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Investigating the fate of iodinated X-ray contrast media iohexol and diatrizoate during microbial degradation in an MBBR system treating urban wastewater

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Abstract The capability of a moving bed biofilm reactor (MBBR) to remove the iodinated contrast media (ICM) iohexol (IOX) and diatrizoate (DTZ) from municipal wastewater was studied. A selected number of clones of microorganisms present in the biofilm were identified. Biotransformation products were tentatively identified and the toxicity of the treated effluent was assessed. Microbial samples were DNA-sequenced and subjected to phylogenetic analysis in order to confirm the identity of the microorganisms present and determine the microbial diversity. The analysis demonstrated that the wastewater was populated by a bacterial consortium related to different members of Proteobacteria, Firmicutes, and Nitrisporae. The optimum removal values of the ICM achieved were 79 % for IOX and 73 % for DTZ, whereas 13 biotransformation products for IOX and 14 for DTZ were identified. Their determination was performed using ultra-performance liquid chromatography–tandem mass spectrometry. The toxicity of the treated effluent tested to *Daphnia magna* showed no statistical difference compared to that without the addition of the two ICM. The MBBR was proven to be a technology able to remove a significant percentage of the two ICM from urban wastewater without the formation of toxic biodegradation products. A large number of biotransformation products was found to be formed. Even though the amount of clones sequenced in this study does not reveal the entire bacterial diversity present, it provides an indication of the predominating phylotypes inhabiting the study site.

Keywords Iodinated contrast media · Moving bed biofilm reactor · Biotransformation products · Microbial colonization · Toxicity testing

Introduction

Iodinated X-ray contrast media (ICM) constitute a group of contaminants of emerging concern, which have been detected at elevated concentrations in the aquatic environment (Seitz et al. 2006a, b; Schulz et al. 2008; Sugihara et al. 2013). These compounds deserve special scientific attention as they constitute one of the most frequently used pharmaceutical classes in hospitals (Gartiser et al. 1996; Hirsch et al. 2000; Drewes et al. 2001; Putschew et al. 2001) and also are reported to be found in high concentrations (at the range of micrograms per liter) in samples collected from urban wastewater treatment plants (UWTPs; Ternes and Hirsch 2000, Drewes et al. 2001). In a study by Carballa et al. (2004) for instance, significant concentrations of pharmaceuticals including X-ray contrast media were found in the influent. Iopromide for example was found in the range 6–7 μg/L in influent.

Several studies have already proven that the ICM are persistent against conventional biological treatment processes

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E. Hapeshi · A. Lambrianides · P. Koutsoftas · C. Michael · D. Fatta-Kassinos
Department of Civil and Environmental Engineering, University of Cyprus, P.O Box 20537, 1678 Nicosia, Cyprus

E. Hapeshi · A. Lambrianides · P. Koutsoftas · C. Michael · D. Fatta-Kassinos (✉)
Nireas International Water Research Center, University of Cyprus, P.O Box 20537, 1678 Nicosia, Cyprus
e-mail: dfatta@ucy.ac.cy

E. Kastanos
Department of Life and Health Sciences, University of Nicosia, 46 Makedonitissas Avenue, 1700 Nicosia, Cyprus

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(Putschew et al. 2001; Fono and Sedlak 2007; Busetti et al. 2008). According to Kalsch (1999), the adsorption and binding of diatrizoate to aerobic-activated sludge was found to be poor, suggesting that this substance is hardly retained in sewage treatment plant sludge.

The study by Carballa et al. (2004) showed that aerobic treatment through activated sludge caused an important reduction in many pharmaceuticals, hormones, and other compounds (between 35 and 75 %), with the exception of ICM (iopromide) which remained in the aqueous phase. Likewise, as reported by Fono and Sedlak (2007), the concentrations of ICM including iopromide, iopamidol, iothalamic acid, and diatrizoic acid ranged between 1.9 and 16.3 μg/L, with a median of 6.5 μg/L, in treated effluent. Biotransformation products of iopromide and diatrizoate have also been detected in effluents (Haiss and Kümmerer 2006; Perez et al. 2006), and presumably similar transformation processes could occur also in environmental media like surface and groundwater (Fono and Sedlak 2007). Joss et al. (2006) investigated the biological degradation of selected ICM (diatrizoate, iohexol, iomeprol, iopamidol, iopromide, iothalamic acid, ioxithalamic acid) and estimated the degradation constants in batch systems with activated sludge from two UWTPs. Biological rate constants were calculated to be between 0.1 and 2.5 L/g_{ss} day. These rates confirm the fact that biological degradation (activated sludge) in urban wastewater contributes only to a quite limited extent to the overall load reduction of ICM.

The effective removal of ICM from wastewater effluents is very important due to their considerable impacts on ecological systems that have been established so far. Their potential toxicological significance and their long-term effects lie in the low-concentration chronic exposure of organisms (Blake and Halasz 1995; Steger-Hartmann et al. 2002).

Since activated sludge process has been proven to be inefficient in removing these compounds, advanced oxidation processes have been applied and examined with respect to their capacity in providing enhanced removal of ICM from wastewater (Ternes et al. 2003; Köhler et al. 2012; Velo-Gala et al. 2012). Seitz et al. (2006a, b) found that during three different ozone doses of 1, 2, and 3 mg/L and at different contact times (2, 4, 6, 8, and 10 min) applied separately, an average removal rate of 30 % was observed for non-ionic ICM. Likewise, Ternes et al. (2003) studied the degradation of diatrizoate (DTZ) from wastewater, removing only 14 % of the initial concentration with O_3, only 25 % with the O_3/H_2O_2 system, and 35 % with an O_3/UV system. The actual mechanism of ICM degradation by ozone is not completely understood, but it has been suggested that direct reaction of molecular O_3 with ICM is unlikely to occur (Huber et al. 2005; Seitz et al. 2008). The degradation of ICM using UV radiation and hydrogen peroxide (H_2O_2) has also been investigated. It was reported that iodine atoms are released and, in the cases of photochemical oxidation, partial degradation takes place, reaching 46 % after 40 h of photolysis (Doll and Frimmel 2003). Sprehe et al. (2001) reported that photochemical oxidation is a process that is able to decrease the adsorbable organic halide concentrations and to increase the degradation potential of ICM in hospital wastewaters. Other studies used other photocatalytic processes such as TiO_2 photocatalysis in order to oxidize the ICM, but the results show that only low partial oxidation can be achieved (Doll and Frimmel 2004, 2005; Sugihara et al. 2013). A report from Benotti et al. (2009) showed that 70 % of iopromide was oxidized in a photocatalytic reactor with UV radiation and titanium dioxide (TiO_2). Doll and Frimmel (2005) noted that the photocatalytic degradation of ICM indicates significant degradation rates of ICM, but restricted mineralization.

Moreover, other advanced processes, like reverse osmosis (RO), were tested based on the high molecular weight of ICM. According to Busetti et al. (2008), microfiltration and RO led to a removal of ICM of 90 %.

The microbial degradation in UWTPs is an important removal process for various compounds, especially for those that are resistant to photolysis and various other chemical oxidation processes (Kunkel et al. 2008). Since ICM are resistant to such processes, then, as discussed also by Ferrai et al. (2010), there is a need to upgrade the existing conventional biological wastewater treatment processes. Biological processes based on biofilms have been proven to offer satisfactory solutions for the removal of organic components from wastewater, avoiding also the problems associated with the technology of activated sludge, such as large reactor size, the need for settling tanks, and biomass recycling (Delnavaz et al. 2010; Li et al. 2011; McQuarrie and Boltz 2011; Calderón et al. 2012). The moving bed biofilm reactor (MBBR) technology introduced almost 30 years ago is considered as one of the best options to replace suspended activated sludge due to its advantages, including simplicity, growth of aerobic and anaerobic organisms in the same system, compactness, smaller tank volume, increased solid retention time for slow-growing organisms, and reduction of hydraulic head losses (Andreottola et al. 2000; Loukidou and Zouboulis 2001; Khan et al. 2011; Shore et al. 2012; Zupanc et al. 2013). These advantages are due to the biomass which grows on especially designed carriers that move within the water volume, making available a greater surface area on which biofilm can grow (Ødegaard 2006; Gapes and Keller 2009; Calderón et al. 2012; Zupanc et al. 2013).

MBBR is regarded as one of the most promising treatment approaches for the removal of contaminants from wastewater because of its affordable cost and high efficiency (Lei et al. 2010). Although in the past decades the MBBR technology has been successfully applied for many industrial wastewater including paper mill wastewaters (Rusten et al. 1994; Hosseini and Borghei 2005), pharmaceutical industry wastewater (Lei et al. 2010), and for the treatment of

municipal wastewater, according to our knowledge, no attempt was made to study the removal of ICM from wastewater using MBBR technology. Only recently, a study by Zupanc et al. (2013) was performed in order to evaluate this technology for the treatment of pharmaceuticals including clofibric acid, ibuprofen, and diclofenac in urban wastewater.

As previously reported, many X-ray contrast media such as DTZ and IOX are not completely mineralized in the environment (Putschew et al. 2001; Ternes and Hirsch 2000), giving rise to stable transformation products that are potentially more harmful than their precursor compounds (Kalsch 1999; Jeong et al. 2010). According to the literature available, a limited number of studies on these biotransformation and metabolic products (Kalsch 1999; Haib and Kümmerer 2006; Pérez and Barceló 2007) exist.

Therefore, the overall aim of this study was to fill the aforementioned relevant gaps by trying to assess the efficacy of the MBBR technology against the removal of IOX and DTZ in urban wastewater. The specific objectives of this study were (1) to investigate the bacterial consortium developed in the MBBR utilizing a molecular approach, (2) to study the capacity of a pilot MBBR system to remove ICM compounds like IOX and DTZ from urban wastewater, (3) to characterize their biotransformation products, and (4) to assess the potential toxicity of the treated effluent.

Experimental

Chemicals

Iohexol (CAS 66108-95-0) and sodium diatrizoate hydrated (CAS 737-31-5 anhydrous) were purchased from Sigma-Aldrich. IOX was used without any further purification. Sodium DTZ hydrated was heated for 4 h in an oven at 105 °C and then kept in a desiccator until further use. The appropriate amount of IOX and sodium DTZ was dissolved in approximately 1 L of primary treated wastewater; after complete dissolution, the resulting solution was transferred quantitatively in the inlet tank of the MBBR. All the analyses for the characterization of the inflow and outflow wastewater were carried out with the appropriate COD kits (Merck® Spectroquant kits, WTW Photolab S6), except BOD_5 which was performed using the 444406 OxiDirect meter. Methanol used for the ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) analysis was Chromasolv LC grade solvents, provided by Sigma-Aldrich (Steinheim, Germany). Formic acid (ACS grade) was obtained from Merck (Darmstadt, Germany).

ICM have been detected in micrograms per liter concentrations in urban wastewater (Ternes and Hirsch 2000; Drewes et al. 2001). However, in the present study, the concentrations applied are higher than the environmentally relevant concentrations in order to convincingly elucidate the biotransformation products.

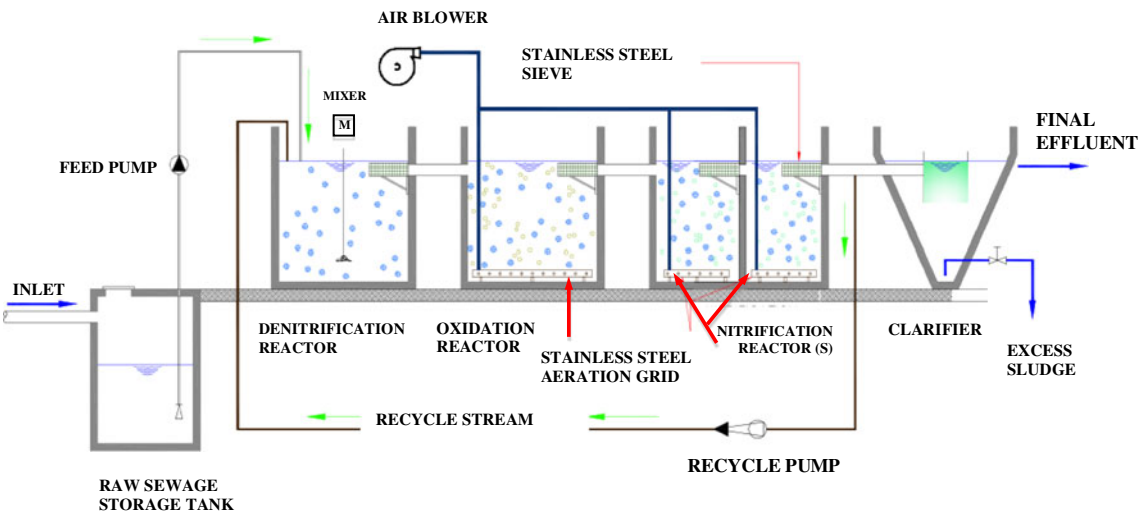
Equipment and methods

The MBBR system

The pilot MBBR unit used was especially designed for the requirements of the present study (Scheme 1) and was manufactured by S.K. Euromarket Ltd., Cyprus.

The pilot-scale plant of MBBR incorporates four subsequent compartments: denitrification, oxidation, and two nitrification reactors, with volumes of 23.4, 24.0, and 9 L for each of the nitrification reactors, respectively. In addition, there is one secondary settling tank in the shape of an inverted cone.

The carrier elements are made of polyethylene with a density of about 0.97 g/cm^3, a density which is slightly



Scheme 1 Pilot MBBR (explanations are provided in the text)

lower than that of water, and a specific biofilm surface of 500 m^2/m^3 (Fig. 1). Circulation of the biofilm carriers inside the denitrification reactor is carried out by mechanical stirring using an automatic mixer, while circulation in the two other reactors, oxidation and nitrification reactors, is achieved by aeration. The filling ratio applied (recommended percentage volumetric filling of plastic elements in an empty reactor) was about 50 %.

Operation of the pilot unit was fully automatic. Influent from a sewage treatment plant was collected (after the primary sedimentation) into the raw sewage storage tank (a plastic container) of 1 m^3 capacity. A peristaltic pump was used to transfer the influent into the denitrification reactor with a rate of 2.0 L/h, where the mixing of raw influent with recirculated nitrified stream was achieved with a mechanical mixer. Following the denitrification stage, influent was conveyed by gravity to the biochemical oxygen demand (BOD) oxidation compartment. Plastic carriers in the denitrification compartment were restricted from passing the BOD oxidation compartment by a perforated sieve that was installed on the vertical wall in between the two compartments. Biodegradable organic carbon was oxidized with a supply of air via medium bubble stainless steel diffusers installed at the bottom of the BOD oxidation compartment. In turn, the oxidized liquid was introduced into two successive nitrification compartments. All ammonia and organic nitrogen was fully oxidized to NO_3^- with a supply of air via medium bubble stainless steel diffusers installed at the bottom of the nitrification compartments.

The nitrified liquid was recirculated to the denitrification compartment with the use of a submersible pump installed at the second nitrification chamber. Experiments were conducted at 20–22 °C, which was the temperature of the wastewater after the primary sedimentation at the treatment station and under controlled conditions of dissolved oxygen concentrations of 2.0–2.5 and 4.0–4.5 mg/L in the BOD oxidation and nitrification compartments, respectively.

After the system reached the steady-state condition, samples from all the compartments and the outflow were taken at regular time intervals for subsequent analyses (including samples for the microbial diversity analyses). The conditions were established as the optimum ones following the general guidelines of the manufacturer of the MBBR system while taking into account other previous experimental results with MBBR systems (Hem et al. 1994; Odegaard et al. 1994; Weiss et al. 2005).

The main qualitative characteristics of the wastewater used throughout the experiments are summarized in Table 3.

DNA extraction and PCR

Prior to the genomic DNA extraction from each sample (50 mL), ultrasonic separation of cells was performed (for 10 min using a Satorius/Sigma 3K30 centrifuge), followed by DNA extraction QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA) according to the instructions supplied by the manufacturer.

The bacterial 16S rDNA fragments were amplified by PCR using the universal bacterial primers 27F and 1525R (Lane 1991). The primers used for the different PCR reactions in this study are presented in Table 1. Amplification was carried out in 20 μL reaction mixtures containing Taq polymerase, 10 pmol of each primer, 5–50 ng template genomic DNA, and water added to obtain a final volume of 20 μL and performed with a Thermal Cycler TECHNETC-412. The cycle parameters and PCR programs are presented in Table 2. The reactions were stopped by cooling the mixture to 4 °C. The quantity and quality of the products were checked on a 1 % agarose gel and visualized with ethidium bromide. Bands with the appropriate size range were cut out of the gel and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA).

Cloning and screening of PCR amplification products

PCR products were TA-cloned into pCR2.1 (Invitrogen, Carlsbad, CA) according to the protocols provided by the manufacturers. Plasmid DNA was isolated and clones were screened for the presence of inserts by PCR using vector-

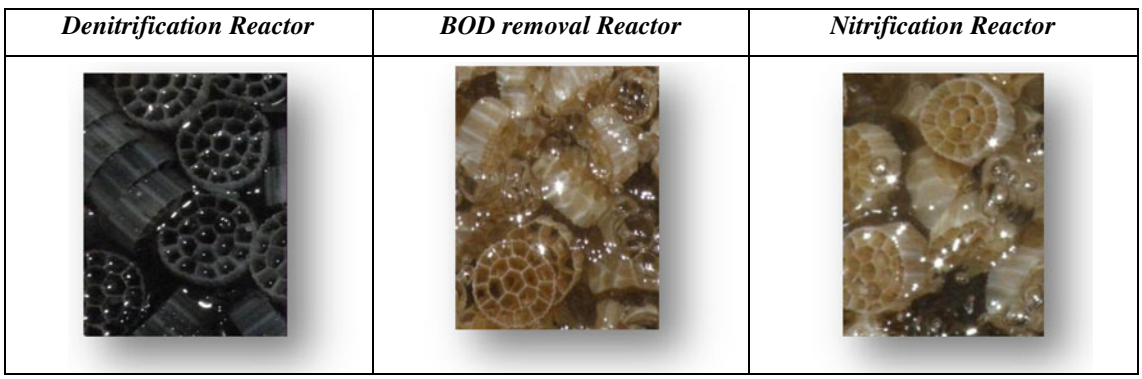


Fig. 1 Images of the samples obtained from each chamber of the reactor (denitrification, BOD removal, and nitrification process)

Table 1 Sequences and types of primers used for PCR

Name	Primer sequence (5′–3′)	Primer type and target	Expected size of product (bp)	Reference
27F	AGAGTTTGATCMTGGCTCAG	Forward universal bacterial primer	1,500	Lane (1991)
1525	AAGGAGGTGWTCCARCC	Reverse universal bacterial primer	1,500	Lane (1991)
M13F	GTTTTCCCAGTCACGACGTTGTA	Forward pUC primer	1,800	Messing (1984)
M13R	CAGGAAACAGCTATGACC	Reverse pUC primer	1,800	Messing (1984)

specific primers, M13F and M13R. Amplification products were digested with restriction endonuclease *Hin*dIII and *Xba*I for 3 h. The reaction was stopped by incubating the samples at 65 °C for 20 min. Ten microliters of the restriction digests was separated using 1 % agarose gel electrophoresis to confirm the sizes. Representative clones were selected for sequencing analysis.

DNA sequencing

Double-strand sequencing was carried out by Eurofins, MWG Operon, Germany. The closest relatives to 16S rDNA sequences were obtained using NCBI's sequence similarity search tool BLASTN 2.2.2. (Basic Local Alignment Search Tool).

UPLC-MS/MS analysis

All analyses were performed on an ACQUITY TQD UPLC-MS/MS system (Waters) using a method specifically developed for this application. A triple quadrupole mass spectrometer TQD (serial no. QBA012) coupled with an electrospray ionization (ESI) source running in positive ion (PI) mode was used for the detection of target analytes. In order to achieve sufficient sensitivity for quantitative analysis, data acquisition was performed in multiple reaction monitoring mode, recording the transitions between the precursor ion and the most abundant fragment ions. The protonated molecular ion $[M+H]^+$ was selected as the precursor ion for the analyte. Precursor and product ions with their associated collision energies and retention times are summarized in Electronic supplementary material (ESM) Table S1), together with the operating MS/MS parameters.

Sample extraction was performed with solid phase extraction (SPE) using ISOLUTE ENV+ (50 mg/3 mL; Separtis, Grenzach-Wyhlen, Germany) cartridges. The sample (10 mL) was filtered through a syringe filter (22-μm pores) prior the SPE. The cartridges were first activated with 6 mL methanol and 6 mL ultrapure water (pH 3) and then the samples were percolated through the cartridges. After extraction, the cartridges were dried for 20 min under vacuum. The compounds adsorbed on the cartridges material were eluted with 5 mL methanol. Ten microliters of the methanolic solution was then injected in the UPLC. The LC analysis conditions were: T_{column}=40 °C, flow rate= 0.3 mL/min, run time=9 min. The column used was the BEH Shield RP18. Analysis was performed using water+ 0.1 % formic acid as eluent A and methanol as eluent B. The elution gradient was: 0 min 5 % B, 1.5 min 5 % B, 2 min 30 % B, 3 min 50 % B, 5 min 70 % B, 6 min 90 % B, 7 min 90 % B, 7.1 min 5 % B, and 9 min 5 % B.

For biotransformation product (BTP) identification, the initial concentration of 10 mg/L of DTZ and IOX was used in order to be able to identify transformation products produced in low concentrations during the biological process. For the identification of the BTPs, data acquisition was performed with ESI in PI mode in full-scan mode (range of *m*/*z*50–1,000) and selected ion recording mode at cone voltage of 50 V.

Toxicity testing

The toxicity tests to *Daphnia magna* were performed according to the ISO 6341:1996 method, and each test sample as well as the controls were tested in quadruplicate. The test is based on the observation of the freshwater species *D. magna* immobilization after 24 and 48 h of exposure in the samples. The water used to activate and hatch the organisms (72–90 h) was synthetic freshwater containing $NaHCO_3$, $CaCl_2$, $MgSO_4$, and KCl. Synthetic freshwater was used as the dilution water. Sufficient amount of dissolved oxygen (~5 mg/L) was achieved by aeration. The dilution water was prepared a day prior to its use in order to provide oxygen saturation and ensure complete salt dissolution and homogenization. Cultures were grown under continuous

Table 2 PCR programs used for the assays

	Bacterial16S rDNA	Vector pCR®2.1
Primers	27F-1525R	M13F-M13R
Size of the amplicon (bp)	1,500	1,800
Initial denaturation	95 °C, 10 min	95 °C, 10 min
Denaturation	95 °C, 60 s	95 °C, 60 s
Hybridization	53 °C, 60 s	50 °C, 60 s
Extension	72 °C, 90 s	72 °C, 90 s
No. of cycles	35	35
Final extension	72 °C, 10 min	72 °C, 10 min

illumination at a constant temperature of 20–22 °C. Two hours before testing, the neonates were fed using *Spirulina* microalgae in order to preclude mortality by starvation, thus avoiding biased test results. Analysis was carried out on specific test plates, which were filled with the examined dilutions. After the transfer of *Daphnia* neonates into the cells, the test plates were incubated at 20 °C in the dark. Observations of test populations were made at 24 and 48 h of exposure; any dead or immobilized neonates were recorded. Mortality data were used to estimate the toxicity of the treated effluent that contained, among others, the biotransformation products of the two ICM as well.

Results and discussion

Efficiency of the MBBR system used in the present study

Before any attempt to study the biodegradation of IOX and DTZ, the MBBR system was set in operation with the primary wastewater to allow for biofilm development, while samples were taken from the inflow and outflow at 3, 8, 15, 25, 35, 42, and 45 days. Outflow samples were taken 24 h after inflow sampling, taking into account the hydraulic retention time. All samples were monitored for the main parameters of the wastewater, i.e., COD, BOD_5, Total-P, Total-N, NH_4–N, TSS, and TS. Values of all the parameters in the outflow showed a gradual reduction from day 8 to day 25, with a sharp decrease of their values on day 35 (data not shown). Forty-two days after the start-up of the MBBR, the system had reached a steady-state condition, as judged by measurements from day 42 onward. The quality of the outflow as can be seen in Table 3 satisfies the standards set by the EU (Directive 91/271/EEC) for urban wastewater treatment and discharges to sensitive areas (BOD< 25 mg/L, COD<125 mg/L, TSS<35 mg/L, total phosphorous<2 mg/L, total nitrogen<15 mg/L).

Table 3 Main characteristic parameters of the inflow (raw) urban wastewater and the outflow (treated) wastewater

Parameter	Inflow	Outflow[a]
pH	6.8–7.3	7.0–7.5
Temperature °C	19–22	21–24
COD mg/L	620–700	25–37
BOD mg/L	300–350	8–16
Total-P mg/L	7.0–7.5	1.3–2.0
Total-N mg/L	65–75	8.5–11.5
NH_4–N mg/L	45–60	0.2–0.3
TS mg/L	200–250	46–52
TSS mg/L	70–94	12–16

[a] After 42 days onward

Phylogenetic analysis of the clone sequences

In order to gain insight into the microbial colonization of the three chambers of the MBBR unit, an investigation utilizing a molecular approach was undertaken. In recent years, several genetic tools other than the classical approach based on the cultivation of bacteria from environmental samples have been applied (Torsvik et al. 1990; Wagner et al. 1993). The most practiced approach to study microbial diversity is the analysis of genes (Alfreider et al. 2002; Kowalchuc et al. 2004; Alfreider and Vogt 2007; Wéry et al. 2010).

DNA sequencing, a process of determining the nucleotide order of a DNA fragment, was used as an initial measure of diversity in the seven clones selected. Different types of biomass were developed in each part of the reactor (Fig. 1). Samples were DNA-sequenced (Fig. 2) and subjected to phylogenetic analysis in order to confirm the identity of the microorganisms present and determine the microbial diversity. The sequences were initially aligned and verified manually and then analyzed with a similarity search program from GenBank, which implements the BLAST algorithm (Altschul et al. 1990).

Although there are no exact sequence similarity limits for the phylogenetic resolution of the 16S rDNA approach and for the definition of specific taxa such as genus and species, the closest relatives to 16S rDNA sequences with similarities >95 % in the phylogenetic tree are listed in Table 4. The wastewater was found to be populated by a bacterial consortium related to different members of Proteobacteria, Firmicutes, and Nitrisporae. In detail, our analysis revealed that clones from samples obtained from the denitrification chamber of the MBBR reactor were most closely related to *Clostridium*, the genus of Gram-positive bacteria belonging to the *Firmicutes*. Denitrifying bacteria such as *Clostridium* use nitrate as the terminal electron acceptor during anaerobic respiration and are presumed to be the microorganisms through which denitrification is achieved (Lim et al. 2005). In general, analysis of the microbial community related to nitrogen removal is a prerequisite for understanding the nitrogen removal process in depth.

In the case of the nitrification chamber of the MBBR reactor, clone 13N was identified, which is a member of the genus *Aeromonas*, Gram-negative bacteria within the gamma subclass of Proteobacteria. *Aeromonas* are widespread in environmental habitats such as water and soil (Trakhna et al. 2009). In concurrence with the results obtained from other studies, Proteobacteria harbor the important populations under aerobic or nitrate-reducing conditions (Alfreider and Vogt 2007). One sequence (19N), which originated from the nitrification chamber of the MBBR, clearly belonged to the genus *Bacillus*, which are Gram-positive bacteria that are members of phylum Firmicutes.

Two clone sequences that were recovered from the BOD chamber of the MBBR reactor are related to the genus

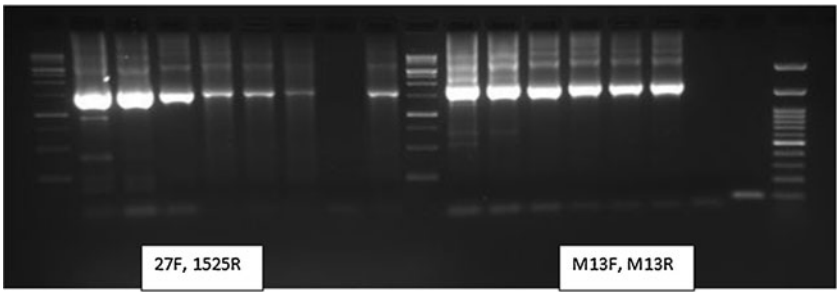


Fig. 2 Agarose gel electrophoresis on polymerase chain reaction samples performed using selected wastewater samples. The gel confirms the existence of 16S rDNA using primers 27F–1525R. Bands of 1,500 bp were visible in all lanes, at the expected position. Negative control (−) samples were added (dH_2O) where no bands were visible. Bacterial genomic DNA was used as a positive control with primers 27F and 1525R. pUC19 was used as a positive control with primers M13F and M13R. As a result, a band of the expected size (200 bp) was visible

Stenotrophomonas, Gram-negative bacteria within the gamma subclass of Proteobacteria, and to the genus *Nitrispora*, which are nitrite-oxidizing bacteria, among the most diverse and widespread nitrifiers in natural ecosystems and biological wastewater treatment. In this context, it is of interest that *Nitrispora*, forming a deeply branching lineage in the bacterial phylum Nitrisporae, in addition to their wide distribution in natural habitats such as soils, sediments, oceans, and hot springs, are the predominant bacteria in wastewater treatment plants and thus belong to the microorganisms most relevant for biotechnology.

The MBBR system as a batch reactor

The MBBR system was modified from a continuous flow reactor to a batch reactor for 1 day in order to obtain information on the degradation kinetics of IOX and DTZ. In the modified reactor, the feed pump was set off and the recirculation pump on in order to ensure that the wastewater would follow all the stages of treatment in the MBBR system. Appropriate amounts of IOX and DTZ were spiked in the reactor in order to obtain a final concentration of 10 mg/L. Figure 3 shows the biotransformation of IOX and DTZ for 24 h of operation time.

It should be noted that in the raw wastewater used for the treatment assessment, an amount of 1.92 μg/L DTZ was quantified, whereas no IOX was detected. This concentration of DTZ was quite low and did not affect the concentration spiked into the samples that was of a higher level.

During the first 2 h of operation, a removal of 34 % of the initial concentration of IOX was observed, while after 8 h the removal increased to 48 %. At the end of the 24-h period of operation of the MBBR, biodegradation reached 63 %. The concentration of the IOX in the reactor at the end of the experiment was 3.5 mg/L.

The same experiment was performed with spiked DTZ; the results are shown in Fig. 3. As can be seen, the removal of DTZ is much slower than IOX and less efficient. At the end of the experiment, i.e., 24 h of operation, only 33 % of the spiked amount of the parent compound was removed.

Analysis of the sludge in both experiments gave negligible amounts of adsorbed DTZ or IOX: 0.93 μg/g sludge on dry base and 5.85×10^{-3} μg/g, respectively. This was an expected fact for highly hydrophilic compounds such as IOX and DTZ. Moreover, laboratory experiments with aqueous 10 mg/L solutions of IOX and DTZ under stirring for 24 h at room temperature proved that no hydrolysis of the compounds occurred. Therefore, it was concluded that the reduction of the concentration of IOX and DTZ observed in the reactor should be attributed only to their biotransformation.

The MBBR system as a continuous flow reactor

The removal efficiency of the MBBR system operating under regular conditions, i.e., as a continuous flow reactor, was investigated by spiking the compounds in the raw

Table 4 Summary of the phylogenetic analysis of the 16S rDNA sequences obtained from the selected clones from wastewater samples

Clones were assigned the shortcuts "D", "N," and "B" indicating the sample identity, which corresponds to denitrification, nitrification, and BOD removal reactors

Name of clone	Accession no.	Closest relative	Similarity (%)
1D	NC017174.1	*Clostridium difficile*	95
3D	NC017174.1	*Clostridium difficile*	96
10N	NC004557.1	*Clostridium tetani*	95
13N	NC009348.1	*Aeromonas salmonicida*	99
19N	NC012472.1	*Bacillus cereus*	99
33B	NC010947.1	*Stenotrophomonas maltophilia*	100
34B	NC014355.1	*Candidatus Nitrispira defluvii*	99

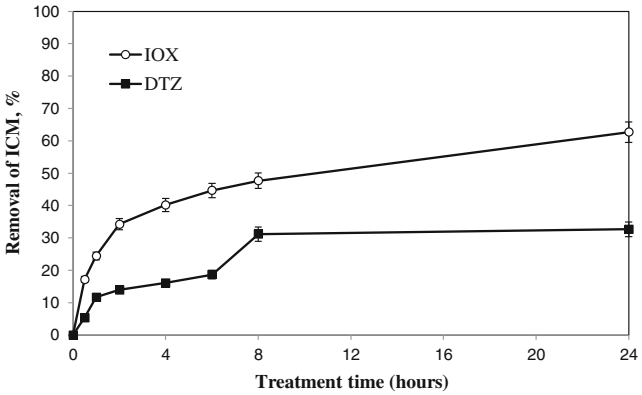


Fig. 3 Percentage removal of spiked IOX and DTX (in 10 mg/L) vs. treatment time

sewage storage tank of the reactor in four concentrations ranging from 0.1 to 20 mg/L.

Figure 4a, b illustrates the results for IOX and DTZ, respectively, obtained for 5 days of operation of the reactor. Five days was considered as a suitable time for the familiarization of the microorganisms in the reactor with these substrates. From Fig. 4, it is obvious that for IOX, during the first day of operation, and for all the concentrations tested, the system reached a removal level, which did not increase significantly during the subsequent 4 days. For the lower concentration (0.1 mg/L IOX), removal was increased only by 5 % from day 1 to day 5; for the highest concentration (20 mg/L IOX), the increase in the percentage removal was 7 % for the same period. A similar elimination efficiency was observed with the MBR treatment. MBR treatment showed only a low degradation capacity with the lowest value for IOX (5 %) with an average influent concentration of 2.32 μg/L (Köhler et al. 2012).

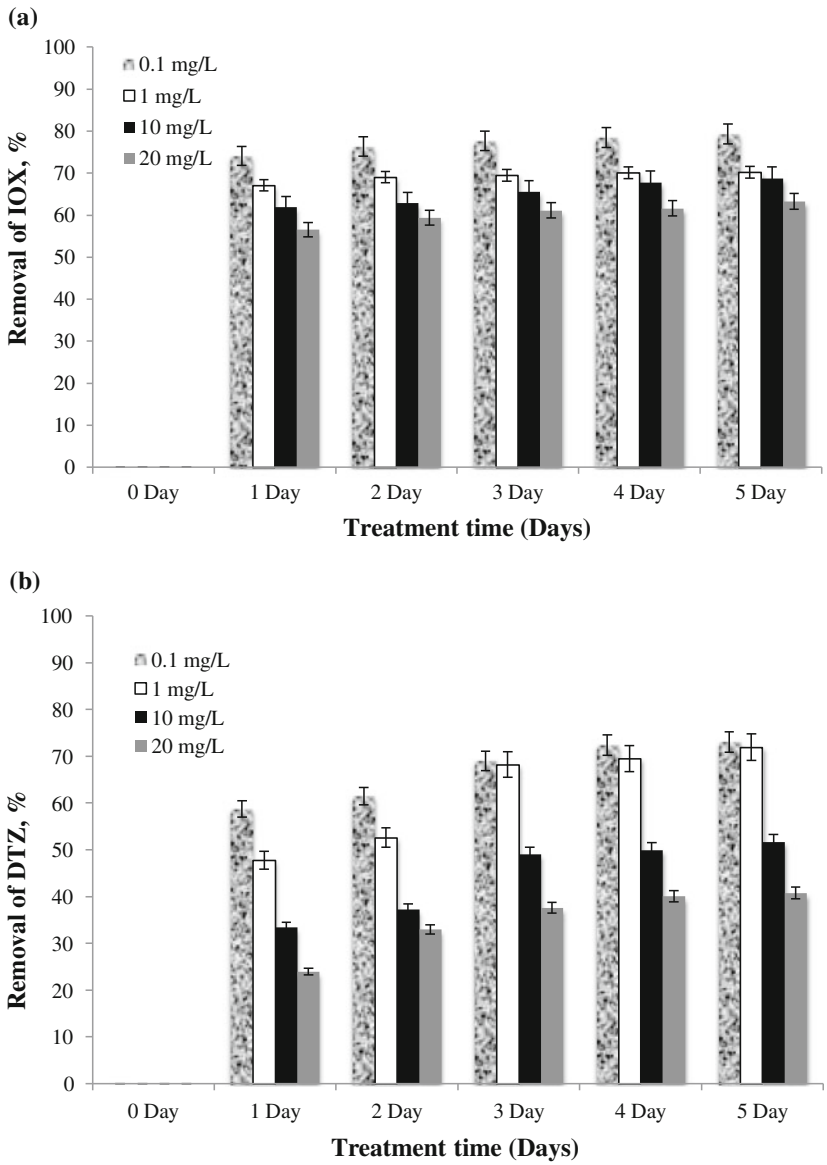


Fig. 4 Percentage removal of IOX (**a**) and DTZ (**b**) at concentrations 0.1, 1.0, 10, and 20 mg/L during 5 days of operation of the MBBR (continuous mode of operation)

The behavior of DTZ in the treatment reactor (Fig. 4b) was, however, different. From day 1 to day 5, the percentage of its removal, for initial concentrations of 0.1, 1, 10, and 20 mg/L, increased to 14, 24, 18, and 17 %, respectively.

Little is known about the elimination and biodegradability of ICM in the environment and especially about DTZ. Kalsch (1999) reported some degradation of DTZ in a non-standardized laboratory-scale batch system containing sediments. Aerobic degradation as well as binding to aerobic-activated sludge of DTZ for 54 h in fresh, unadapted sludge was negligible, suggesting that this substance is hardly retained in sewage treatment plants. Meanwhile, Haib and Kümmerer (2006) reported that DTZ degradation during the course of Zahn–Wellens test (ZWT) started between the 16th and the 23rd day. Determination of DTZ revealed that 83–88 % of the initial concentration of DTZ in the test vessel was found up to the 16th day.

According to the literature, generally, aerobic treatment (activated sludge) is shown to cause a poor reduction in ICM which were detected in influents such as iopromide. The results obtained for the investigated ICM indicated that there is no significant removal of this compound throughout the treatment. In fact, these compounds are designed to be highly stable, so they are not readily biodegradable (Carballa et al. 2004).

It should also be noted that for both compounds, the decrease of their concentration during the MBBR treatment is linearly related to their initial concentration, as can be seen in Fig. 5a, b. This confirms that the biodegradation of IOX and DTZ follows first-order reaction rate kinetics.

The results obtained clearly show that at lower initial ICM concentrations, the removal percentage is higher. Therefore, one could conclude that under real conditions, where the concentrations of such compounds are in the micrograms per liter range, the removal percentage will at least reach the removal percentages obtained in the present study (i.e., 79 % for IOX and 73 % for DTZ). As previously reported by Putschew et al. (2001) and Ternes and Hirsch (2000), the concentration levels of ICM frequently exceed 1 μg/L in both the raw influents and the final effluents. Median concentrations of some ICM were determined between 1.6 and 13 μg/L in the raw influents examined in this study, while the median concentration for DTZ and IOX was found to be higher than 3.3 μg/L.

Identification of BTPs

Biotransformation products of DTZ

In the present study, an attempt was made to elucidate the reaction pathways and mechanisms through the identification of BTPs, formed during the treatment of X-ray contrast media in a MBBR reactor performing in a continuous flow mode. To date, a limited number of studies exist with regard to the transformation products of ICM (Kalsch 1999; Haiss and Kümmerer 2006; Pérez and Barceló 2007; Jeong et al. 2010; Velo-Gala et al. 2012). In the framework of this study, UPLC-MS/MS was used to identify the possible transformation products that are generated during the MBBR treatment. Identification was based on the analysis of total ion chromatograms (TIC). Direct quantification of the biotransformation products of each X-ray contrast media was not feasible since reference standards are not available for these compounds.

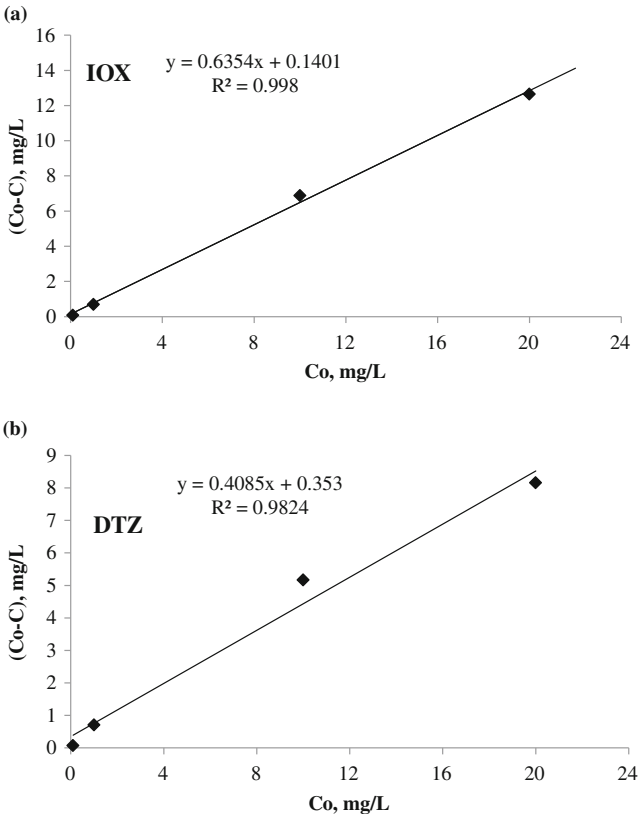
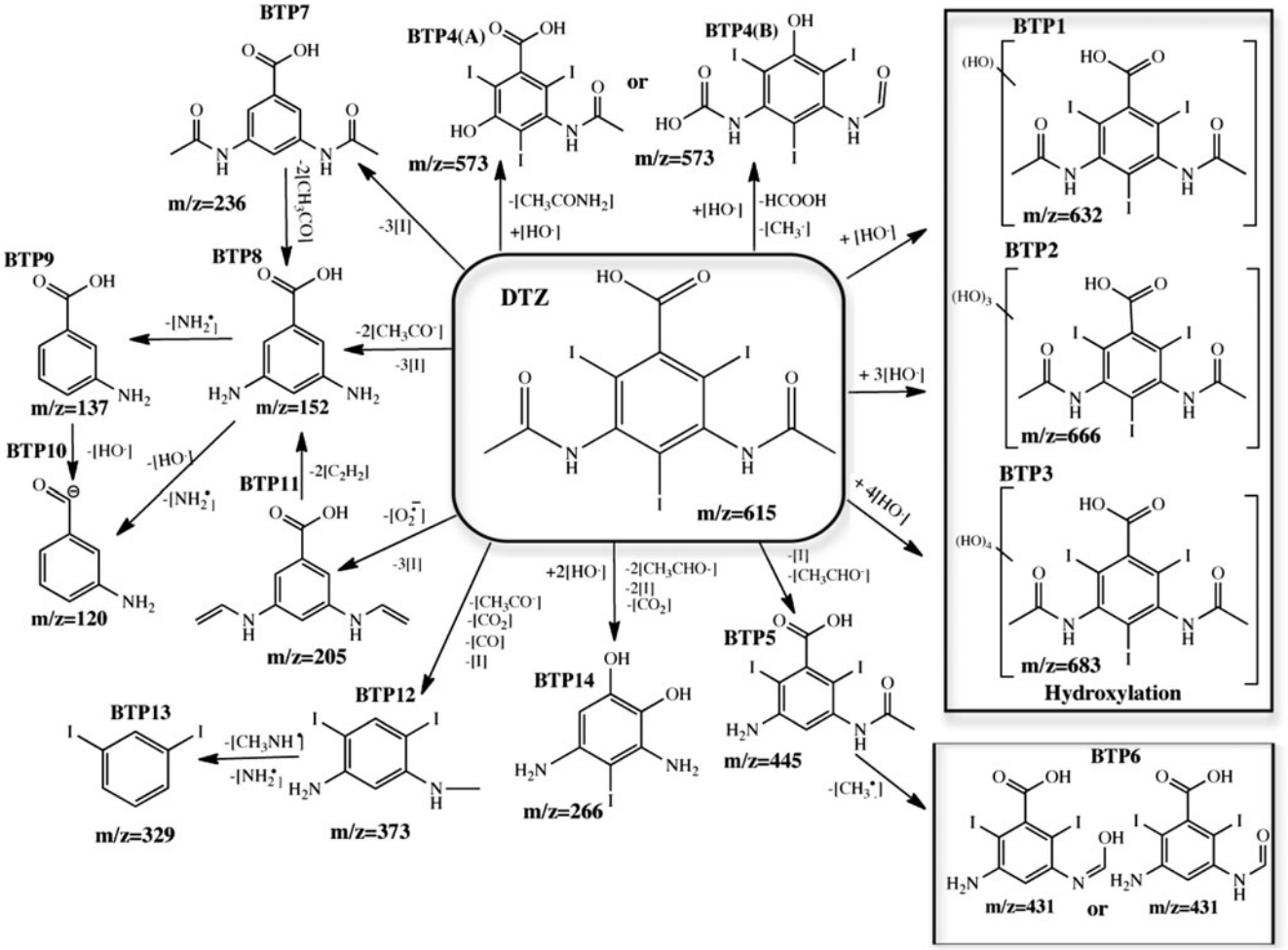


Fig. 5 Degradation kinetics of IOX (**a**) and DTZ (**b**)

Scheme 2 illustrates the postulated chemical structures and the exact mass values of the detected ions for the degradation products identified during the DTZ degradation process. A typical TIC of the mass spectra obtained for the biotransformation products after MBBR treatment of DTZ in the treated wastewater presents four peaks at about 0.5, 5, 5.8, and 6.3 min (referred to as peaks A, B, C, and D; ESM Fig. S1). As demonstrated, several main product ions with different *m*/*z* (with relative intensities (RIs) between 10 and100%) are observed in the average mass spectra of peaks A, B, C, and D, respectively (ESM Fig. S2). According to the chromatographic retention times, the biotransformation products, which correspond to peak B, are slightly more polar than the other BTPs, whereas the products with peaks C and D are slightly less polar.

Overall, as many as 14 compounds were tentatively identified as BTPs, with their peaks being >10 % of the highest



Scheme 2 Proposed reaction pathway for the biotransformation of DTZ

peak intensity, thus allowing the precise identification of BTPs; these are shown in Scheme 2 (BTP1–BTP14), along with the proposed reaction pathway of DTZ biodegradation. On the basis of the results presented in this study and also previous studies on the DTZ degradation, several competing pathways are suggested, in which dealkylation, hydroxylation, decarboxylation, deiodination, loss of amine functions, loss of acetyl functional group, and oxidation of hydroxyl groups, amongst others, are described as major transformation mechanisms (Kalsch 1999; Haib and Kümmerer 2006; Jeong et al. 2010; Velo-Gala et al. 2012).

Some of the findings in this study are in accordance with those obtained by previously reported studies in which the treatment of DTZ and other X-ray contrast media with different technologies were investigated and similar transformation mechanisms (e.g., deiodination) were observed (Hennebel et al. 2010; Jeong et al. 2010; Velo-Gala et al. 2012; Sugihara et al. 2013).

Based on previous reports, the main mechanism followed in the metabolic degradation of halogenated compounds in solution is radical chemistry (Mönig and Asmus 1984; Mao et al. 1991). As Mao et al. (1991) reported, support for the establishment of the radical mechanism has been provided by radiation chemical studies which revealed that the free radical chemistry of halogenated hydrocarbons in solution leads to the same products as the ones obtained after metabolic degradation of these compounds. Considering that the degradation products from free radical chemistry and metabolism are the same, it can be assumed that the underlying transformation mechanism in biological treatment does indeed involve radicals (Mönig and Asmus 1984; Mao et al. 1991). As previously reported in the literature, these radical species are assumed to play a significant role in the metabolism of halogenated compounds since they are highly reactive species. Although the product patterns in biological systems differ, depending on the concentration of oxygen in the environment, a distinction must be made accordingly between an anaerobic and an aerobic metabolism (Mönig and Asmus 1984). In particular, considerable interest has focused on halogenated organic radicals owing to the significant role that these species may play in biochemical reaction mechanisms associated with environmental problems (Mönig et al. 1983a, b).

Even though transformation mechanisms similar to those proposed by other studies are proposed herein, some biotransformation products have not been previously reported, indicating that a plethora of biotransformation products may occur depending on the experimental and analytical setup. Based on the literature, amongst the X-ray contrast media, DTZ can be transformed after advanced oxidation treatment such as TiO_2 photocatalysis, ozonation, gamma radiation, etc., whereas some other X-ray contrast media are more persistent (Steger-Hartmann et al. 2002; Hennebel et al. 2010; Seitz et al. 2008; Jeong et al. 2010; Velo-Gala et al. 2012; Sugihara et al. 2013). Furthermore, the presence of oxygen or another electron receptor (e.g., iodine ions) is necessary for X-ray contrast medium transformation. The structure of most BTPs of DTZ shows preservation of the core structure of the X-ray contrast media.

The results presented in Scheme 2 demonstrate that 10 of the 14 proposed BTPs (BTPs 1–5, 7–8, 11–12, and 14) retain both the nitrogen atoms of the parent compound compared to the other BTPs, which have been formed from the cleavage of one and/or two nitrogen atoms from the main structure of DTZ, in accordance with the biotransformation pathway of DTZ. Therefore, the elucidation of these possible structures of BTPs could also result in the removal of few carbon atoms from the hydrocarbon side chains.

In this study, BTP1, BTP2, and BTP3 are some of the major biotransformation products formed after the treatment, which may be attributed to the hydroxylation of DTZ. Several isobaric compounds (polyhydroxylated compounds), corresponding to positional isomers, may be formed. However, differentiation of positional isomers was not always feasible based solely on the UPLC-MS/MS data. More specifically, the biodegradation of DTZ could lead to the formation of mono-hydroxylated (BTP1, m/z632), trihydroxylated (BTP2, m/z666), and tetra-hydroxylated (BTP3, m/z683) products, such as HO-DTZ, 3-HO-DTZ, and 4HO-DTZ, respectively.

Two major analogues of DTZ were formed during the MBBR treatment: BTP4(A) and BTP4(B) 573 m/z, RI=15–50 %. The formation of BTP4(A) may be attributed to the addition of a hydroxyl radical on the aromatic ring at one of the amide positions and the elimination of the amide side chain (Sugihara et al. 2013), while BTP4(B) can be generated by the combination of demethylation, loss of the molecule of formic acid, and, finally, hydroxylation. The results demonstrate that at least one BTP4(A) was formed, which possessed iodine.

The formation of BTP5 (RI=15–50 %) with m/z value 445 could be attributed to the onefold deacetylation and subsequent loss of one atom of iodine from the DTZ structure, while BTP6 with 431 m/z (RI=20–40 %) may be formed after demethylation of the amide group of BTP5.

In the present study, the deiodination step is one of the biotransformation mechanisms observed during the MBBR treatment. The last deiodination step appeared to be the most difficult since the BTP with no iodine atom was only recovered after 4 days of treatment. The fully dehalogenated compound was highly likely the main end product since no other peaks of the other BTPs with one or two iodine atoms could be detected. As reported by Hennebel et al. (2010), the fully deiodinated product is expected to be more biodegradable in the environment due to lower sterical hindrance. LC-MS/MS analysis indicated the formation of BTP7 (m/z236, RI=15–100 %) in experimental conditions, which differed from the parent compound mass only in the number of iodine substituents. The deiodination of DTZ is a process which may involve, in the primary step, the transfer of one electron from enzyme to halogen, leading to the formation of radicals. As previously reported (Haggblom 1990; Kalsch 1999), the carbon–iodine bond is rather unpolar and chemically less stable and, as a result, the aerobic cleavage of iodinated aromatics by bacterial dioxygenases is possible. As Mohn and Tiedje (1992) reported, deiodination is a process which includes reductive dehalogenation at low redox potential, especially under methanogenic conditions, and is frequently observed. More specifically in this reaction, the carbon and halogen involved in the bond have been recognized as electron acceptors of anaerobic electron transport chains (Kalsch 1999).

In addition to the assigned product ions, three further BTPs—BTP8 (152 m/z), BTP9 (137 m/z), and BTP10 (120 m/z)—were observed. BTP8 can be formed through three reaction routes of DTZ→BTP8, DTZ→BTP7→BTP8, and DTZ→BTP11→BTP8. BTP8 can be directly formed by the combination of deidionation and deacetylation of DTZ. BTP8 can also be generated through twofold deacetylation of BTP7. For this reason, BTP7 can be regarded as the promoter for the formation of BTP8 (m/z152). The final elucidation route for the generation of the BTP8 can be attributed to the dealkylation of BTP11 (m/z205). More specifically, the 152 m/z value may indicate the possibility of a loss of two molecules of acetylene from BTP11.

MBBR treatment of DTZ can lead to the formation of two other BTPs, i.e., BTP9 and BTP10 with m/z values of 137 and 120, respectively. As shown, BTP9 (m/z137) may be formed by the loss of a onefold side amine moiety from BTP8 and BTP10 after dehydroxylation from BTP9 and after the loss of NH_3 and hydroxyl radical from BTP8, respectively.

Another analogue, BTP11 (m/z205, RI=10–30 %), may be formed following iodide liberation through C-I fragmentation and losing $O_2^{\cdot-}$ moiety from the amide groups. Subsequent DTZ modification at positions C-1 and C-4 of the aromatic ring (decarboxylation and deiodination) and loss of a carbonyl moiety and acetyl group at the two amide side groups can lead to the formation of its analogue, BTP12 (m/z373, RI=20–70 %), whose further reduction can yield

BTP13 (RI=15–40 %). BTP113 may be formed due to cleavage at positions C-3 and C-5 of the BTP12 structure, giving a 2,6-diiodobenzoate derivative.

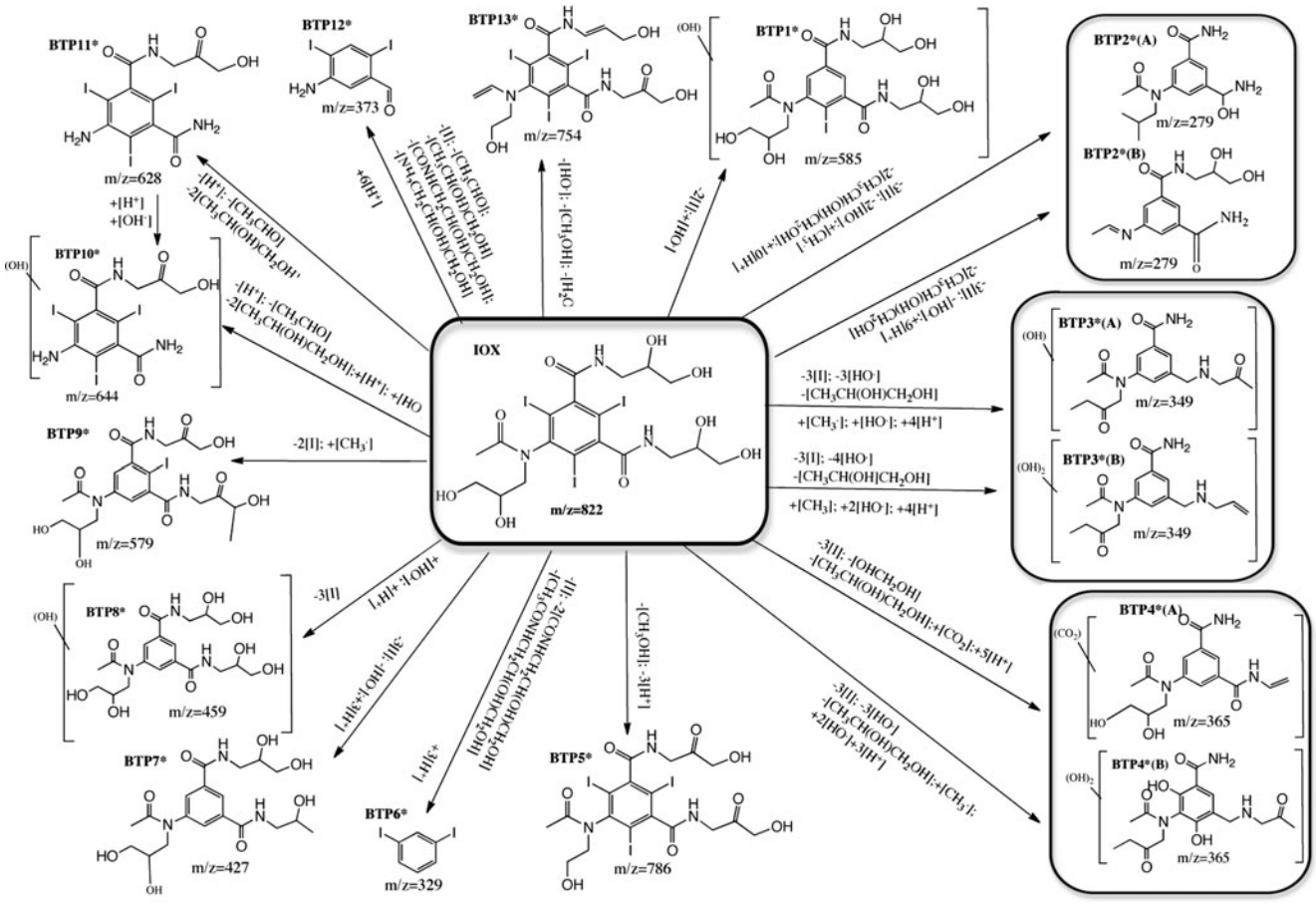
Finally, according to the elucidation of the DTZ pathway, BTB14 (*m/z*266, RI=25–60 %) contains multiple structural changes consistent with the oxidation of the amide side chains, together with dehalogenation and decarboxylation processes. As Jeong et al. (2010) reported in their study, an electrophilic aromatic substitution mechanism wherein the ipso addition of HO at the carboxylate moiety followed by decarboxylation leads to a phenolic product (BTP14). More specifically, BTP14 (*m/z*266), a hydroxylated analogue, created from DTZ after the substitution of iodine (C-2) by water or hydroxyl anion through C-I fragmentation, was isolated during the MBBR treatment.

Biotransformation products of IOX

Thirteen compounds were tentatively identified as BTPs formed during the MBBR treatment in treated effluents of IOX (822 [M+H] *m/z*). The precise identification of the BTPs of IOX has been elucidated based on their peaks, which are >10 % of the highest peak intensity. A typical TIC of the mass spectra and the average mass spectra obtained for the BTPs of IOX in the treated wastewater are presented in ESM Figs. S3 and S4, respectively. Scheme 3 (BTP1*–BTP13*) presents the proposed reaction pathway of IOX biodegradation during the MBBR treatment.

As in the case of DTZ, on the basis of the data presented in this paper, several competing pathways are suggested in which hydroxylation, deacetylation, deiodination, loss of amine functions, and loss of alcohol functional groups, amongst others, are described as major transformation mechanisms. It should be noted that most of the proposed biotransformation products generated during the treatment maintained the aromatic ring IOX structure and that major changes primarily occurred in the substituents at positions C-1, C-3, and C-5 of the IOX structure. More specifically, on the basis of the results and Scheme 3 presented in this study, two major pathways are suggested as the major transformation mechanisms, which are deiodination and hydroxylation.

As previously reported for both DTZ and IOX, several BTPs are formed (BTP2*(A)–(B), BTP3*(A)–(B), BTP4*(A)–(B), BT6*, and BTP7*) through the deiodination step. In parallel to the deiodination, hydroxylation reactions were



Scheme 3 Proposed reaction pathway for the biotransformation of IOX

also demonstrated by the presence of protonated molecules. BTP1* (*m*/*z*585), BTP3*(A)–(B) (*m*/*z*349), BTP4*(B) (*m*/*z* 365), and BTP8* (*m*/*z*458) are the major biotransformation products formed during the deiodination–hydroxylation mechanisms.

Subsequent hydroxyl radical addition could lead to the formation of mono-hydroxylated (BTP1*, *m*/*z*585; BTP3* (A), *m*/*z*349; BTP8*, *m*/*z*459; and BTP10*, *m*/*z*644) and bi-hydroxylated (BTP3*(B), *m*/*z*349 and BTP4*(B), *m*/*z*365) products. As shown in Scheme 3, several isobaric compounds corresponding to positional isomers were detected. However, differentiation of positional isomers was not feasible based solely on the UPLC-MS/MS data. More specifically, BTP1* (*m*/*z*585, RI=30–100 %) can be formed through the loss of two atoms and the subsequent addition of a hydroxyl radical in the structure of IOX.

Subsequently, MBBR treatment of IOX can lead to the formation of two other analogues of IOX, i.e., BTP2*(A) and BTP2*(B) with *m*/*z* value of 279. As shown, BTP2*(A) (*m*/*z*329, RI=15–30 %) may be formed by the combination of deiodination, loss of two hydroxyl radicals, loss of two groups of $C_3H_8O_2$ at two of the amide positions (C-1 and C-3), and, finally, addition of one methyl group. BTP2*(B) (RI=20–30 %) may be formed after deiodination, loss of two $C_3H_8O_2$ at positions C-3 and C-5 of the aromatic group, and dehydroxylation from the IOX.

As previously referred to, BTP3*(A) (*m*/*z*349, RI=10–60 %) and BTP3*(B) (*m*/*z*349, RI=10–45 %) are two of the hydroxylated products which were formed during the MBBR treatment. As shown, both the formation of BTP3*(A) and BTP3*(B) can be generated by deiodination, loss of $C_3H_8O_2$, dehydroxylation, and then by further addition of a methyl group. Furthermore, one and two hydroxyl radicals were added, resulting in the formation of BTP3*(A) and BTP3*(B), respectively. BTP4*(B) (*m*/*z*365, RI=15–55 %) resulted from the loss of some alcohol functional groups (i.e., $CH_3CH(OH)CH_2OH$, $OHCH_2OH$) on the IOX structure and then by a further addition of a molecule of carbon dioxide, forming a carboxylate derivative.

One additional BTP with *m*/*z* value 786 (BTP5*; RI=10–70 %) was observed. The 786 *m*/*z* ion could be formed from the loss of CH_3OH. Another biotransformation product, BTP6* (*m*/*z*329, RI=10–30 %), may be formed following iodide liberation, through C-I fragmentation, and losing two groups of $C_4H_9O_3N$ and $C_5H_{10}O_3N$ at positions C-1, C-3, and C-5 of the aromatic ring, respectively. The formation of BTP8* (RI=25–100 %) with *m*/*z* value 459 could be attributed to the deiodination and subsequent hydroxylation from the IOX structure, while BTP7* with 429 *m*/*z* (RI=20–40 %) may be formed after dehydroxylation of BTP8*.

Two major analogues of IOX were formed during the MBBR treatment: BTP9* (*m*/*z*579, RI=20–80 %) and BTP10* (*m*/*z*644, RI=15–50 %). The species with *m*/*z*579 (BTP9*) corresponds to the loss of two atoms of iodine and parallel addition of one methyl group in the IOX. BTP10* can be formed through IOX and BTP11*. For this reason, BTP11* can be regarded as the promoter for the formation of BTP10* (*m*/*z*644). Actually, BTP10* can be generated through the loss of two molecules of $CH_3CH(OH)CH_2OH$ and one molecule of acetaldehyde with subsequent hydroxylation. The second reaction route which is followed is DTZ→BTP10*→BTP9*. More specifically, the 644 *m*/*z* value may indicate possible hydroxylation from BTP11*. The formation of BTP11* (RI=10–30 %) with *m*/*z* value 628 could be attributed to the onefold deacetylation and subsequent loss of two groups of $C_3H_8O_2$ from the IOX structure.

Subsequent IOX modification at positions C-1, C-3, and C-5 of the aromatic ring (loss of $C_4H_9O_3$ (C-1), $C_3H_9O_2N$ (C-3), and $C_3H_8O_2$ and deacetylation (C-5)) and loss of an atom of iodide could lead to the formation of its analogue, BTP12* (*m*/*z*373, RI=20–70 %). Finally, according to the elucidation of the IOX pathway, BTP13* (*m*/*z*754, RI=25–60 %) was created from IOX after substitution of a molecule of water, loss of a hydroxyl radical, and loss of an alcohol group (CH_3OH) at the amide group at position C-5 of the aromatic ring.

The toxicity of the treated effluent was also examined. No significant differences were observed in the immobilization of *D. magna* between the control (treated effluent without IOX and DTZ) and the spiked treated effluent (using the highest concentration of 20 mg/L of IOX or DTZ tested).

In order to determine the toxicity of the matrix alone, a set of control toxicity tests was performed by exposing *D. magna* to the wastewater samples. The control tests did not show any toxicity to *D. magna* after 24 and 48 h of exposure; after 24 h of exposure, the immobilization was 8.3± 0.6 %, while after 48 h the immobilization of daphnids reached 14±1.5 %.

A higher toxicity to *D. magna* for 48 h compared to 24 h of exposure of the treated wastewater already spiked with 20 mg/L IOX was observed, where immobilization for 24 h of exposure was 10±0.5 % and for 48 h of exposure was 17.5±1.3 %. The immobilization, however, for DTZ was around the same level for both exposure times (i.e., 12.5± 1.0 % for 24 h and 12.5±0.6 % for 48 h).

Conclusions

The MBBR technology was proven to be a technology able to sufficiently remove substances belonging to the ICM group, such as IOX and DTZ, from urban wastewaters without the formation of toxic biodegradation products.

The amount of clones sequenced in this study do not reveal the entire bacterial diversity present; nevertheless,

they provide an indication of the predominant phylotypes inhabiting the study site. To our knowledge, this is the first analysis of the diversity of bacteria in an MBBR treatment plant for the treatment of pharmaceuticals like ICM.

The optimum removal values achieved were 79 % for IOX and 73 % for DTZ, whereas a large number of BTPs were tentatively identified (13 and 14 BTPs for IOX and DTZ, respectively). For both DTZ and IOX, on the basis of the data presented in this paper, several competing pathways are tentatively suggested, in which hydroxylation, deacetylation, deiodination, loss of amine functions, and loss of alcohol functional groups, amongst others, were determined as the major transformation mechanisms. The toxicity of the treated effluent tested to *D. magna* showed no statistical difference compared to that without the addition of the two ICM, indicating, hence, the absence of toxicity generated by the biologically transformed products.

Acknowledgments This work has been implemented within the framework of the project UPGRADING/DURABLE/0308/07, "Fate, Effect and Removal Potential of Xenobiotics Present in Aqueous Matrices (IX-Aqua)" and NIREAS-International Water Research Center activities (project NEA IPODOMI/STRATH/0308/09), both co-funded by the Republic of Cyprus and the European Regional Development Fund through the Research Promotion Foundation of Cyprus.

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