Journal of Environmental Chemical Engineering 6 (2018) 3242–3248

Contents lists available at ScienceDirect

Journal of Environmental Chemical Engineering

journal homepage: www.elsevier.com/locate/jece

Diclofenac biodegradation by newly isolated *Klebsiella* sp. KSC: Microbial intermediates and ecotoxicological assessment

Kyriakos Stylianou[a], Evroula Hapeshi[b], Marlen I. Vasquez[a], Despo Fatta-Kassinos[b], Ioannis Vyrides[a,*]

[a] Cyprus University of Technology, Department of Environmental Science and Technology, 95 Irinis Str., P.O. Box 50329, 3603, Lemesos, Cyprus
[b] Nireas-International Water Research Center and Civil and Environmental Engineering Department, University of Cyprus, P.O. Box 20537, 1678 Nicosia, Cyprus

ARTICLE INFO

Keywords:
Biodegradation
Biotransformation
Diclofenac
Ecotoxicity assay
Klebsiella sp.

ABSTRACT

The presence of diclofenac, a frequently used analgesic drug in the environment, can be harmful to a variety of organisms like algae, crustaceans and fish. In this study, a bacterial strain of *Klebsiella* sp. KSC (Gen Bank, accession number KX500307) able to biodegrade high concentration of diclofenac was isolated from livestock soil and identified. The exposure of *Klebsiella* sp. KSC to 70 mg/L of diclofenac resulted in diclofenac mineralization after 72 h. This is the first study that points out substantial biodegradation of high concentration of diclofenac (70 mg/L) in less than 72 h; and this shows the potential of this strain to be bioaugmented in contaminated sites or to bioreactors. The chemical structure of twelve biotransformation products of diclofenac are proposed. Its transformation pathway may involve hydroxylation, dehydroxylation, decarboxylation and dechlorination of the central ring of diclofenac. Acute ecotoxicity assay with *Vibrio fischeri* test showed that the main biotransformation product (TP298) of diclofenac by *Klebsiella sp*. KSC was less toxic than the parent compound.

1. Introduction

Diclofenac (2-{2-[(2,6-dichlorophenyl)amino]phenyl}acetic acid) is a regularly used analgesic, antiarthritic and antirheumatic non-steroidal anti-inflammatory drug, whose global consumption quantities of 940 t per year [1]. Diclofenac has a low removal efficiency ranging from 5 to 81% in conventional sewage treatment [2–5], and has been found in wastewater treatment plant effluents in many countries worldwide like Germany [6], Spain [7], and Canada [8].

Diclofenac is considered as a contaminant of emerging concern. It has been included in the list of priority hazardous compounds [9]. Diclofenac has been found to be toxic at the low μg/L levels in chronic exposures of different organisms like fish and mussels, as reviewed in Vasquez et al. [10]. Interestingly, a permanent shift of the microbial community composition of a wastewater treatment bioreactor was observed after an exposure to diclofenac at 50 pg/L [11]. It has also been found to influence the nature of a fluvial bacterial community at the 10–100 μg/L range [12].

The advanced technologies for diclofenac treatment such as advanced oxidation and membrane-based technologies can remove diclofenac at a higher rate compared to biological treatment. However, their high operational cost and the potential formation of toxic transformation products (for advance oxidation technologies) is a serious drawback [13]

Quintana et al. [14] used fresh activated sludge as inoculum with 20 mg/L of diclofenac as the only carbon source in phosphate buffer under aerobic condition and no transformation was obtained for diclofenac under these conditions over 28 days. The addition of external carbon source in order to achieve co-metabolism did not have any positive effect on diclofenac biodegradation either.

Tran et al. [15] in a review regarding biodegradation of emerging contaminants stated that diclofenac is poorly removed by bacterial consortia (i.e. mixed culture/activated sludge). The study of Bouju et al. [16] found that 40% diclofenac was removed after 18 days of biological MBR biomass batch incubation. The analyses of metabolites have shown that the hydroxylation of diclofenac to 4′-hydroxydiclofenac was identified as one major bottleneck in diclofenac biotransformation [16]. Gröning et al. [17] showed under aerobic conditions in a fixed-bed column bioreactor that biofilms of river sediment partially mineralised diclofenac to a stable end product 5-hydroxydiclofenac-pbenzoquinone imine.

Recently, Facey et al. [18] used forest soil and found that diclofenac 100 mg/L could be biodegraded in less than 10 days. The aforementioned studies [16–18,14], however, used a mixed culture during

* Corresponding author.
E-mail address: ioannis.vyrides@cut.ac.cy (I. Vyrides).

https://doi.org/10.1016/j.jece.2018.04.052
Received 5 November 2017; Received in revised form 11 April 2018; Accepted 19 April 2018
Available online 26 April 2018
2213-3437/

biodegradation of diclofenac and no information was pointed out regarding the microbial population and the dynamics of the tested soil. Therefore, one of this work's objective was to isolate the microorganisms that can biodegrade diclofenac as a sole carbon source. This, apart from fundamental knowledge can have a potential in practical applications; by adding the microorganisms (diclofenac utilizers) in environment or system can enhance diclofenac biodegradation.

Recently, Bessa et al. [19] has isolated from activated sludge the strain *Brevibacterium* sp. D4 that was able to biodegrade 35% of 10 mg/L of diclofenac as a sole carbon source in 30 days. Aissaoui et al. [20] has also isolated from activated sludge the *Enterobacter hormaechei* D15 strain that was able to biodegrade 52.8% of diclofenac as a sole carbon source in 48 h.

In another recent study, Moreira et al. [13] used *Labrys portucalensis* F11 and found 70% biotransformation of diclofenac (10 mg/L) in 30 days. This strain was previously isolated from industrial contaminated cited and can biodegrade fluorobenzene as a sole carbon source [21].

However, up to date, no study has attempted to isolate bacteria from environmental contaminated sites that can biodegrade substantially higher concentration of diclofenac as a sole carbon compared to other studies. The potential microbial isolate strain can point out fundamental aspect regarding diclofenac metabolites during its biodegradation. Also, it can be used for bioremediation in soil or aquatic environments and/or it can be bioaugmented in bioreactors or in biological systems in WWTPs.

Therefore the objectives of this study was (i) to isolate and identify bacteria capable to biodegrade high concentration of diclofenac (70 mg/L) in relatively short time (ii) to investigate diclofenac transformation pathways, and (iii) to assess the ecotoxicity of biotransformation products of diclofenac.

2. Materials and methods

2.1. Isolation of pure strain from environmental samples

Samples were collected from various sources such as from soil exposed to livestock in Aradipou area (Cyprus), sludge from a lake (Paralimni lake, Cyprus) and from surface water from a dam (Liopetri, Cyprus). The aforementioned samples could have been potentially exposed to recalcitrant compounds from various sources and therefore microbial population able to diclofenac biodegradation could have been potentially developed. The samples (5 g) were used as an inoculum for the enrichment culture, which was cultivated in a sterilized mineral salts medium (MSM) modified as follows: (0.5 g/L $(NH4)_2SO_4$, 0.5 g/L K_2HPO_4, 0.2 g/L $MgCl_2$, 0.007 g/L $CaCl_2{\cdot}H_2O$, 0.5 g/L NaCl, (chemicals were purchased from Sigma–Aldrich). Enrichment of microorganisms was carried out in 100 ml flasks with 50 ml working volume, which were supplemented with 0.1 g/L of diclofenac (from ethanol stock solution) and inoculated with 5 g of sample under sterile conditions for the initial activation of the pollutant degraders. The samples were incubated for a period of 7 days at 30 °C, at pH 7 and 100 rpm shaking. The isolation and characterization of diclofenac degrading bacteria was performed by the removing 0.1 ml sample from each flask that exhibited high turbidity, and then serially diluted by a factor of 10^{-1} to 10^{-10}. The diluted samples were plated by the streak method on the MSM medium containing agar and 0.1 g/L diclofenac. The inoculated Petri dishes were incubated for 7 days at 30 °C. Following the incubation period, single colonies were picked and re-cultivated in MSM agar media following the same procedure as stated above. This procedure was repeated four times until pure identical colonies were observed.

2.2. Strain characterization by 16S rRNA sequence analysis

DNA extraction was done by exposing the microbial pellet to 1% Sodium Dodecyl Sulfate (SDS) (Sigma–Aldrich) and 0.2 M NaOH (Sigma–Aldrich) solutions for 15 min Drakou et al. [22]. The primers 27 F and 1492 R were used to amplify the 16S rRNA region. Sequencing products were resolved by Macrogen (the Netherlands) on an Applied Biosystems model 3730 XL automated DNA sequencing system (Applied BioSystems, USA).The 16S rRNA gene nucleotide sequences of isolated strain were analysed using BLAST (http://ncbi.nlm.nih.gov/BLAST).

2.3. Phylogenetic tree diagram

Alignment for phylogenetic analysis of 16S rDNA was made by using Clustal W. Phylogenetic tree was constructed using the neighbour joining method with MEGA 6.06 software. Bootstrapping analysis for 10000 replicates was performed to estimate the confidence of tree topologies. The analysis involved 9 nucleotide sequences.

2.4. Biodegradation of diclofenac

A 49 ml MSM growth medium (pH 7) placed in a 100 ml sterilized bottles and 1 ml of diclofenac was added (from a stock solution in ethanol) as a sole carbon source so the final diclofenac concentration was 70 mg/L. The bottles were placed in 30 °C and 100 rpm and the initial OD600 of inoculated microorganism was 0.07. Experiments were performed in duplicate under sterile conditions and control assays were set under the same conditions with no microorganism's inoculation. Samples were taken at 6 h, 18 h, 28 h, 48 h and 72 h. Cell growth was monitored spectrophotometrically (PerkinElmer Lambda 25 UV/VIS Spectrometer) by measuring the optical density at 600 nm in the batch experiments. A preliminary screening of diclofenac concentrations was also done by absorbance measurement at 278 nm with a calibration curve recorded between 1 and 30 mg/L ($R^2 = 0.99$). The samples were diluted in order to be within the calibration curve. At the 72 h, a UV scan of the samples from 240 to 340 nm took place for monitoring of the diclofenac pick at 278 nm or any other pick that could be form.

2.5. Acute toxicity testing with Vibrio fischeri

The toxicity of diclofenac and the biotransformation products created by the isolated strain was evaluated. Nine sterilized bottles (100 ml) were filled with 50 ml of MSM growth medium. All bottles were placed in a shaking incubator in which the conditions were stabled (32 °C and 100 rpm). Samples were taken at 0 h (immediately after addition of the diclofenac), 6 h, 24 h and 72 h, filtered through 0.22 μm, pH adjusted and kept at −20 °C until analysis. The bioluminescent marine bacteria *V. fischeri* were used for acute toxicity testing as described elsewhere [23]. The results are expressed as the inhibition percentage of the bioluminescence of the bacteria. The variability of this assay was in the range of ± 10% deviation.

2.6. UPLC–MS/MS analysis

Samples were taken from serum bottles at 0 h, 6 h, 24 h and 72 h, filtered through 0.22 μm and kept at −20 °C until analysis. Solid phase extraction (SPE) for pre-concentration of target analytes in samples was carried out using Oasis HLB cartridges (3 cc, 60 mg) from Waters Corporation (Milford MA, USA) Gros et al., 2009. Analyses of biotransformation products of diclofenac were performed by Ultra Performance Liquid Chromatography coupled with mass spectrometry on an ACQUITY TQD UPLC–MS/MS system (Waters) using a method specifically developed for this application. A triple quadrupole mass spectrometer TQD (serial number QBA012) coupled with electrospray ionization (ESI) source running in positive ion (PI) mode was used for the detection of analysis. Data acquisition was performed with MassLynx™ software.

For the identification of biotransformation products at first, a full scan mode (range of m/z 50–1000) and a selected ion recording (SIR)

mode at cone voltage of 50 V were performed. Additionally a multiple-reaction monitoring mode (MRM) were used to aquire Mass (MS) spectra to confirm the biotransformation products based on the observed fragmentations.

In this framework of the study, several analytical data such as information about MS/MS fragmentation pattern and the exact mass values of the protonated molecule [M + H] + which was selected as the precursor ion for the collision-induced dissociation fragmentation to give MS2 product were used for the most accurate determination of the unknown structures of the formed TPs. For the further MSn analysis the ion with the highest relative abundance in intensity was chosen.

Chromatographic separation was carried out using BEH Shield RP C18 column (50 mm × 2.1 mm I.D., particle size 1.7 μm) (Waters). An optimized gradient was used at a constant flow rate of 0.3 ml/min using water + 0.1% formic acid (solvent A) and methanol (solvent B). The gradient elution was: 0 min, 5% B; 0–2 min linear from 5 to 30% B; 2–3 min, 50% B; 3–5 min, 70% B; 5–7 min, 95% B; 7–9 min linear from 95 to 5% B. The temperature of column was T_{column} = 40 °C and run time 9 min.

3. Results and discussion

3.1. Isolation and characterisation of bacteria

During the isolation procedure diclofenac was provided at a higher concentration (70 mg/L) compare to the concentrations found at the environment in order to avoid any substrate deficiency and to select the strains that diclofenac can be used as a sole carbon source.

After three months of enrichment, only 3 pure colonies were found to grow well in the Petri dish with 100 mg/L of diclofenac as the sole carbon source. Then, the ability of individual colonies to degrade 70 mg/L of diclofenac at liquid culture was tested and only 1 of the 3 strains grew well in 70 mg/L after 72 h. The initial source of the tolerant isolated strain to diclofenac (KSC) was from soil exposed to livestock in Aradipou area. The selected nucleotide sequence of KSC (1418 bp) was analysed for bacterial species homology through the NCBI database by BLASTn tool analysis. Following a comparison of the strain's homology with that of other microorganisms in the NCBI database, a 99.8% homology with *Klebsiella oxytoca* sp. CAV1752 was found. 16S rRNA sequences of isolated strain of *Klebsiella* sp. KSC was submitted to Gen Bank and the accession number KX500307 was given. Phylogenetic tree (Fig. 1) was constructed with a reliability branch percentage value of more than 70%. *Citrobacter freundii* ATCC 8090 (NR_028894.1) as well as *Escherichia coli* (J01859.1) were used as outgroups to root the tree. According to obtained phylogenetic tree, the sequences of 16S rRNA of the strain *Klebsiella* sp. KSC has a high level reliability (94%) and forms a single cluster and possesses 99% sequence similarity with the corresponding nucleotide sequence of the strain *K. oxytoca* JCM1665 (NR_112010.1).

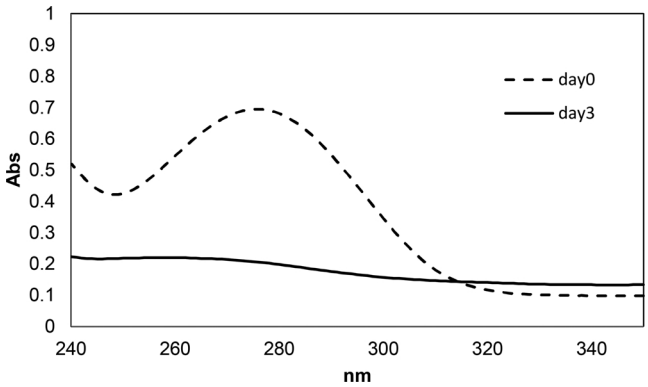


Fig. 2. UV absorbance of diclofenac at day 0 (broken line) and after the inoculation with Klebsiela sp. KSC at day 3 (continuous line).

3.2. Biodegradation of diclofenac by pure Klebsiela sp. KSC

During the first 28 h a slight reduction of the concentration of diclofenac was detected by the isolated *Klebsiela* sp. KSC. However, after 28 h the absorbance at 278 nm significantly decreased and at 72 h it was almost eliminated. As can be seen from Fig. 2 no pick is detected at 278 nm after 72 h, indicating a significant reduction of the core compound. A UPLC–MS/MS analysis took place in order to point out the microbial intermediates and the final products.

3.3. Biotransformation products of diclofenac during Klebsiela sp. KSC biodegradation

Table 1 presents the proposed twelve biotransformation products of diclofenac (i.e. TP144, TP177, TP254, TP259, TP294, TP312, TP310, TP328, TP298, TP282, TP286 and TP301) with a peak height greater than 15% the highest peak intensity. Most of the biotransformation products of diclofenac demonstrate preservation of the core of diclofenac structure (i.e. TP254, TP294, TP312, TP310, TP328, TP298, TP282, TP286 and TP301). Hydroxylation may be the driving mechanism to form TP328, TP298, TP286 and TP312. This is in accordance with previous reports, in which hydroxylation and decarboxylation of the aromatic rings of diclofenac was observed [24]; Sargado et al., 2013. According to Facey et al. [18], one of the biodegradation mechanisms, which is observed at diclofenac, is the a bioactivated hydroxylation by the hepatic cytochrome P450 enzymes in humans and animals, producing several hydroxylation biotransformation products such as TP312 and TP328 [25–28,18]. It is significant to mention that the TP312, TP328 and TP310 are reported by Facey et al. [18]. As a result of these mechanisms, alcohols and ketones may be formed. More specifically, the biodegradation of diclofenac could lead to the formation of mono-hydroxylated compounds (HO-diclofenac, i.e. TP312, TP254, T177 and TP144), di-hydroxylated compounds (2-HO-diclofenac, i.e. TP328 and TP259), tri-hydroxylated product (3-HO-

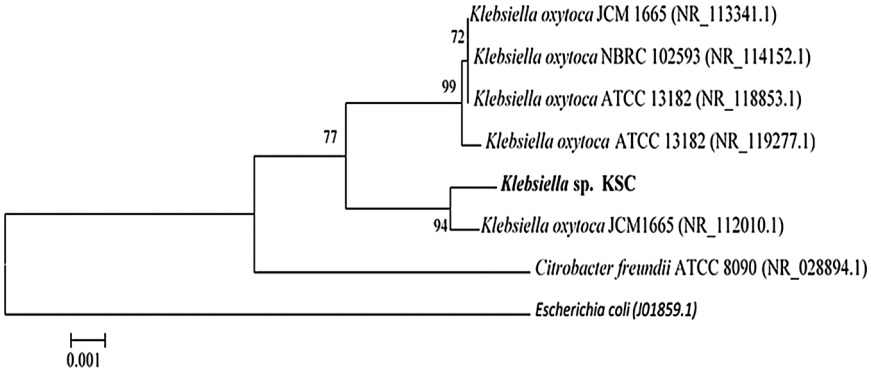
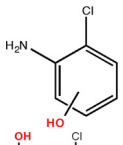
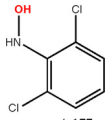
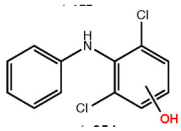
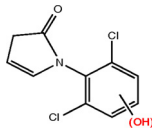
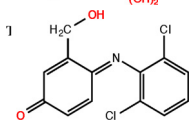
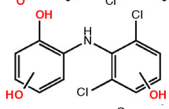
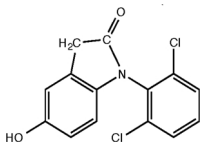
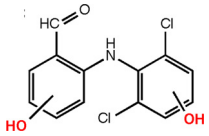
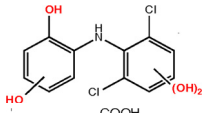
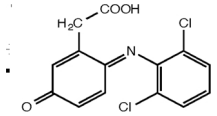
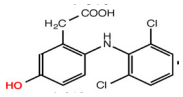
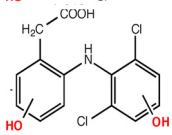


Fig. 1. Phylogenetic position of new isolated *Klebsiella* sp. KSC. The evolutionary history was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA 6.

Table 1
Identification of the transformation products of diclofenac.

Compound	*m/z*	Structure
TP144	144	
TP177	177	
TP254	254	
TP259	259	
TP282	282	
TP286	286	
TP294	294	
TP298	298	
TP301	301	
TP310	310	
TP312	312	
TP328	328	

diclofenac, i.e. TP298 and TP286) and tetra-hydroxylated compound (4-HO-diclofenac, i.e. TP301). TP298 can be formed through the joint removal of a carboxyl group and two hydrogens from the diclofenac and addition of three hydroxyl groups to the parent compound. TP286 can originate from the detachment of the acetate group from the structure of the parent substance and subsequently tri-hydroxylation of the diclofenac. TP282 may occur by the concurrent elimination of carboxylic group, dihydroxylation and dehydrogenation of the core molecule. TP294 may be obtained due to (i) the sequential loss of hydroxyl group and cyclization between the carboxyl group and the atom of nitrogen and (ii) addition of hydroxyl group at the benzene ring of diclofenac. TP259 can be formed through the reaction pathway diclofenac → TP294 → TP259, through the elimination of the C_4H_6O group and hydroxylation of the di-chlorobenzene. Loss of acetate group of the aromatic ring of diclofenac, as well as the addition of the hydroxyl group at the structure of diclofenac can lead to the formation of TP254. TP301 (*m/z* 301) may be possibly formed from the elimination of the acetic acid group and the addition of four hydroxyl groups or through the reaction diclofenac → TP286 → TP301 in which a hydroxyl group is added to the benzene moieties of TP286. TP177 and TP144 may be generated by the cleavage of the non-chlorinated aromatic ring. More specifically, TP177 may be formed by the cleavage between the amine group and the benzene ring followed by the addition of the hydroxyl moiety at the atom of nitrogen. TP144 can result from the cleavage between the amine atom and the aromatic ring, the elimination of the chlorine group and subsequently addition of a hydroxyl group. TP312 may be formed by the addition of hydroxyl group to the aromatic ring. It is considered as one of the major metabolites of the oxidative diclofenac metabolism in humans [28]; [26] and one of the main transformation products during the (sono)photocatalysis of diclofenac [28]. TP310 can result from dehydrogenation of the hydroxyl and amine group of TP312. Cytochromes P450 (CYP) are the most significant genes involved in the hydroxylation of diclofenac in humans [29] and are known to be produced by bacteria as well [30].

Fig. 3(A), shows the fragmentation spectrum of diclofenac (*m/z* 296) which presents the characteristic fragment ions at *m/z* 278, *m/z* 250 and *m/z* 215 which are obtained due to the (i) sequential loss of hydroxyl group and cyclization between the methyl group and the nitrogen moiety, (ii) loss of the carboxyl group and, (iii) loss of carboxyl group and chlorine from the central ring of diclofenac. The biotransformation products were generated through the combination of several mechanisms such as hydroxylation, decarboxylation and dechlorination. It should be noted that this study clearly demonstrates that all the biotransformation products of diclofenac are chlorinated compounds, showing that the complete reduction of chlorine moiety is difficult to occur.

Fig. 3(B) shows the fragmentation pattern of TP301. This MS/MS pattern confirmed the postulated structure of TP301 showing the different fragments at *m/z* 254 and *m/z* 205, which correspond to the: (i) loss of an acetic acid moiety and subsequently addition of a hydroxyl group, and (ii) loss of a chlorine group and one acetic acid group. According to the formation ratio of the detected TPs of ERM based on the area of the peaks of the chromatograms

Fig. 4 illustrates the evolution of the formation ratio of the main biotransformation products of diclofenac based on the area of the peaks of the chromatograms. The amount of each biotransformation product is expressed as a percentage of the peak area obtained over the concentration of diclofenac. After 72 h of treatment, the range of the estimated transformation ratio of diclofenac and its biotransformation products ranged between 5 and 90%. Diclofenac has shown incomplete removal within the 72 h treatment. TP298 was the dominant intermediate product over the other detected biotransformation products. It appears after 6 h of treatment appeared while its concentration continuously decreases until 72 h of treatment. TP177 was found at constant concentration from the start until the end of treatment reaction. In contrast, the concentrations of the other biotransformation products were reduced but remained steady until the end of the treatment.

3.4. Acute toxicity assessment with bioluminescent bacteria

As presented in Fig. 5, diclofenac was severely toxic to *Vibrio fischeri* at the concentrations tested (mg/L range) both 5 and 15 min exposure time. The same trend was observed in the control experiments (MSM + diclofenac) of 24 and 72 h incubation, indicating that diclofenac is not transformed by other abiotic factors during the experiment.

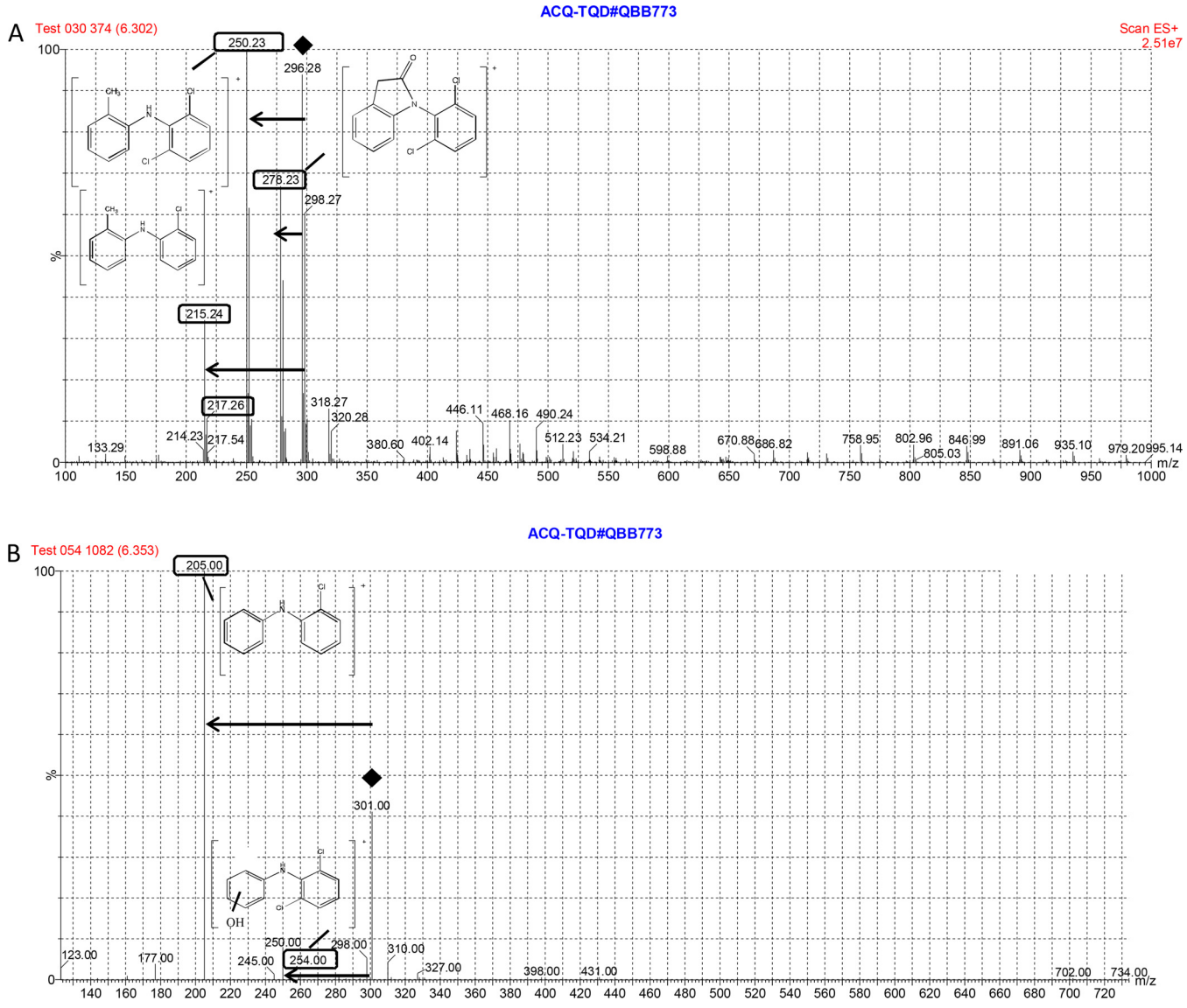


Fig. 3. (A) Product ion mass spectra (MS/MS spectrum) of the diclofenac and (B) Product ion mass spectra (MS/MS spectrum) of the TP301.

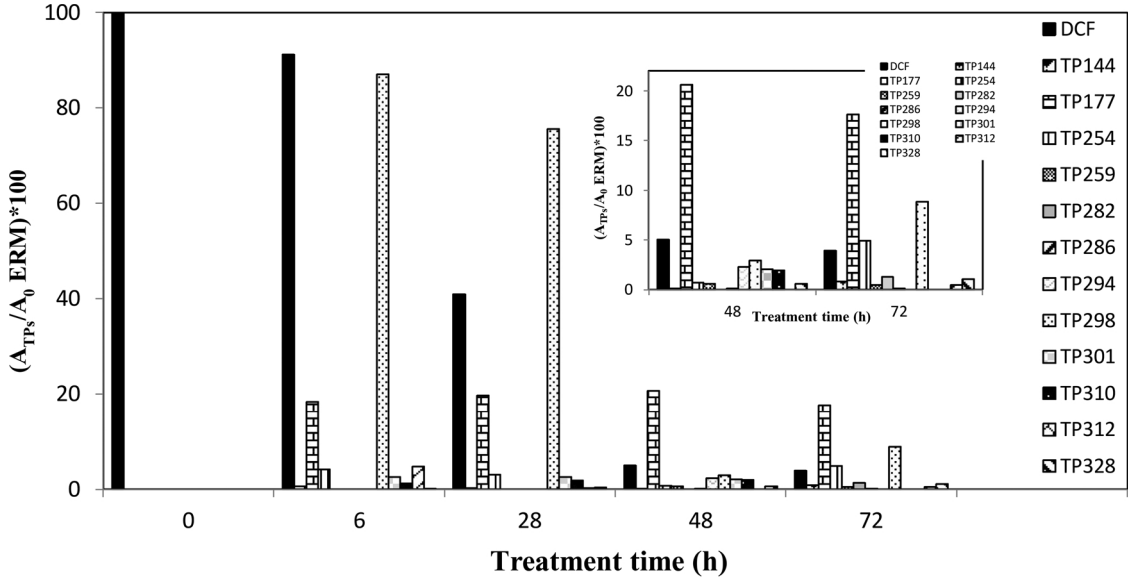
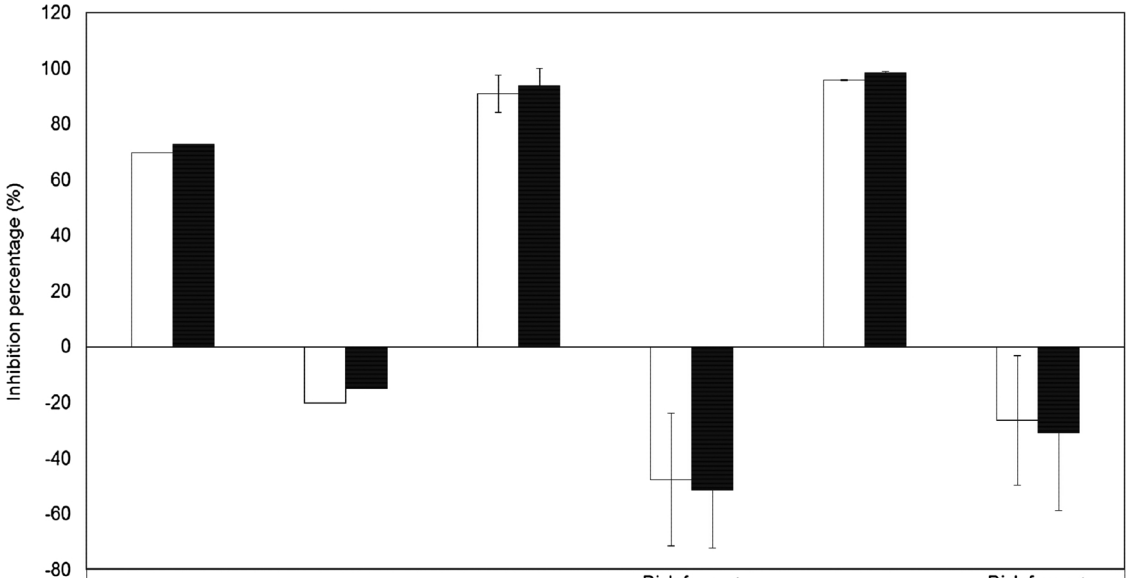


Fig. 4. The formation ratio based on the area of the chromatographic peaks of the identified biotransformation products during the transformation of diclofenac.



	Diclofenac (0 h)	Diluent	Diclofenac (24 h)	Diclofenac + Klebsiela sp. KSC (24 h)	Diclofenac (72 h)	Diclofenac + Klebsiela sp. KSC (72 h)
□5 min	69,6	-20,4	90,8	-47,8	95,7	-26,5
■15 min	72,8	-15,2	93,7	-51,3	98,4	-30,9

Fig. 5. Toxicity of diclofenac and diclofenac to *Vibrio fischeri* at the concentrations tested (mg/L range) both 5 and 15 min exposure time.

The bioluminescence inhibition of the control experiments ranged between 69 and 98%. The incubation of diclofenac with the isolated strain *Klebsiela sp. KSC* resulted to the biodegradation of diclofenac to non-toxic/hormetic compounds both at 24 and 72 h. The bioluminescence inhibition of *V. fischeri* after a 5- and 15-min exposure time to the samples incubated for 24 h (MSM + diclofenac + microorganisms 24 h) was −47.8% and −51.3%, respectively. Greater bioluminescence inhibition was observed when the incubation period was 72 h with −26.5% (5 min) and −30.9% (15 min), inhibition percentage. This is an indication that the reaction intermediates stimulated the microbial growth. The differences between exposure times (5 and 15 min) and incubation periods (24 and 72 h) are not statistically significant. The diluent (ethanol) at the concentrations used in this study was not found to cause toxic effects.

4. Conclusions

Soil and sludge samples from various environmental sources in Cyprus such as livestock, lake and dam were screened for microbial isolates able to biodegrade high concentration of diclofenac (70 mg/L). The strain able to biodegrade diclofenac (70 mg/L of diclofenac in 72 h) was isolated from livestock soil and was identified as *Klebsiella* sp. KSC (Gen Bank, accession number KX500307). This is the first study that points out an environmental isolate strain able to biodegrade high concentration of diclofenac (70 mg/L) in less than 72 h and this shows the potential of this strain to be bioaugmented in contaminated sites or to bioreactors. The main degradation pathways of diclofenac appear to involve hydroxylation, decarboxylation and partly dechlorination of the compound. The *V. fischeri* test showed that the biotransformation products of diclofenac by *Klebsiella sp*. KSC, were not able to cause toxic effects in contrast to diclofenac.

References

[1] D. Domaradzka, U. Guzik, D. Wojcieszyńska, Biodegradation and biotransformation of polycyclic non-steroidal anti-inflammatory drugs, Rev. Environ. Sci. Biol. Technol. 14 (2015) 229–239.
[2] Y. Luo, W. Guo, H.H. Ngo, L.D. Nghiem, F.I. Hai, J. Zhang, S. Liang, X.C. Wan, A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment, Sci. Total Environ. 473 (2014) 619–641, http://dx.doi.org/10.1016/j.scitotenv.2013.12.065.
[3] N. Paxeus, Removal of selected non-steroidal anti-inflammatory drugs (NSAIDs), gemfibrozil, carbamazepine, b-blockers, trimethoprim and triclosan in conventional wastewater treatment plants in five EU countries and their discharge to the aquatic environment, Water Sci. Technol. 50 (5) (2004) 253–260.
[4] N. Kreuzinger, M. Clara, B. Strenn, H. Kroiss, Relevance of the sludge retention time (SRT) as design criteria for wastewater treatment plants for the removal of endocrine disruptors and pharmaceuticals from wastewater, Water Sci. Technol. 50 (2004) 149–156.
[5] Y. Zhang, S.U. Geißen, C. Gal, Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies, Chemosphere 73 (2008) 1151–1161.
[6] D. Stülten, S. Zühlke, M. Lamshöft, M. Spiteller, Occurrence of diclofenac and selected metabolites in sewage effluents, Sci. Total Environ. 405 (2008) 310–316.
[7] R. Rosal, A. Rodríguez, J.A. Perdigón-Melón, A. Petre, E. García-Calvo, M.J. Gómez, et al., Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation, Water Res. 44 (2010) 578–588.
[8] L. Lishman, S.A. Smyth, K. Sarafin, S. Kleywegt, J. Toito, T. Peart, B. Lee, M. Servos, M. Beland, P. Seto, Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada, Sci. Total Environ. 367 (2006) 544–558.
[9] European Commission, Proposal for a Directive of the European Parliament and of the Council Amending Directives 2000/60/EC and 2008/105/EC as Regards Priority Substances in the Field of Water Policy, (2012), pp. 1–35.
[10] M.I. Vasquez, I. Michael, K. Kümmerer, D. Fatta-Kassinos, Bioassays currently available for evaluating the biological potency of pharmaceuticals in treated wastewater, Wastewater Reuse and Current Challenges, Springer, Cham, 2016, pp. 49–80.
[11] T. Kosjek, E. Heath, B. Kompare, Removal of pharmaceutical residues in a pilot wastewater treatment plant, Anal. Bioanal. Chem. 387 (2007) 1379–1387, http://dx.doi.org/10.1007/s00216-006-0969-1.
[12] T. Backhaus, T. Porsbring, A. Arrhenius, et al., Single-substance and mixture toxicity of five pharmaceuticals and personal care products to marine periphyton communities, Environ. Toxicol. Chem. 30 (2011) 2030–2040, http://dx.doi.org/10.1002/etc.586.
[13] I.S. Moreira, V.S. Bessa, S. Murgolo, C. Piccirillo, G. Mascolo, P.M. Castro, Biodegradation of Diclofenac by the bacterial strain Labrys portucalensis F11, Ecotoxicol. Environ. Saf. 152 (2018) 104–113.
[14] J.B. Quintana, S. Weiss, T. Reemtsma, Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor, Water Res. 39 (2005) 2654–2664.
[15] N.H. Tran, T. Urase, H.H. Ngo, J. Hu, S.L. Ong, Insight into metabolic and cometabolic activities of autotrophic and heterotrophic microorganisms in the biodegradation of emerging trace organic contaminants, Bioresour. Technol. 146 (2013) 721–731.
[16] H. Bouju, P. Nastold, B. Beck, J. Hollender, P.F.X. Corvini, T. Wintgens, Elucidation of biotransformation of diclofenac and 4′ hydroxydiclofenac during biological

wastewater treatment, J. Hazard. Mater. 301 (2016) 443–452.
[17] J. Gröning, C. Held, C. Garten, et al., Transformation of diclofenac by the indigenous microflora of river sediments and identification of a major intermediate, Chemosphere 69 (2007) 509–516, http://dx.doi.org/10.1016/j.chemosphere.2007.03.037.
[18] S.J. Facey, B.A. Nebel, L. Kontny, M. Allgaier, B. Hauer, Rapid and complete degradation of diclofenac by native soil microorganisms, Environ. Technol. Innov. 10 (2018) 55–61.
[19] V.S. Bessa, I.S. Moreira, M.E. Tiritan, P.M.L. Castro, Enrichment of bacterial strains for the biodegradation of diclofenac and carbamazepine from activated sludge, Int. Biodeterior. Biodegrad. 120 (2017) 135–142.
[20] S. Aissaoui, H. Ouled-Haddar, M. Sifour, K. Harrouche, H. Sghaier, Metabolic and co-metabolic transformation of diclofenac by enterobacter hormaechei D15 isolated from activated sludge, Curr. Microbiol. 74 (3) (2017) 381–388.
[21] M.F. Carvalho, R. Ferreira Jorge, C.C. De Marco, P. De Marco, P.M. Castro, Isolation and properties of a pure bacterial strain capable of fluorobenzene degradation as sole carbon and energy source, Environ. Microbiol. 7 (2) (2005) 294–298.
[22] E.M. Drakou, M. Koutinas, I. Pantelides, M. Tsolakidou, I. Vyrides, Insights into the metabolic basis of the halotolerant Pseudomonas aeruginosa strain LVD-10 during toluene biodegradation, Int. Biodeterior. Biodegrad. 99 (2015) 85–94.
[23] M.I. Vasquez, D. Fatta-Kassinos, Is the evaluation of traditional physicochemical parameters sufficient to explain the potential toxicity of the treated wastewater at sewage treatment plants? Environ. Sci. Pollut. Res. Int. 20 (2013) 3516–3518, http://dx.doi.org/10.1007/s11356-013-1637-6.
[24] L.A. Perez-Estrada, S. Malato, W. Gernjak, A. Aguera, E.M. Thurman, I. Ferrer, A.R. Fernandez-Alba, Photo-fenton degradation of diclofenac: identification of main intermediates and degradation pathway, Environ. Sci. Technol. 39 (2005) 8300–8306.
[25] R. Webster, M. Pacey, T. Winchester, P. Johnson, S. Jezequel, Microbial oxidative metabolism of diclofenac: production of 4(-hydroxydiclofenac using *Epicoccum nigrum* IMI354292, Appl. Microbiol. Biotechnol. 49 (1998) 371–376.
[26] S. Shen, Davis MR. Marchick, G.A. Doss, L.R. Pohl, Metabolic activation of diclofenac by human cytochrome P450 3A4: role of 5-Hydroxydiclofenac, Chem. Res. Toxicol. 12 (1999) 214–222.
[27] J.S. Van Leeuwen, G. Vredenburg, S. Dragovic, T.F.J. Tjong, J.C. Vos, N.P.E. Vermeulen, Metabolism related toxicity of diclofenac in yeast as model system, Toxicol. Lett. 200 (2011) 162–168.
[28] I. Michael, A. Achilleos, D. Lambropoulou, V. Osorio Torrens, S. Perez, V. Petrovic, D. Barcelo, D. Fatta-Kassinos, Proposed transformation pathway and evolution profile of diclofenac and ibuprofen transformation products during (sono)photocatalyis, Appl. Catal. B: Environ. 147 (2014) 1015–1027.
[29] R. Bort, K. Macé, A. Boobis, et al., Hepatic metabolism of diclofenac: role of human CYP in the minor oxidative pathways, Biochem. Pharmacol. 58 (1999) 787–796, http://dx.doi.org/10.1016/S0006-2952(99)00167-7.
[30] A.W. Munro, H.M. Girvan, K.J. McLean, Cytochrome P450–redox partner fusion enzymes, Biochim. Biophys. Acta (BBA)- Gen. Subj. 1770 (3) (2007) 345–359.

Further reading

T. Testing, Toxicity Testing “sensitive, rapid & cost effective” Applications of the Microtox System, Fisheries (Bethesda) (1988).