Assessment of Biodegradability of Diclofenac under Methanogenic Conditions

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Abstract
Diclofenac is a recalcitrant non-steroidal anti-inflammatory drug detected in the environmental samples. The biodegradation potential of diclofenac was assessed with three mixed fermentative/methanogenic cultures enriched from contaminated sediments and anaerobic digestion sludge of a municipal wastewater treatment plant. Glucose and methanol served as the electron and carbon source. Diclofenac concentration in both liquid and solid phases were determined by solid phase extraction (SPE) followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Although diclofenac partially biodegraded in all enrichment cultures, complete degradation was not observed. Extraction of diclofenac from the solid phase in the sludge samples reflected that less than 6% of the diclofenac was sorped on the solid phase and the rest was either biotransformed (25-40%) or remained in the liquid phase (55-70%). The results show that diclofenac did not readily biodegraded under anaerobic conditions. Thus, additional precautions are required in wastewater treatment plants to prevent the mitigation of diclofenac as well as other similar micropollutants into the receiving bodies.

Keywords
Anaerobic biodegradation; non-steroidal anti-inflammatory drugs; pharmaceuticals; sediment

INTRODUCTION

Diclofenac is one of the most commonly used non-steroidal anti-inflammatory drugs (NSAIDs) (Calasan et al., 2011). Due to its high consumption and poor degradability, in many cases diclofenac is not completely eliminated in wastewater treatment plants (Zorita et al., 2009; Fatta-Kassinos et al., 2011). Diclofenac can therefore be detected in receiving waters, sediments and sludges (Langford et al., 2011; Kunkel and Radke, 2012). Diclofenac has been of environmental concern due to not only the potential threats to drinking water sources but also the potential harmful effects on non-target organisms at environmentally relevant concentrations (Cleuvers, 2004; Oaks et al., 2004; Gros et al., 2010). There is a lack of information on the fate of diclofenac under anaerobic conditions typically encountered in water-saturated soils and freshwater sediments. The objective of the research reported here was to assess the reductive biotransformation of diclofenac in a mixed, methanogenic culture derived from sediments and anaerobic digestion sludge.

MATERIALS AND METHODS

Chemicals and materials

Diclofenac sodium salt (98%; CAS 15307-79-6) and diclofenac-d_4 were purchased from Sigma-Aldrich (Steinheim, Germany) and C/D/N Isotopes Inc. (Canada), respectively. All chemicals used for the preparation of solutions and chemical analyses were of high purity. All glassware used in the experiments were soaked in methanol, rinsed with Milli-Q water and baked at 450°C for 1 hour.

Cultures Development

Two enrichment cultures were developed from sediment samples taken from Alibeykoy Creek (Sediment A) and Kagithane Creek (Sediment B) in Istanbul, Turkey. Another enrichment culture

was also developed from an anaerobic digestion sludge of a municipal wastewater treatment plant located in Istanbul, Turkey. Diclofenac were not detected in the sediment samples used as inoculum in this study (detection limit 5 ng/g). The cultures were initiated by diluting 80 g of the sediment or 200 mL digestion sludge in 2 L of mineral media (Okutman Tas and Pavlostathis, 2007) in a N_2-flushed, 3 L glass flask reactor, capped with a Teflon-lined stopper. At the beginning of each 7 day feeding cycle, glucose, yeast extract, and diclofenac in methanol were added resulting in initial concentrations of 300 mg/L, 17 mg/L, 10 µg/L, and 53 mg/L, respectively. The fresh media was added to the reactor by wasting 333 mL culture from the completely mixing reactor every two weeks. The cultures were kept in the dark in a 22°C constant temperature room and were stirred once a day. The pH of the cultures were kept around 7 with $NaHCO_3$ addition.

The microbial activity of the cultures was monitored by measuring gas production, gas composition, pH, Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), Volatile Fatty Acids (VFAs), Total Organic Carbon (TOC) and Dissolved Organic Carbon (DOC) concentrations.

Instrumental analyses

Diclofenac. Quantification of diclofenac was performed with a LC-MS/MS (Thermo Accela UPLC coupled with Thermo Quantum Access tandem MS, USA). Liquid samples were purified and concentrated using OASIS HLB SPE cartridges (200 mg, 6 cc) (Waters, Millford, MA, USA). Cartridges were preconditioned with 10 mL acetonitrile, 5 mL methanol, and 5 mL water, respectively. Samples were filtered from cartridges at 5 mL/min flowrate under vacuum control and washed with 5 mL water. Cartridges were dried for 1.5 hours under the N_2 stream. Diclofenac adsorbed onto cartridges were eluted by using 2 mL acetonitrile and 2 mL methanol, respectively. Solvents were evaporated under N_2 stream (TurboVap II, Caliper LifeSciences) to dryness. Diclofenac was re-dissolved in 1 mL methanol:water (10:90) and quantified with LC-MS/MS. Values given for the samples correspond to the average value of two aliquots for each sample.

Gas composition. Methane and carbondioxide concentrations were determined by a GC unit (Agilent Technologies, Model 7890A) equipped with two columns and two thermal conductivity detectors.

RESULTS AND DISCUSSION

The characterization results of the sediment and the digestion sludge samples were provided in Table 1.

Table 1. Characterization results of sediment and anaerobic digestion sludge samples

	pH	Total Solids (%)[a]	Volatile Solids (%)[a]	Total Organic Carbon (mg/g)[a]	Diclofenac ng/g[a]
Sediment A	7.4	63±0.6	4.6±0.3	29.1	< 5
Sediment B	7.2	52±2	6.8±0.1	42.3	< 5
Anaerobic Sludge	7.4	4	35	35	< 5

[a] dry weight basis

In all the enrichment cultures, acetate concentration was higher than that of propionate and both VFAs were almost completely consumed at the end of a feeding cycle (Figure 1). The steady-state pH of the enrichment cultures were 7.2±0.5. The CH_4 and CO_2 partial pressures were 0.73 and 0.27; 0.72 and 0.28; 0.71 and 0.29; for the cultures derived from Sediment A, Sediment B, and anaerobic digestion sludge, respectively. The theoretical feeding concentration profiles and measured concentration of diclofenac before every feeding cycle during the culture enrichment are shown in Figure 2. Although microbial transformation, direct or cometabolic, is considered to be the most important elimination process, particularly for polar acidic pharmaceuticals (Quintana et al., 2005),

diclofenac was degraded in some extend in all of the enrichment cultures. Complete degradation was not observed in none of the reactors. The observed biodegradation of diclofenac in the enriched cultures ranged between 25-40%.

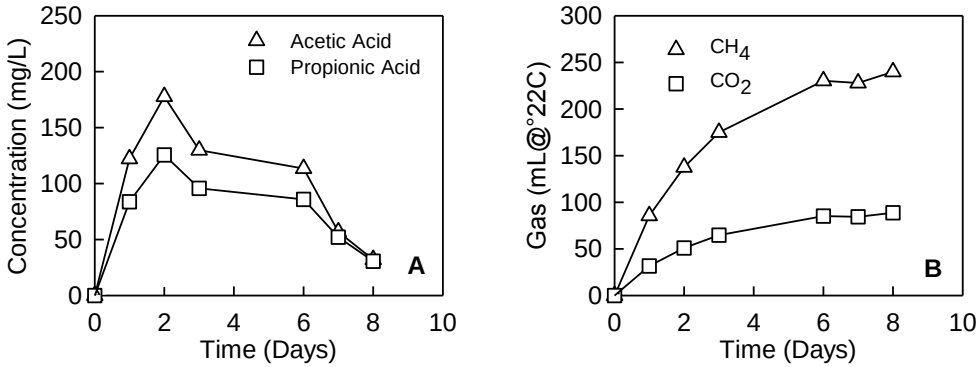

Figure 1. VFAs production and consumption (A), as well as cumulative methane and carbon dioxide production (B) in the culture derived from Sediment A for a feeding cycle.

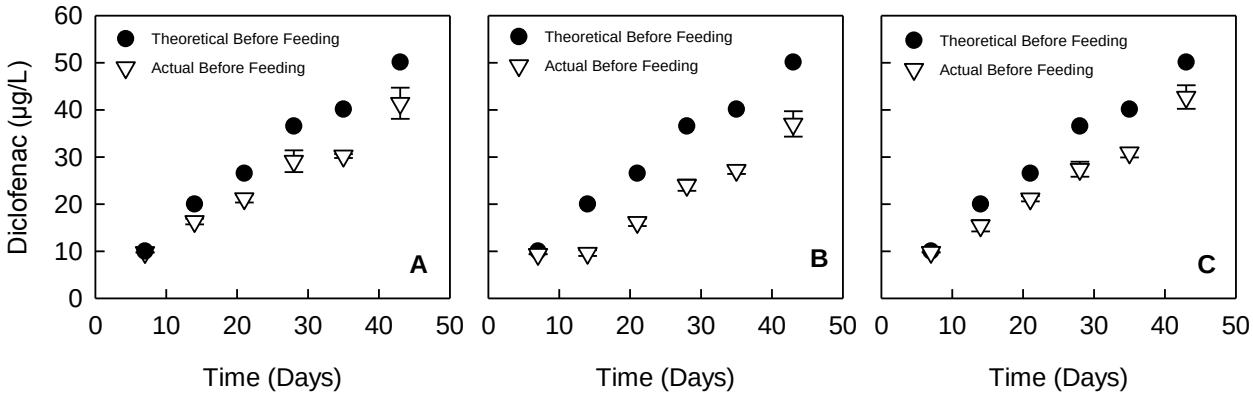

Figure 2. Time course of diclofenac in enrichment cultures; (A) culture derived from Sediment A; (B) culture derived from sediment B; (C) culture derived from anaerobic digestion sludge (multiple feeding cycles) (error bars represent mean values ± one standard deviation).

In the reactors, the mass balance in terms of the diclofenac removal should be carefully investigated. Although some of the diclofenac can be eliminated by direct microbial degradation, the eliminated amount due to sorption on the sludge should not be underestimated. Therefore, in order to quantify complete biodegradation, it is important to determine also the amount of diclofenac absorbed on the sludge. In this study, quantification of the diclofenac in the sludge phase was also performed in the samples taken and mass balances in the reactors were provided in Figure 3. In the enrichment cultures developed from the sediments, 1 and 2 % of the diclofenac introduced to the reactors were sorbed by the solid phase. The sorption of the diclofenac on the solid phase increased to 6% in the culture developed from anaerobic digestion sludge. The increase in sorption is related to the organic matter content of the reactor. The ratio of VSS/SS in the cultures enriched from the Sediment A and Sediment B were 0.23 and 0.12, respectively. Whereas, the biomass content of the culture developed from anaerobic digestion sludge was higher than the sediment derived cultures and the ratio of VSS/SS was 0.50. The sorption of diclofenac on the solids depends basically on its physico-chemical properties, such as lipophilicity or acidity. However, even in the presence of the high biomass content, the sorption of the diclofenac was not the predominant mechanism. Similar to our results, Kimura et al. (2007) reported that the sorption is not the dominant mechanism in the elimination of the acidic pharmaceuticals (i.e., diclofenac) in biological wastewater treatment systems.

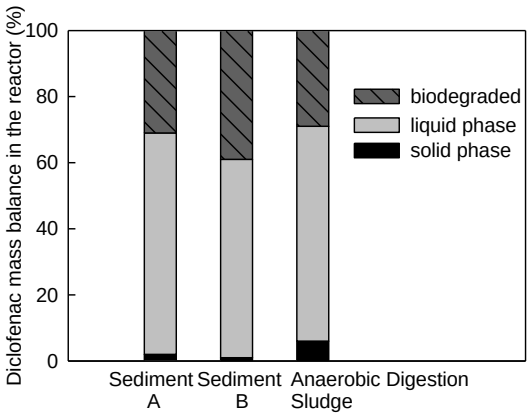

Figure 3. Mass balances in the enrichment cultures reflecting the biodegradation and sorption potential of diclofenac

CONCLUSIONS

The results of this study indicate that diclofenac is not completely removed through biodegradation processes and may reach sediments and subsurface environments. As such, these findings have significant environmental implications in terms of the fate of diclofenac in receiving environments. Taking into account that diclofenac present in wastewater, appear to be not readily biodegradable, enhancing its removal in the wastewater treatment plants is an important strategy to minimize the negative effects on the environmental systems. Therefore, cautions should be taken to eliminate the release of diclofenac and similar micropollutants through wastewater treatment plants. Further elucidation of alternative wastewater treatment processes is necessary in order to completely understand and improve the degradation and/or removal of recalcitrant micropollutants.

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REFERENCES

Calasan, J. Mijatovic, V., Horvat, O., Varga, J., Sabo, A. Stilinovic, N. 2011 The outpatient utilization of non-steroidal anti-inflammatory drugs in South Backa District, Serbia. *Int J. Clin Pharm* **33**, 246-251.

Cleuvers, M. 2004 Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. *Ecotoxicol. Environ. Safe* **59**(3), 309-315.

Fatta-Kassinos, D., Hapeshi, E., Achilleos, A., Meric, S., Gros, M., Petrovic, M., Barcelo, D. 2011 Existence of pharmaceutical compounds in tertiary treated urban wastewater that is utilized for reuse applications. *Water Resour. Manage*. **25**, 1183-1193.

Gros, M., Petrović, M., Ginebreda, A., Barceló, D. 2010 Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. *Environment International* **36**, 15-26.

Kimura, K., Hara, H., Watanabe, Y. 2007 Elimination of selected acidic pharmaceuticals from municipal wastewater by an activated sludge system and membrane bioreactors. *Environmental Science and Technology* **41**, 3708-3714.

Kunkel, U., Radke, M. 2012 Fate of pharmaceuticals in rivers: Deriving a benchmark dataset at favorable attenuation conditions. *Water Research* **46**(17), 5551–5565.

Langford, K.H., Reid, M., Thomas, K.V. 2011 Multi-residue screening of prioritised human pharmaceuticals, illicit drugs and bactericides in sediments and sludge. *Journal of Environmental Monitoring* **13**(8), 2284-2291.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J., Arshad, M., Mahmood, S., Ali, A., Khan, A.A. 2004 Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature* **427**, 630-633.

Okutman Tas, D., Pavlostathis, S.G. 2007 The influence of iron reduction on the reductive biotransformation of pentachloronitrobenzene. *European Journal of Soil Biology* **43**, 264-275.

Quintana, J.B., Weiss, S., Reemtsma, T. 2005 Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. *Water Res.* **39** 2654-2664.

Zorita, S., Martensson, L., Mathiasson, L. 2009 Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden. *Science of the Total Environment* **407**, 2760-2770.