygen concentrations in all bottles did not fall below 0.5 mg/L at any time. Oxygen depletion in the blanks after 28 days was 0.58 mg/L and therefore less than 1.5 mg/L.

The biodegradation values for CPR as determined in the CBT by monitoring the oxygen concentration were 1.3% and 2.4% at test

Table 3
Analytical data of chlorpromazine (CPR; bold in italics) and its photolysis products (RT, retention time; *m/z*, mass to charge ratio; S/N, signal/noise ratio). For gray and bold marked lines mass spectra up to MS^4 were acquired, allowing structural elucidation; see supplementary material.

Photolysis product #	RT [min]	Maximum [*m/z*]	Peak area	S/N	Photolysis product #	RT [min]	Maximum [*m/z*]	Peak area	S/N
1	**1.2**	**367.9**	5.4E+07	**70**	**30**	**22.7**	**631.4**	2.2E+07	**28**
2	**1.8**	**413.3**	1.9E+07	**114**	31	24.4	560.3	1.2E+07	40
3	2.1	320.0	1.2E+07	74	32	24.7	318.3	3.8E+06	13
4	2.4	332.3	3.1E+07	42	33	26.4	324.9	1.9E+07	30
5	3.0	361.5	1.2E+07	56	34	27.2	318.3	9.0E+06	15
6	3.1	475.2	1.2E+07	27	**35**	**27.8**	**599.4**	1.3E+07	**55**
7	**3.8**	**315.6**	1.1E+08	**260**	36	28.2	286.4	8.4E+06	32
8	4.3	373.2	1.7E+07	51	**37**	**29.0**	**317.8**	2.2E+07	**51**
9	**4.7**	**317.5**	3.4E+06	**217**	**38**	**29.6**	**629.9**	4.4E+07	**156**
10	**5.4**	**333.4**	4.1E+06	**31**	**39**	**30.1**	**316.3**	4.8E+07	**73**
11	5.6	331.6	1.3E+06	37	40	31.2	335.9	4.6E+07	85
12	**6.6**	**317.3**	5.8E+08	**1194**	**41**	**31.8**	**615.7**	2.1E+08	**282**
13	7.7	301.7	2.2E+07	33	42	32.5	321.3	2.6E+07	44
14	8.4	349.2	1.4E+07	22	***43***	***32.7***	***319.6***	*2.8E+07*	***87***
15	**10.0**	**315.4**	2.2E+08	**435**	44	33.7	318.0	2.5E+07	32
16	**11.7**	**319.7**	6.2E+06	**10**	45	33.9	297.2	1.9E+07	23
17	**12.2**	**319.1**	2.4E+06	**26**	**46**	**34.4**	**615.4**	1.3E+07	**51**
18	**12.9**	**335.4**	5.5E+08	**614**	47	34.9	583.4	2.0E+07	33
19	14.0	285.7	1.2E+07	29	48	35.3	411.5	1.8E+07	54
20	14.9	286.7	1.1E+07	45	**49**	**35.9**	**613.7**	8.3E+07	**153**
21	15.5	325.5	8.8E+06	14	**50**	**37.2**	**599.6**	1.2E+08	**264**
22	**17.0**	**335.7**	6.7E+06	**17**	51	38.1	319.6	3.9E+07	45
23	17.5	302.1	2.1E+07	92	**52**	**39.8**	**301.4**	4.9E+06	**34**
24	**18.1**	**317.2**	5.0E+07	**64**	**53**	**40.4**	**301.7**	5.3E+06	**45**
25	**19.2**	**318.2**	1.5E+07	**27**	54	41.3	319.5	3.2E+07	35
26	**20.0**	**367.5**	1.4E+07	**22**	**55**	**41.6**	**301.4**	3.0E+07	**241**
27	**20.9**	**318.5**	9.5E+06	**22**	56	43.1	307.4	1.2E+07	22
28	**21.2**	**615.5**	3.1E+07	**33**	57	43.2	502.6	8.0E+06	34
29	22.0	324.9	2.0E+07	32	58	43.6	451.0	8.0E+06	42

end after 28 days (Fig. SI-6 and Table 2). These values characterize CPR as being *not readily biodegradable*. 50.9 ± 1.7% of the toxicity control was degraded at test end, indicating no toxicity of CPR [47].

LC–MS analysis of samples taken at days 0 and 28 demonstrated a negligible removal of CPR (Fig. 6). LC–MS^n data showed emersion of a new peak at 335.7 *m/z* in the chromatogram. Peak data were checked with the photolysis products found in Section 3.1 and matched with *m/z* values and retention time, therefore explaining the negligible elimination of CPR (2.2% and 2.5%) by photodegradation. The only two remaining peaks belonged to CPR and its main photoproduct (Fig. 7 – first line). Therefore,

Table 4
Analytical data (chromatographic and mass-spectrometric) of the seven identified transformation products in the abiotic and biotic degradation tests; TRP, transformation product, RT, retention time, n.d., not determined.

Degradation test/TRP	TRP [*m/z*]	RT [min]	MS/MS [*m/z*] (precursor in bold)	MS^3 [*m/z*] (precursor in bold)	MS^4 [*m/z*] (precursor in bold)
Photolysis TRP # 1	**317.3**	**6.6**	**317.3** > 228.0, 242.3, 255.7, 272.0	**228.0** > 199.9 **242.3** > 213.2, 209.6, 135.9 **255.7** > 227.3, 213.9, 161.9 **272.0** > 238.9, 211.8, 133.0	**209.6** > 181.0 **227.3** > 136.0 **238.9** > 210.9, 194.0
Photolysis TRP # 2	**335.4**	**12.9**	**335.5** > 304.3, 290.2, 255.3	**304.3** > 233.4, 272.9, 211.9 **290.2** > 273.2, 255.3, 242.1 **255.3** > 227.3, 221.6, 199.9	**233.4** > 197.9 **273.2** > 231.9 **255.3** > 239.8, 227.0, 198.8 **227.3** > 198.8 **211.6** > 192.9 **242.1** > 207.7
Photolysis TRP # 3	**301.4**	**16.8**	**301.3** > 256.5, 227.9, 86.2	**227.9** > 226.1, 196.1, 166.9 **256.5** > 228.0, 196.0, 138.9, 215.0	**n.d.**
Zahn–Wellens TRP # 1	**250.3**	**20.8**	**252.0** > 233.6, 233.0, 215.9	**233.5** > 197.9 **215.7** > 181.9, 154.0	**n.d.**
Zahn–Wellens TRP # 2	**333.5**	**29.4**	**334.2** > 274.4, 246.5, 239.6	**274.4** > 245.9, 239.6, 213.9 **246.5** > 213.9, 211.0 **239.6** > 224.0, 206.0	**n.d.**
Zahn–Wellens TRP # 3	**377.5**	**30.7**	**377.6** > 144.1, 116.2, 239.6, 274.5	**116.2** > 74.1 **144.1** > 116.0, 84.2, 58.4 **239.6** > 205.9, 212.6 **274.5** > 239.0, 214.0, 246.0	**n.d.**
Anaerobic degradation TRP # 1	**285.3**	**24.0**	**285.2** > 240.0, 211.9, 86.2	**240.0** > 211.9, 180.0, 117.0 **211.9** > 180.0 **86.2** > 58.7	**n.d.**

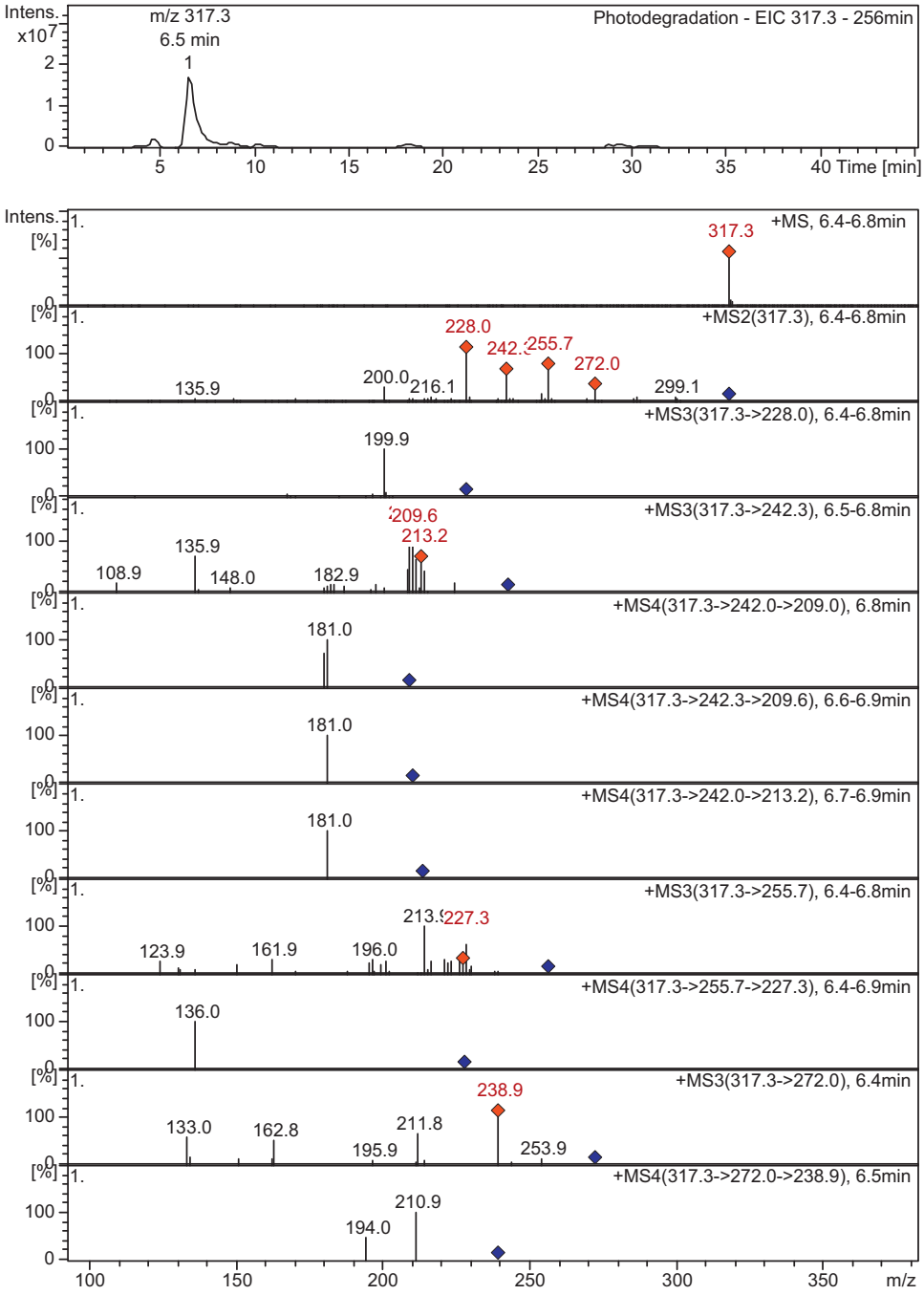

Fig. 4. Extracted ion chromatogram (EIC) and MS^{1-4} fragmentation spectra of the 1st main photolysis product of chlorpromazine 317.3 m/z at t_r = 6.5 min after 256 min irradiation with a xenon lamp.

it can be concluded that CPR was not at all biodegraded in the CBT.

3.2.2. Aerobic degradation: Manometric Respiratory test (OECD 301 F)

The MRT was valid [50] since more than 60% ThOD of the quality control substrate was biodegraded within ten days (Fig. SI-7). Biodegradation results in the two bottles that contained CPR differed significantly. While biodegradation in bottle A was −2.9%, it was found to be −25.2% in bottle B (Table 2). Negative biodegradation values result from subtracting BOD values of each test bottle from the corresponding blank values. If endogenous oxygen consumption in a test bottle is prevented by toxic properties of the test compound, the biodegradation degree can turn negative. Biodegradation in the toxicity control bottle should be about 50%, but turned out to be only 33.7%. According to the guideline (<25% ThOD), this value is still too high to explain toxicity nevertheless supports the finding in bottle B. No degradation (1.0%) was found in the sterile control sample.

LC–MS measurements confirmed the biodegradation values by means of very low CPR primary elimination (5.9% and 7.4%) in all bottles (Table 2 and Fig. 6). Again, evaluation of the mass spectrometric data showed formation of some photolysis products as was already observed in Section 3.2.1. After TIC subtraction with the

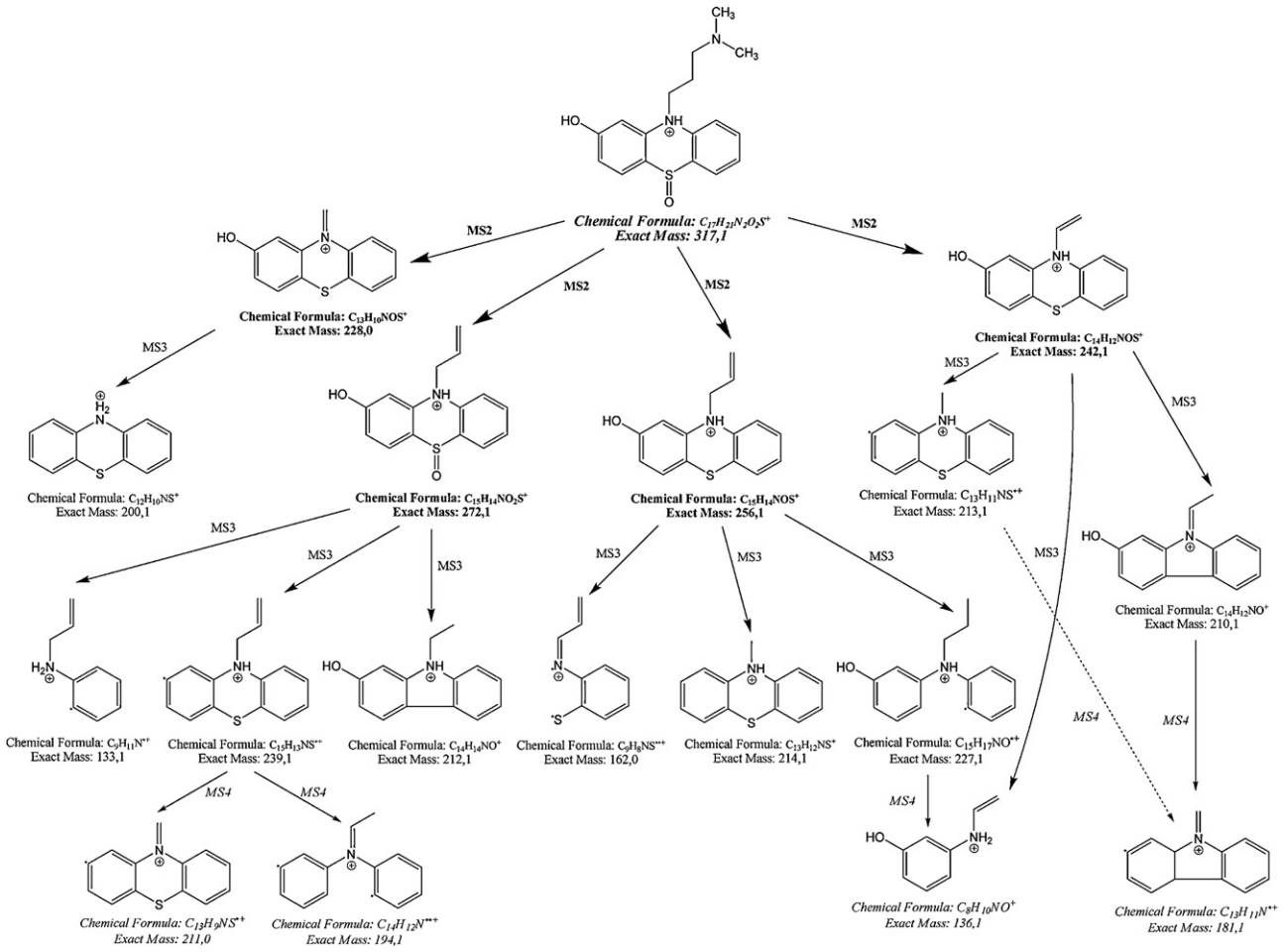

Fig. 5. Fragmentation scheme for the 1st main photolysis product of chlorpromazine 317.3 m/z according to the acquired MS^{1-4} spectra.

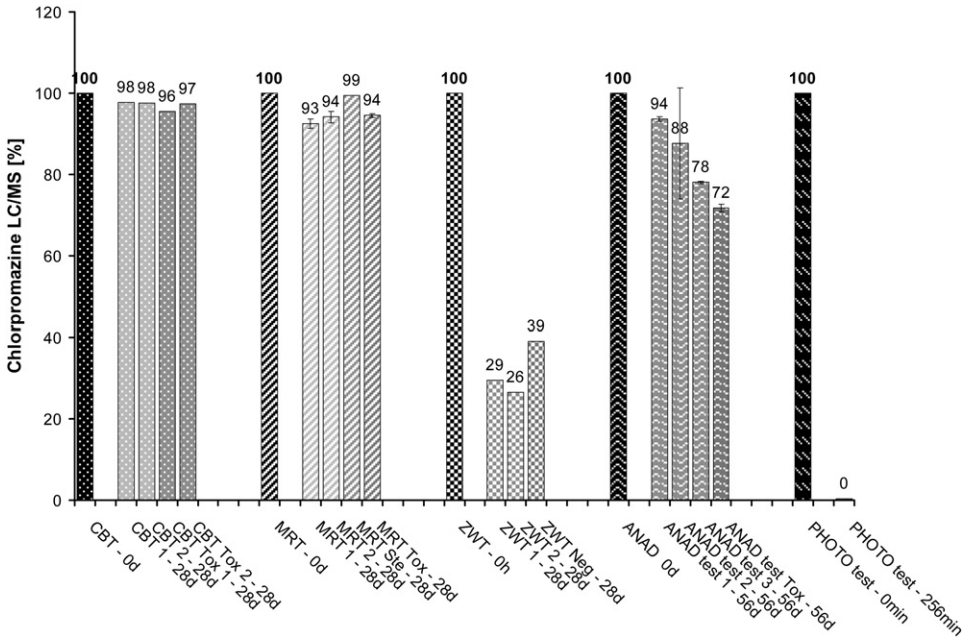

Fig. 6. Summary of LC–MS results of all degradation tests (CBT, Closed Bottle test, MRT, Manometric Respiratory test, ZWT, Zahn–Wellens test, ANAD, anaerobic degradation; PHOTO, photolysis).

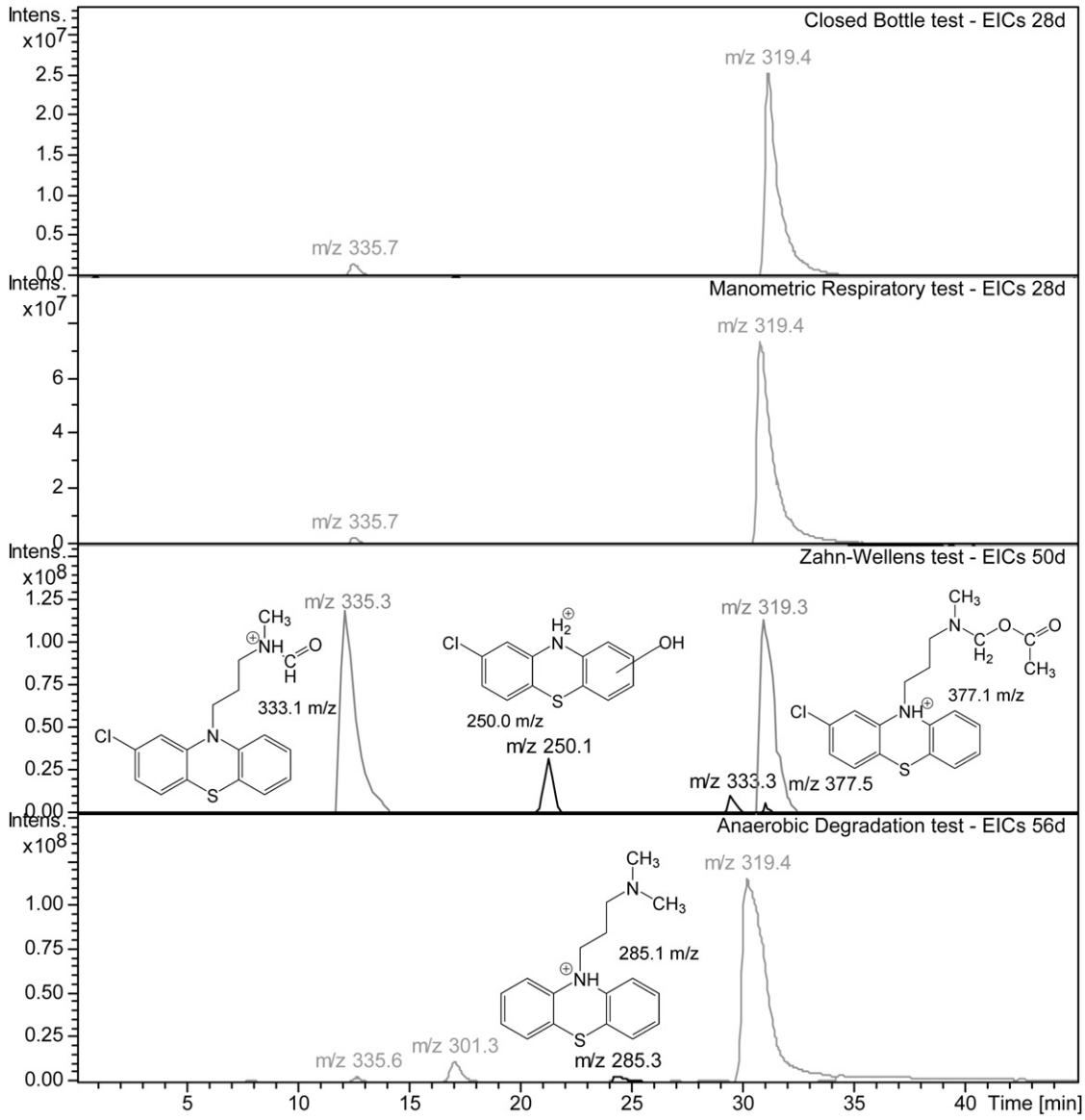

Fig. 7. Extracted ion chromatograms (EICs) of transformation products at test end in the *Closed Bottle* test (first line), *Manometric Respiratory* test (second line), *Zahn–Wellens* test (third line) and anaerobic degradation test (fourth line) and suggested molecular structures for the identified biodegradation products. Gray peaks belong to chlorpromazine (m/z = 319) and abiotic photoproducts (m/z = 301 and 335).

MD software and comparison with the photodegradation spectra, only CPR and the photoproduct 335.6 m/z remained (Fig. 7 – second line). A partial substance loss of CPR can also be attributed to adsorption effects ($\log p_{ow}$ = 5.35).

3.2.3. Aerobic degradation: Zahn–Wellens test (OECD 302 B)

The ZWT met the OECD validity criteria [51]: the quality control vessel contained ethylene glycol as the only (easily biodegradable) carbon source. DOC was eliminated to a degree of 97.9% at test end, which is more than 70% within 14 days (Fig. SI-8). In both test vessels, CPR was not eliminated according to DOC analysis (−2.1% and −9.2%, respectively, Table 2). 3 h after sludge addition, a high DOC loss was observed which was caused by adsorption of CPR to the sludge. Carbon elimination curves of the two test vessels as well as the negative control vessel turn negative in a parallel manner toward test end (Fig. SI-8). This behavior can be explained by the formation of polar photodegradation products, which do not adsorb to sludge and pass the 0.45 μm membrane filtration more easily. These products were also found in the LC–MS analysis (see also Sections 3.2.1 and 3.2.2). However, the elimination curves do not end at the same negative point. Instead, a difference of −37% remains between the negative control and the mean values of the two test vessels, which indicates the formation of biological transformation products.

LC–MS analysis detected only CPR in both test vessels and the negative control vessel at test begin. During the course of the test, new peaks emerged in the chromatograms. At test end after 28 days, the TIC curves of the two test vessels and the negative control looked more or less the same and showed mainly peaks at the retention times of photoproducts. CPR recovery was 29% in vessel 1 and 26% in vessel 2 (Fig. 6). The recovery in the negative control vessel was higher (39%). This suggests that a huge part of CPR elimination in the ZWT was due to photodegradation. As the test was not performed in complete dark but at diffuse room-light, much more photoproducts were found in comparison to the CBT and MRT. However, a small fraction, namely the difference between test vessels and negative control (10% and 13%), might have been due to biodegradation. In order to detect biological

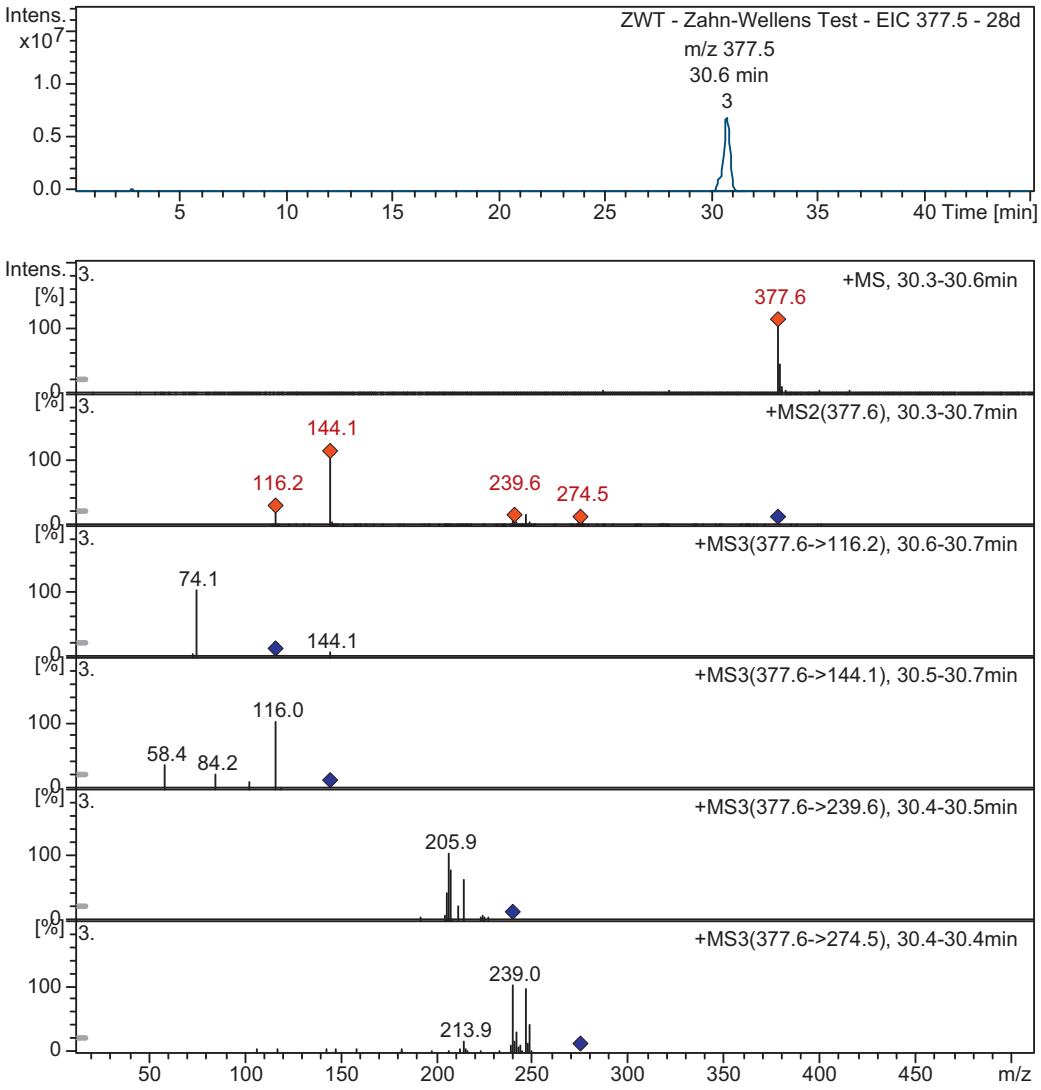

Fig. 8. Extracted ion chromatogram (EIC) and MS^{1-4} fragmentation spectra of the 1st main aerobic biodegradation product of chlorpromazine 377.5 m/z at t_r = 30.6 min after 28 days incubation in the *Zahn–Wellens* test.

transformation products of small peak intensity, the MD software was used again. The LC–MS data of the test vessels was subtracted from the blank. In the remaining TIC, some peaks at the same retention times and with m/z-values identical to those of photoproducts could be identified. However, three non-photolytic peaks remained in the chromatographic window of both test vessels' LC–MS data. The retention times were $t_{r,1}$ = 21 min, $t_{r,2}$ = 29 min and $t_{r,3}$ = 31 min (Fig. 7 – third line). The corresponding mass spectra showed peaks at 250.3 m/z, 333.5 m/z, and 377.5 m/z, respectively. These peaks were studied with MS^n experiments and resulted in different product ions (Table 4). The most intense peak at m/z = 377.5 had a difference of 58 m/z compared to CPR and was studied with MS^{2-3} experiments. The resulting fragmentation data (Fig. 8) suggests the formation of a CPR-acetylation adduct during the course of the Zahn–Wellens test. Fig. 9 shows a proposed fragmentation pathway for the obtained mass spectra, which were acquired in MS^2 and MS^3 modus. The structures of the two other, less intensive, transformation products were elucidated the same way. The compound m/z 250.3 at $t_{r,1}$ = 21 min possibly is single hydroxylated and chlorosubstituted phenothiazine, which was formed by complete degradation of the CPR side chain (Figs. SI-9 and SI-10). By contrast, the 2nd transformation product m/z 333.5 seems to result from demethylation and following carboxylation at the nitrogen atom of the side chain (Figs. SI-11 and SI-12). The molecular structures and their corresponding extracted ion chromatograms EIC of all three bacterial aerobic transformation products, which were elucidated in the ZWT, are shown in Fig. 7 (third line).

3.2.4. *Anaerobic degradation test (ISO 11734: 1995)*

Validity criteria of the ISO norm were met [47]. None of the test bottles turned pink during or at the end of the test, guaranteeing the absence of oxygen, therefore also assuring correct measured pressure values. All three reference substances were degraded to more than 60%: PEG 400 (68.2 ± 0.8%), sodium benzoate (71.6 ± 5.5%), and phenol (64.1 ± 5.9%). Calculated degradation degrees for CPR, however, were negative (−83.7%, −83.3%, −80.3%).

This can only be explained through intoxication of the anaerobic sludge, which resulted in the loss of biogas production. The calculation of biodegradation degrees is based on biogas production and subtraction from the blanks. The absence of any biogas production in the test vessels therefore leads to negative degradation when the blanks are still active (Table 2). These findings are supported by the results of the toxicity control, where PEG 400 was not biodegraded, therefore also leading to a negative degradation degree of −81%. Fig. 10 illustrates these calculations: Whereas the three blank bottles already had a strong self-activity, reaching pressure values

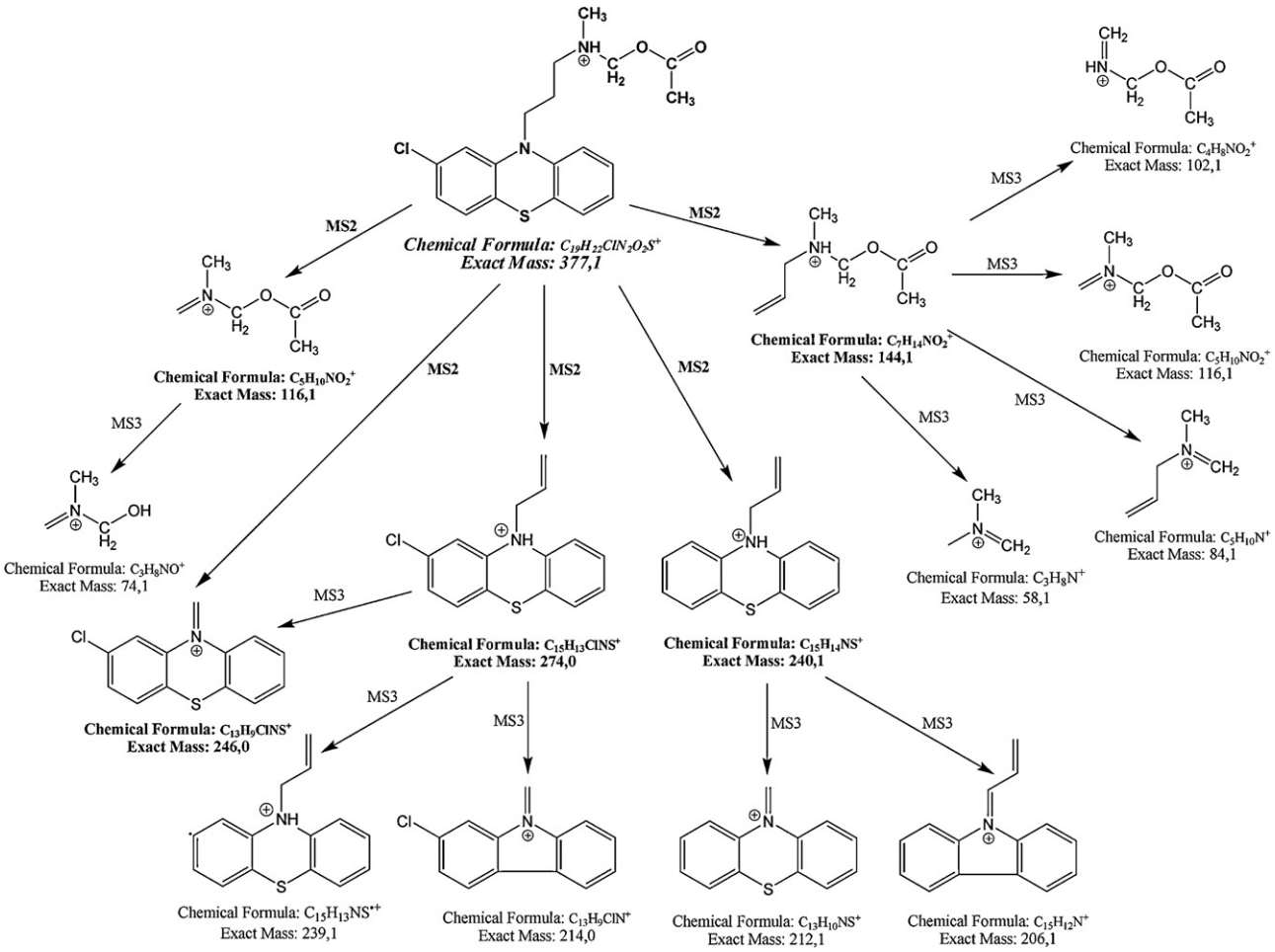

Fig. 9. Fragmentation scheme for the 1st aerobic biodegradation product of chlorpromazine 377.5 m/z in the *Zahn–Wellens* test according to the acquired MS^{1-4} spectra.

around 170 mbar at test end, the absolute pressure values for the CPR bottles did not pass 30 mbar (Fig. 10). Biodegradation of PEG 400 started shortly after test start, whereas degradation of the two aromatic compounds had a lack phase of five days in case of sodium benzoate, and 20 days in case of phenol, respectively.

Analysis of LC–MS samples showed a CPR recovery between 72% and 94% with highest CPR elimination in the toxicity control (Fig. 6). The MD software was used again to subtract mass spectra deriving from the anaerobic sludge or system contaminations. Evaluation of the chromatogram revealed only one peak, which was not due to photodegradative processes: $m/z = 285.3$ at $t_r = 24$ min (Fig. 7 – fourth line). Isolation and fragmentation in the MS^n mode resulted in product ions up to MS^3 (Fig. SI-13). The fragmentation data indicate the formation of dehalogenated CPR (Fig. SI-14). Comparison with purchased promazine (another antipsychotic drug of the phenothiazine family) standards showed the same retention time and fragmentation pattern, confirming this single anaerobic degradation product of CPR as promazine, due to anaerobic dehalogenation.

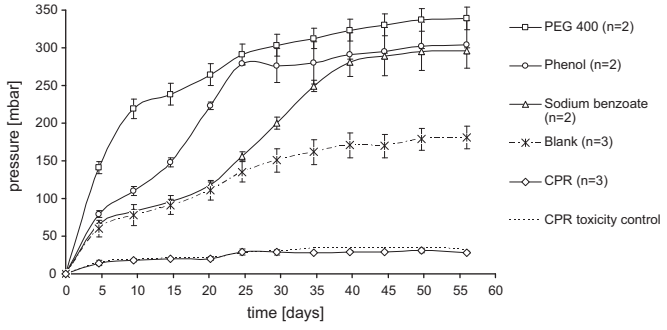

Fig. 10. Anaerobic biodegradation of chlorpromazine (CPR) and pressure curves of the different test bottles during the incubation time of 56 days; PEG, polyethylene glycol.

4. Discussion

The assessment of the environmental degradation of CPR with five different test systems gave different results comparing (bio/photo)degradation and primary elimination (Table 2 and Fig. 6). Identification of transformation products was challenging, because of the huge number of photoproducts and the low intensity of biodegradation products. A chromatographic separation method with a special column allowed full peak separation of almost all of the transformation products. Further, the application of a special peak finding algorithm, as well as the use of the MD software, allowed the detection of peaks which only belonged to the biotic or abiotic degradation of CPR and not to any other compounds deriving from the inoculum, medium or system contaminations. Finally, fragmentation experiments of higher order, using the Auto MS^n mode of the mass-spectrometric software, allowed to elucidate molecular structures of all biotic and the three main photolytic transformation products.

Abiotic photodegradation showed first order kinetics (Fig. SI-1) with almost complete elimination of CPR after 256 min irradiaton (Fig. 2). The last measurement point at 256 min was not considered in the kinetics calculation, because only residual concentrations of CPR had been detected after only 128 min of irradiation. Interestingly, the TIC curves changed their peak patterns during the course of the radiation. While at test begin, CPR and dehalogenated, single hydroxylated CPR (301.4 m/z at t_r = 16.8 min) dominated (Fig. 1), at test end, mainly mutliple hydroxylated and tentatively dimer species were found. These analytical findings correlate with the change of color during the experiment from clear to ruby as dimers are mainly responsible for light absorption [34]. 57 photoproducts were tentatively identified at test end according to LC–MS data, among them many dimers at higher retention times than CPR itself.

For 28 photoproducts, MS^n experiments of higher order were performed, therefore allowing structural elucidation of these compounds. Fragmentation pathways and proposals for the molecular structures were given for the three most abundant ions which were formed during the course of the test. 317.3 m/z at $t_{r,1}$ = 6.5 min was identified as 2-hydroxypromazine sulfoxide (Figs. 1 and 4), i.e. as product of dechlorination, followed by hydroxylation and sulfoxidation. 335.4 m/z at $t_{r,2}$ = 14 min was identified as CPR sulfoxide (Fig. 1 and Fig. SI-3). 301.1 m/z at 16.8 min was formed in the early irradiation time stages and was shown to be 2-hydroxypromazine (Fig. 1 and Fig. SI-5), therefore being a possible photoproduct precursor of 317 m/z. In another study, excitation with wavelengths ≥330 nm was shown to produce 2-hyxdroxypromazine as well as CPR sulfoxide, whereas only irradiation under UVB exposure (≤270 nm) led to the formation of the CPR cation radical and therefore possibly hydroxylated CPR derivates [56]. These findings correlate well with the results of our study, as the xenon lamps' emission spectrum ranges from 300 nm to 800 nm. The identity of the formed isomer cannot be derived from MS^n data. It can however be presumed that hydroxylation occurred first at the dehalogenated position 2 of the aromatic ring system since the fragmentation schemes showed cleavage of the side chain without adducted oxygen (Fig. 5 and Figs. SI-3 and SI-5). The structures of the 25 additional photolysis products can be reconstructed the same way, using the acquired MS^{1-4} data (Tables SI-1 and SI-2). Additional structural information can be taken from the acquired fluorescence and UV spectra (Fig. SI-15).

Whereas some toxic effects are exerted by CPR itself, for instance the ability to promote bilayer condensation [57] or binding to MDR-1 transport channels [23], phototoxicity and photoallergy due to photoproducts of CPR have been reported several times in the literature. The radical cation of CPR seems to play a crucial role in toxicity, since cleavage of the chlorine atom forms a very reactive intermediate, which can bind easily to many cell compounds [38]. By contrast, the formation of dimers seems to result in interactions with cell membranes, leading to cell lysis [37,58]. Viola and Dall'Acqua [41] have described the photosensitization of biomolecules by CPR and found direct effects mediated by the photoproducts as well as indirect effects due to the formation of reactive oxygen species. Matsuo et al. [59] reported cell death of *Escherichia coli*, caused by phototoxicity of CPR. Since *E. coli* is a prototype of most bacterial strains in sewage effluent, ecotoxicological effects on bacteria in the environment can be expected and were shown according to the toxicity control results in the ANAD test. Phototoxicity of CPR on the aquatic protozoan *S. ambiguum* has already been reported [45]. Yet most reactive species of CPR, which might mediate certain toxic or allergic effects are still not identified [40].

CPR was not biodegraded in the aerobic OECD tests applied here [47,50,51], classifying it as *neither readily nor inherently biodegradable* (Table 2). So this substance cannot be expected to be completely biodegraded in the aerobic aquatic environment and in STPs. LC auto MS^n analysis of the CBT and MRT samples showed only formation of photo- but no biodegradation products (Fig. 7 – first and second line). Even though the tests themselves were performed in the dark, handling of the test bottles at test begin and test end could not guarantee total absence of light. These photoproducts were detected in the ion trap with 335.7 m/z at the same retention time (12.9 min) as the 2nd main photolytic degradation product in the PHOTO test. Together with similar fragments (data not shown here), this implies that hydroxylation of CPR not only occurs under strong xenon lamp irradiation, but also at diffuse room light. As LC–MS analysis of the sterile bottle in the MRT showed almost complete recovery of CPR, any elimination by hydrolysis can be excluded.

LC–MS results of the ZWT samples showed a higher CPR elimination than in the CBT and MRT (Fig. 6). Again, the main photoproduct 335.7 m/z was found, which at test end already possessed a taller peak than CPR itself (Fig. 7 – third line). Some elimination could also be attributed to sludge adsorption (3 h value in Fig. SI-8). The MD software could further identify three aerobic CPR biodegradation products; though of low signal (TIC) intensity only (Fig. 7 – third line). Whereas transformation products of the photolysis were found to be hydroxylation products at the aromatic rings, reactions in the ZWT were different. The most intense peak at 377.5 m/z was identified as ((3-(2-chloro-10H-phenothiazin-10-yl)propyl)(methyl)amino)methylacetate according to MS^n data, as a result of a enzymatical monooxygenation, followed by acetylation (Fig. 9). This would include a monooxygenation of the methyl moiety at the nitrogen atom of the side chain as first step. Hydroxyl groups can further be targets for bacterial acetyltransferases like it was also shown for the popular drug sulfamethoxazole [60]. Monooxygenations of xenobiotics are typically performed by members of the cytochrome P450 family, whereas acetyl addition is executed via acetyltransferases and Acetyl-CoA. Acetylation can detoxify toxic compounds and can be responsible for antibiotic resistance as it was shown in the case of chloramphenicol [61]. Even though acetylation gives no catalytic benefit, this reaction can have detoxification effects on its substrate. As CPR also possesses antibiotic properties, it would be of interest to investigate whether 377.5 m/z is less toxic than CPR. The two other transformation products of the ZWT were found in smaller intensity. 250.3 m/z was a result of complete degradation of the side chain and identified with MS^n data as single hydroxylated phenothiazine (Fig. SI-10). However, as it is known that the aromatic ring structure can be subject to photo-induced hydroxylation, it remains unclear whether the observed hydroxylation was of enzymatic or abiotic origin. In mammal and fungal metabolism, position 7 of CPR was identified as major site of hydroxylation [33,62]. 333.5 m/z was identified according to MS^n data as N-(3-(2-chloro-10H-phenothiazin-10-yl)propyl)-N-methylformamide, probably caused by bacterial N-demethylation, followed by carboxylation (Fig. SI-11).

In the ANAD test [52], only one anaerobic transformation product could be identified. 285.3 m/z was identified as promazine, according to MS^n data (Fig. SI-14) and by comparison with purchased promazine standards which showed the same retention time and product ions. However, it remains unclear whether this product was a result of bacterial anaerobic dehalogenation, or if the cleavage of the chlorine atom was an abiotic reaction, as is reported in literature for CPR in anaerobic milieus [35,63]. Since the ANAD test, which could clarify this question, was conducted without a sterile control (containing only medium and CPR), there can be no final conclusion about this. LC–MS analysis further showed the presence of the photoproduct 335.6 m/z (same as CBT, MRT, ZWT and PHOTO test) and a second photoproduct at 301 m/z, which showed identical retention time and fragments as the 3rd identified degradation product 2-hydroxypromazine in the PHOTO test

(Fig. 7 – fourth line). This implicates that these two photoproducts can also be formed under anaerobic conditions without presence of molecular oxygen.

Summarizing biodegradation, even though some transformation was observed, the phenothiazine core was not mineralized in any of the tests, which shows its stability against to bacterial degradation or indicates bactericide properties. These findings correlate with degradation and metabolism studies on phenothiazine [30,64,65].

CPR showed no toxicity to aerobic bacteria in the CBT and MRT according to the test guidelines. However, it must be considered that sodium acetate was already degraded to more than 50% after two days in these tests. By contrast, the bactericide properties of CPR are more of bacteriostatic origin [23], which might take some time before revealing an effect on bacteria. Additionally, one test bottle in the MRT showed a strong decrease in oxygen consumption, which can be explained by toxic effects through CPR or its photoproducts 335 m/z. Becuase no toxicity control vessel was used in the ZWT, nothing can said about CPR toxicity. Yet the fact that side-chain degradation to 250.3 m/z was not the only biotic metabolite might indicate that some photoproducts of CPR are toxic to aerobic sludge. This presumption is supported by a look at the measured pH values during the test. At test begin, pH was 7.6 in the blank vessels as well as the CPR vessels. After 50 days, pH in the blank vessels containing only sludge decreased to an average pH 6.7, whereas final averaged pH in the CPR vessels was 7.5. This shows that test vessels containing CPR and sludge had almost no bacterial activity. Whether this toxicity was caused by CPR itself or any photolytic or biological degradation product cannot be clarified. However, determination of the toxic properties of the main photolytic degradation product 335 m/z, which is already formed at diffuse room light under both aerobic and anaerobic conditions, is strongly recommend.

While no toxicity according to the guideline was found in the aerobic OECD tests, but strong indicators for toxicity showed up, the toxicity control substrate PEG 400 in the ANAD test could not be degraded. CPR (or its main photoproducts 335 m/z and 301 m/z) is (are) therefore classified toxic to anaerobic sludge according to ISO 11734.

5. Conclusion

Considering 60 years of CPR usage, many transformation products of this drug might have been formed in the aquatic environment, even when taking into account that excreted amounts of non-metabolized CPR are small in individual cases. Since the phenothiazine core of CPR was shown to be absolutely stable to any biotic or abiotic mineralization [30,64,65] (even though under conditions as they are found in a sewage treatment plant), concern has to be raised on the environmental fate of CPR and its transformation products. The rapid and numerous formation of CPR photoproducts, which showed in literature to have complex toxic properties, urgently implicates that irradiation techniques should not be employed in water treatment or freshwater preparation. This especially accounts for tropical development countries without sewage treatment, where drugs rapidly enter open surface water bodies and where CPR photoproducts can easily be formed by natural sunlight as well as during UV disinfection. As phototoxicity was not only reported in literature, but also found in some of the test bottles in the different degradation tests of the present study, the authors strongly recommend further environmental research. Especially an evaluation of the ecotoxicological potential of the photodegradation products seems an important task. Initial experimental work on these questions is in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2012.01.022.

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