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Enzymatic and microbial transformation assays for the evaluation of the environmental fate of diclofenac and its metabolites

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ABSTRACT

Diclofenac has been of environmental concern due to the potential harmful effects on non-target organisms at environmentally relevant concentrations. In this study, we evaluated the transformation kinetics of diclofenac and its two major metabolites in two laboratory-scale experiments: the transformation of diclofenac in the presence of rat liver S9 fraction with co-factors, and the transformation of diclofenac, 4′-hydroxy-diclofenac and diclofenac β-*O*-acyl glucuronide in the inoculum used for the OECD 301C ready-biodegradability test. 4′-Hydroxy-diclofenac was identified as the major phase I metabolite and diclofenac β-*O*-acyl glucuronide was identified as the major phase II metabolite in the S9 assay. Transformation of diclofenac in the microbial degradation test did not occur significantly for 28 d, whereas 4′-hydroxy-diclofenac degraded slowly, indicating that the biological removal of diclofenac is not likely to occur in conventional STPs unless sorptive removal is significant. However, diclofenac β-*O*-acyl glucuronide deconjugated to form equimolar diclofenac within 7 d, in the microbial degradation test. The mixture of diclofenac and its two metabolites, formed after incubating diclofenac in S9 medium for 2 h, was spiked in the inoculum to link both assays. The concentrations of diclofenac and its metabolites, measured over time, agreed well with predicted values, using rate parameters obtained from independent experiments. The results show that phase II metabolites generated in mammals may deconjugate easily in conventional STPs to form a parent compound and that these processes should be considered during the environmental monitoring and risk assessment of diclofenac.

1. Introduction

The potential environmental risks posed by residual pharmaceuticals discharged from sewage treatment plants (STPs) has been addressed in recent studies (e.g., Halling-Sørensen et al., 1998; Ternes, 1998; Kolpin et al., 2002; Ashton et al., 2004; Kim et al., 2007; Coetsier et al., 2009). Although the concentrations of pharmaceuticals in aqueous samples have usually been lower than the $\mu g\,L^{-1}$ level, potential long-term adverse effects on humans and the ecosystem have been suspected (e.g., Halling-Sørensen et al., 1998; Celiz et al., 2009; Mehinto et al., 2010). With recent advances in environmental sampling and monitoring techniques using mass spectrometry, many studies have been conducted worldwide to monitor pharmaceuticals at the $ng\,L^{-1}$ level, with emphasis on the removal efficiency in STPs (e.g., Clara et al., 2005; Joss et al., 2005; Santos et al., 2005; Gómez et al., 2007; Kim et al., 2007; Sim et al., 2010). Since pharmaceuticals are biologically active, their transformation in the body and in the environment may be important for assessing the human and ecological risks of parent compounds and their metabolites (Celiz et al., 2009; Escher and Fenner, 2011). However, most studies aimed at the simultaneous determination of the target parent compounds and removal efficiencies in STPs were simply estimated by measuring the disappearance of the target parent compounds during the treatment processes (e.g., Kolpin et al., 2002; Santos et al., 2005; Kim et al., 2007; Pedrouzo et al., 2010; Sim et al., 2010). Only a limited number of studies have identified the presence of metabolites and quantified them in field samples (e.g., Reddersen et al., 2002; Weigel et al., 2004; Stülten et al., 2008; Langford and Thomas, 2011).

Diclofenac (DCF), a non-steroidal anti-inflammatory drug, is one of the most extensively studied pharmaceuticals, because of its potential toxic effects on non-target organisms. Population decline of Pakistan vulture have been attributed to renal failure and visceral gout after the exposure to DCF (Oaks et al., 2004). Other potential toxic effects have also been observed, at environmentally relevant concentrations, on Japanese medaka (Hong et al., 2007), rainbow trout (Schwaiger et al., 2004), and brown trout (Hoeger et al., 2005). DCF and its metabolites have been detected in many

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effluent samples from STPs, ranging from a few ng L^{-1} to a few hundred ng L^{-1}, and the removal efficiency in STPs has been reported by comparing the concentration of DCF both in the influent and the effluent (Clara et al., 2005; Yu et al., 2006; Gómez et al., 2007; Kim et al., 2007; Pedrouzo et al., 2007; Matamoros et al., 2008; Sim et al., 2010; Langford and Thomas, 2011). Unlike compounds that undergo fast microbial degradation, DCF is not thought to be readily removed in STPs employing conventional activated sludge processes. The occasional negative removal efficiency reported in the literature (Clara et al., 2005; Lee et al., 2005; Lishman et al., 2006; Kim et al., 2007; Pedrouzo et al., 2007; Sim et al., 2010) may be due to the inevitable analytical uncertainties associated with field monitoring or environmental fluctuations. However, the possibility of the formation of DCF in STPs cannot be excluded, since DCF did not undergo fast degradation in various biodegradability tests (Zwiener and Frimmel, 2003; González et al., 2006; Lishman et al., 2006; Yu et al., 2006) and the formation of phase II conjugates of DCF has been reported (e.g., Kumar et al., 2002). For estrogens, microbial deconjugation has been reported in the literature (D'Ascenzo et al., 2003; Diniz et al., 2005; Gomes et al., 2009).

In this study, an enzymatic transformation assay and a microbial transformation assay were used to evaluate the transformation kinetics of DCF and its metabolites. Liver S9 fraction isolated from rat was used for evaluation of the *in vivo* metabolic transformation of DCF in mammals. Enzyme kinetic parameters were obtained for two DCF metabolites, 4′-hydroxydiclofenac (4′-OH DCF) and 1-β-*O*-acyl glucuronide of diclofenac (DCF-G). A modified closed-bottle biodegradability test (OECD 301C) was performed for quantification of the microbial transformation of DCF and its two major metabolites. A two-step enzymatic and microbial transformation experiment was also conducted to compare the measured concentrations of DCF and its two metabolites with the predicted values from kinetic parameters obtained from independent experiments.

2. Materials and methods

2.1. Materials

Diclofenac sodium salt (98%; CAS 15307-79-6) was purchased from Sigma–Aldrich (St. Louis, MO, USA). A major phase I metabolite of diclofenac, 4′-hydroxy-diclofenac (4′-OH DCF) was synthesized using a previously published method (Kim et al., 2010). The purity of 4′-OH DCF was greater than 98%, as determined by high-performance liquid chromatography–mass spectrometry (HPLC–MS). A phase II metabolite of diclofenac, β-*O*-acyl glucuronide (DCF-G, 98%; CAS 64118-81-6) was purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Liver S9 fraction isolated from rats (20 mg mL^{-1}) was purchased from Gibco (Rockville, MD, USA). Adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate (PAPS,>83%; CAS 109434-21-1), Trizma hydrochloride (⩾99.0%; CAS 1185-53-1), β-nicotinamide adenine dinucleotide phosphate hydrate (NADPH, 96%; CAS 53-59-8), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA, ⩾98%; CAS 63700-19-6), d-glucose-6-phosphate potassium salt (G6P, 98%; CAS 103192-55-8), alamethicin (from *Trichoderma viride*; CAS 27061-78-5) were purchased from Sigma–Aldrich. All solvents and salts used for the preparation of solutions and chemical analyses were of high purity.

2.2. Inoculum

One liter of fresh aqueous samples containing sludge and surface soil were collected from four domestic wastewater treatment plants, four industrial wastewater treatment plants, and seven rivers. The samples were mixed thoroughly and floating matter was removed by filtration through a Whatman No. 2 filter paper before use as inoculums. The pH of the filtered supernatant was adjusted to 7 ± 1 with phosphate buffer and aerated for approximately 24 h. An equal volume of a solution containing 0.1% mineral medium (Bacto-peptone, potassium phosphate, and dextrose; Difco Lab., Spark, MD, USA) was added to the inoculums and maintained under aerobic conditions. This procedure was repeated once per day for 30 d for the pre-conditioning.

2.3. Enzymatic transformation assay using rat liver S9 fraction

The enzymatic transformation assay was performed in 50 mM Tris buffer solution (adjusted to pH 7.8 using concentrated sodium hydroxide) containing 10 mM $MgCl_2$. The ionic strength of the buffer solution was adjusted to 0.154 M by addition of potassium chloride. Stock solutions of all co-factors were prepared freshly in sterilized buffer solution. Enzyme cocktail was prepared by adding S9 mixture and all co-factors into the buffer solution immediately after preparation of all stock solutions to make 2.0 mg mL^{-1} S9 mixture, 1 mM NADPH, 4 mM G6P, 2 mM UDPGA, 0.1 mM PAPS, and 25 μg mL^{-1} alamethicin. Diclofenac, dissolved in DMSO, was then added to a vial containing the enzyme cocktail to the desired initial concentrations, ranging from 5 to 150 μmol L^{-1}. The total volume of the assay medium was 1000 μL. After the addition of DMSO, the solution was mixed immediately using a vortex mixer and the reaction vial was placed in a shaking incubator maintained at 37 °C. After 15 min, a 150 μL aliquot was taken from the reaction vial and the aliquot was transferred to a centrifuge tube containing 1350 μL ice-cold acetonitrile for quenching enzymatic reactions. The solution was then centrifuged for 10 min at 5000 rpm (4500*g*) to sediment coagulated proteins. A 1200 μL aliquot of the supernatant was carefully withdrawn and filtered using a 0.45 μm hydrophilic PTFE filter (Advantec, Tokyo, Japan) for instrumental analyses. The concentrations of the two major metabolites formed were determined using HPLC–MS. Michaelis–Menten parameters, V_{max} and K_m, were determined using a non-linear regression using GraphPad Prism 3.0 (La Jolla, CA, USA).

2.4. Microbial transformation assay using a modified OECD 301C test

Microbial transformation of DCF and its metabolites was evaluated using a closed-bottle test for the assessment of ready biodegradability of chemicals (OECD, 1992), with slight modifications. Stock solutions were prepared in methanol and 150 μL or 300 μL (for DCF-G) of the stock solution was added into the solution containing activated sludge and essential nutrients (0.37 mM K_2HPO_4, 0.19 mM KH_2PO_4, 0.37 mM Na_2HPO_4, 0.095 mM NH_4Cl, 0.27 mM $MgSO_4$, 0.74 mM $CaCl_2$, 0.0028 mM $FeCl_3$), according to the OECD 301C guidelines. The total volume of the solution was 150 mL. Bottles were then incubated in a shaking incubator at 25 °C at 150 rpm. For evaluation of the abiotic hydrolysis of DCF-G, a control experiment was conducted following the same procedure in the autoclaved mineral medium, without activated sludge. Ten milliliter of aqueous samples was taken and filtered through a glass fiber filter (0.8 μm) after defined time intervals and the filtrate was passed through a 60 mg Oasis HLB™ solid-phase extraction cartridge (Waters, Milford, MA, USA), pre-conditioned by flowing 3 mL methanol and 3 mL deionized water. Solid-phase extraction cartridges were washed by flowing 4 mL of 5% methanol, and adsorbed chemicals were eluted using 4 mL methanol. The methanol solution was evaporated to dryness under a gentle nitrogen stream and the residue was re-dissolved in 2 mL acetonitrile for HPLC–MS analysis. All microbial degradation experiments were conducted in triplicate.

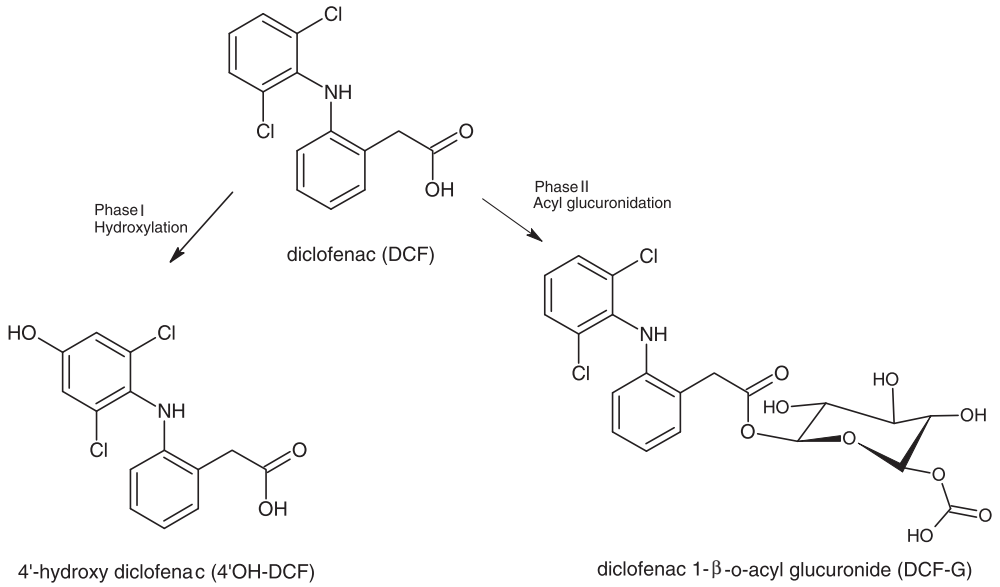

Fig. 1. Major transformation products of diclofenac by rat liver S9 fraction.

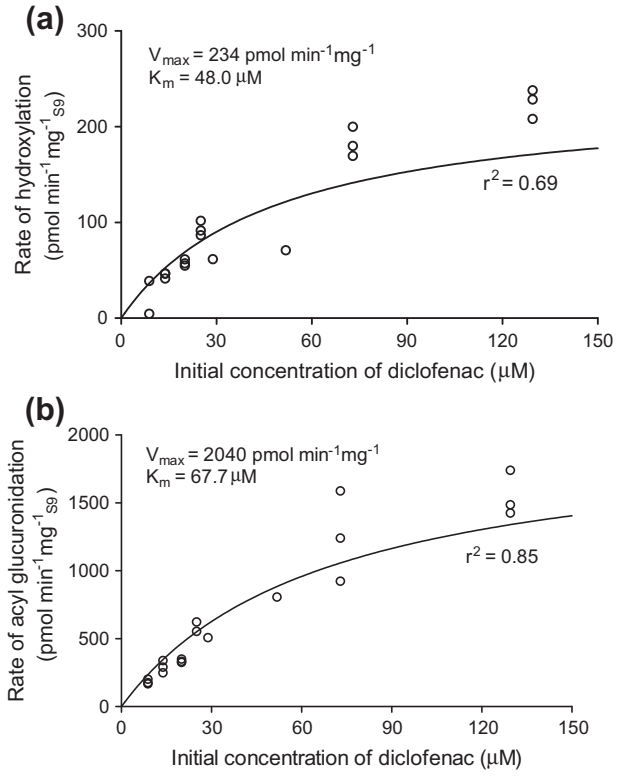

Fig. 2. Determination of Michaelis–Menten kinetic parameters of: (a) hydroxylation to 4′-hydroxy diclofenac and (b) for β-*O*-acyl glucuronidation. Solid lines represent best-fit using Michaelis–Menten kinetics.

For the evaluation of the extraction recovery in the inoculum, DCF and its two metabolites were added into the medium described above using the same spiking procedure. Immediately after spiking and mixing, 10 mL of the solution was taken for solid-phase extraction. Recoveries were calculated at three different concentrations.

To ensure the microbial activity of the inoculum used in this study, the microbial population was counted. Triplicate portions (each 200 μL) were taken from the inoculum sample for microbial assay and were suspended in 9 mL of sterilized deionized water. A 200 μL aliquot of the suspension was taken and diluted 10^7–10^8-fold. Triplicate aliquots (each 100 μL) were taken from the serial dilutions and placed on a nutrient broth agar plate, which was prepared by dissolving 8 g of nutrient broth agar and 15 g of Bacto agar in 1 L of deionized water. The plates were then incubated at 30 °C for 3 d. The total number of colonies was recorded using the dilution plate method (Chang et al., 2007). The number of microorganisms per mL of inoculum was calculated. The microbial activity of the inoculums used in the assay was also checked by the MITI test method (OECD 301C) using aniline as a positive control, and biological oxygen demand (BOD) was measured using an automatic electrolytic respirometer (WTW, OxiTop® IS 06). The inoculum was added to give a concentration of approximately 10^7–10^8 cells L^{-1} (approx. 30 mg L^{-1}) and aniline was treated at 100 mg L^{-1} in the mineral medium. Oxygen uptake was measured using a BOD meter at 25 ± 2 °C under darkness conditions for 14 d.

2.5. Two-step enzymatic and microbial transformation assay

Aqueous samples obtained directly from the enzymatic transformation assay were spiked into the inoculums used for microbial transformation assay. This two-step assay was used to evaluate the predictability of transformation kinetics using the rate parameters obtained from two separate experiments. Seventy-five micromolar DCF was spiked with DMSO into the medium for the enzymatic transformation assay as described previously, except that the total volume was 2 mL. The medium was incubated at 37 °C for 2 h and 1.5 mL was taken and added to a bottle prepared for the modified OECD301C test. The changes in aqueous concentrations of DCF and 4′-OH DCF were measured for 28 d using the method described in Section 2.3.

2.6. Instrumental analyses

Concentrations of diclofenac and its metabolites were measured using an HPLC–MS system (LCMS-2010EV; Shimadzu, Tokyo, Japan) equipped with a single quadrupole/ESI interface. The HPLC system consisted of a LC-20AD solvent delivery module, a DGU-20 A3 degasser, an SIL-20A autosampler, a CTO-20A column oven, and an SPD-M20A UV/VIS photodiode array detector. Samples were

Table 1
Solid-phase extraction recoveries of DCF and 4′-OH DCF.

Chemical	Spiked concentration (μmol L^{-1})	Extraction recovery (%, mean ± standard deviation)
DCF	0.5	105.6 ± 3.6
	1	112.1 ± 8.2
	2	119 ± 2.7
4′-OH DCF	0.5	61.2 ± 6.7
	1	69.2 ± 3.3
	2	73.7, 80.6[a]

[a] Standard deviation was not calculated because recovery was obtained from only two samples.

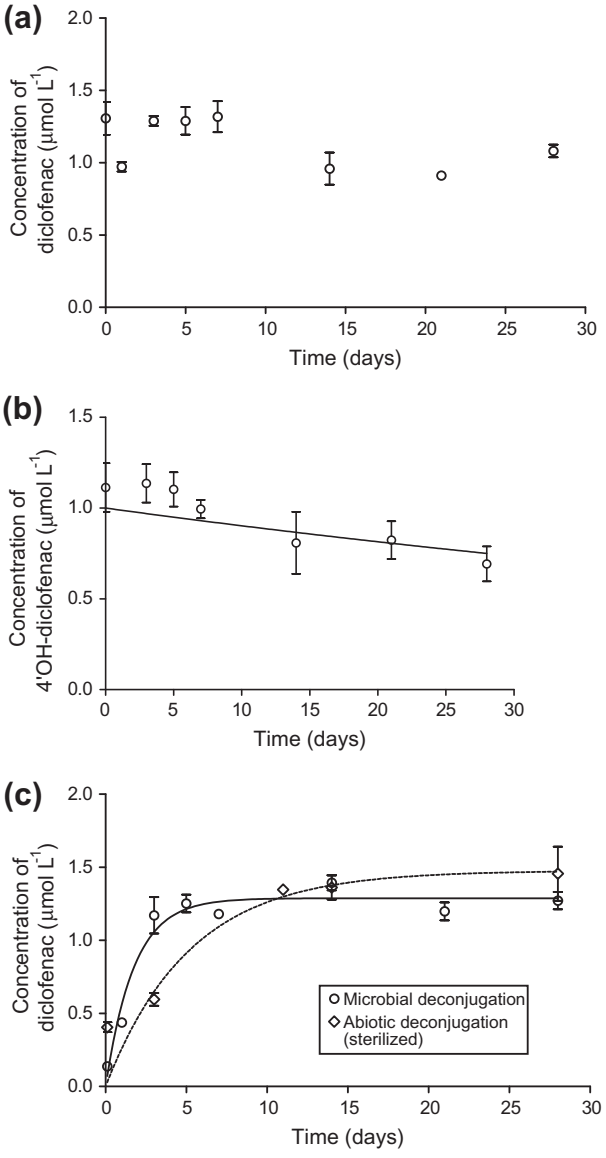

Fig. 3. Microbial transformation of: (a) diclofenac (DCF), (b) 4′-hydroxy diclofenac (4′-OH DCF) and (c) β-*O*-acyl glucuronide of diclofenac (DCF-G). Error bars denote standard deviations of triplicates and they are not shown when they are smaller than the symbol size. The solid line in (b) indicates the best fit using first-order decay kinetics. Open circles and open diamonds in (c) show the concentration of DCF from DCF-G, in the inoculums and without inoculums, respectively. Solid and dashed lines show the best-fit using first-order kinetics.

separated on a Zorbax Eclipse XDB-C18 column (150 mm × 4.6 mm i.d., particle size 5 μm; Agilent Technologies, Seoul, Korea) in the isocratic mode using acetonitrile in 0.1% formic acid (A) and de-ionized water in 0.1% formic acid (B) (v/v, 6:4) solutions at the flow rate of 0.3 mL min^{-1}. Chemicals were identified using the negative-ion mode. The ESI-MS conditions were as follows: capillary temperature, 200 °C; capillary voltage, 1.5 kV; nebulizer gas, 1.5 L min^{-1}; CDL temperature, 250 °C; and heat block, 200 °C.

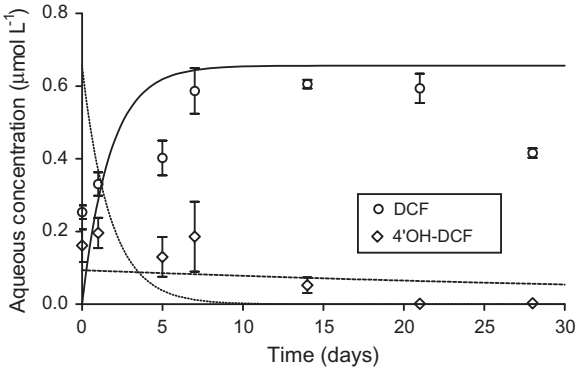

Fig. 4. Changes in the concentration of diclofenac (DCF) and its two major metabolites, 4′-hydroxy diclofenac (4′-OH DCF) and β-acyl glucuronide of diclofenac due to the microbial transformation, after the addition of samples incubated with rat liver S9 fraction for 2 h at 37 °C to the inoculum. Error bars denote standard deviations of triplicates. Solid, dashed, and dotted lines represent the predicted concentration of DCF, 4′-OH DCF, and DCF-G, respectively, using kinetic parameters obtained from earlier experiments.

3. Results and discussion

3.1. Enzymatic transformation of diclofenac

4′-Hydroxy diclofenac (4′-OH DCF) and diclofenac-β-*O*-acyl glucuronide (DCF-G) were identified as two major metabolites in the rat S9 enzymatic transformation assay (Fig. 1). The sum of the total molar concentrations of DCF, 4′-OH DCF, and DCF-G was close to the initial molar concentration of DCF for most samples, indicating that the formation of other metabolites could be neglected. This is consistent with earlier studies, although other oxidative metabolites, such as 5-hydroxy, 4′-5-dihydroxy, and 3-hydroxy diclofenac, and their conjugates with acyl glucuronide or sulfate were also identified in small fractions compared to the two major metabolites in microsomes (Kumar et al., 2002), human hepatocytes (Bort et al., 1999), and in fish bile (Kallio et al., 2010). Fig. 2 shows the rate of formation of (a) 4′-OH DCF and (b) DCF-G with increasing initial DCF concentrations. Michaelis–Menten kinetic parameters, V_{max} and $K_{m,}$ for 4′-hydroxylation were determined as 234 pmol min^{-1} mg^{-1} and 48.0 μM and those for glucuronidation were 2040 pmol min^{-1} mg^{-1} and 67.7 μM, respectively, indicating that acyl glucuronidation was most dominant, followed by 4′-hydroxylation. The measured K_m values agreed well with the range of K_m values in rat, dog, and human microsomes obtained by Kumar et al. (2002), in which the K_m for 4′-hydroxylation in rat microsomes was reported to be 14 μM and that for β-*O*-acyl glucuronidation was 20 μM. The V_{max} values reported by Kumar et al. (2002) were higher than those in this study by a factor of 1.9 to 6.8, showing higher reactivity of liver microsomes than S9 fraction.

3.2. Microbial transformation of diclofenac and its metabolites

The measured extraction recovery of the solid-phase extraction for DCF, 4′-OH DCF, and DCF-G is shown in Table 1. Whereas the extraction recoveries of DCF were slightly above 100% and 4′-OH DCF were close to 70%, regardless of the spiked concentration, little DCF-G was recovered. It is likely that hydroxylation and acyl glucuronigation of DCF makes the compound more polar and

Table 2
Concentration of DCF in the influent and the effluent of various pilot and full-scale sewage treatment plants (STPs) and its removal efficiency (taken from selected literature).

Sampling location	Concentration in influent water ($\mu g\ L^{-1}$)	Concentration in effluent water ($\mu g\ L^{-1}$)	Removal efficiency (%)	Reference
Pilot MBRs[a]	0.01	0.022–0.025	−120 ~ −150	Kim et al. (2007)
Pilot MBRs	2.8	1.9	23 ± 30	Quintana et al. (2005)
Three rural STPs, Austria	0.90–4.1	0.78–3.5	−35 ~ 52.73	Clara et al. (2005)
Urban STP, Spain	1.25 ± 0.11		73–96	Matamoros et al. (2008)
Urban STP, Spain	n.d. ~ 0.55	n.d.~0.46	−∞ ~ 100	Pedrouzo et al. (2007)
Urban STP, Spain	0.2–3.6	0.14–2.2	59±17	Gómez et al. (2007)
STP, Germany.	0.62 ± 0.11	0.47 ± 0.08	24	Bernhard et al. (2006)
MBR, Germany	2.08 ± 0.28	0.88 ±0.17		Bernhard et al. (2006)
Ten municipal STPs, Korea	0.018 ± 0.013	0.023 ± 0.012	−300 ~ 88	Sim et al. (2010)
Eight STPs, Canada	0.05–2.45	0.07–0.25	−220 ~ −50	Lee et al. (2005)
Municipal STP, Japan	0.251 ± 0.1	0.145 ± 0.032		Kimura et al. (2007)
Twelve municipal STPs, Canada.	n.d. ~ 1.01	n.d. ~ 0.748	−143 ~ 77	Lishman et al. (2006)
Seven municipal STPs, Finland		0.35 ± 0.10	9–60	Lindqvist et al. (2005)
Three STPs, Germany	3.02	2.51	17	Heberer (2002)

[a] MBR = membrane bioreactor.

difficult to sample, although the solid-phase extraction cartridge (HLB™) used in this study was designed for sampling both hydrophobic and hydrophilic compounds. Although HLB™ is one of the most common sampling materials used for the simultaneous monitoring of many polar organic compounds, it may not be appropriate for sampling highly hydrophilic metabolites, such as glucuronides.

The microbial populations of the inoculum used in this study was 3.7×10^7 cells mL^{-1} and the percentage degradation of aniline as a positive control, calculated from the oxygen consumption, was 60.9% after 7 d and 68.8% after 14 d, satisfying the OECD 301C criteria (60%). Fig. 3 shows changes in the concentrations of DCF (Fig. 3a), 4′-OH DCF (Fig. 3b), and deconjugated DCF from DCF-G (Fig. 3c) in the microbial transformation assay medium with time. The extracted concentration of DCF did not change significantly over 28 d. Similarly, the percentage transformed DCF in a batch biodegradation study employing a similar method during 50 d was approximately 30% (Yu et al., 2006) and slow biodegradation was reported using other biological reactors (Zwiener and Frimmel, 2003; González et al., 2006). This suggests that the removal efficiency of DCF in STPs would not be high, unless the removal is dominated by sorption to sewage sludge. The logarithm of the sorption coefficient between organic carbon and water ($\log K_{oc}$) of diclofenac has been reported to be between 1.90 and 3.74 (Scheytt et al., 2005). The wide range of $\log K_{oc}$ makes it difficult to assess the contribution of the sorption to sewage sludge in STPs because the expected sorptive removal varies significantly with $\log K_{oc}$ between 2 and 4 (Heidler and Halden, 2008; Kim et al., 2009). In contrast, the phase I metabolite 4′-OH DCF degraded slowly with a pseudo-first order rate constant of 0.018 d^{-1} (Fig. 3b). Although the change in the concentration of DCF-G with time could not be evaluated due to the poor recovery of the solid-phase extraction, the formation of DCF via deconjugation of DCF-G was observed (Fig. 3c). Assuming that this hydrolysis reaction is first-order, the deconjugation rate constants were calculated as 0.28 d^{-1} in the inoculum and 0.12 d^{-1} under sterile conditions. Abiotic hydrolysis can also be important in the deconjugation of DCF-G, although microorganisms may accelerate the process. In addition, almost equimolar DCF was identified in a test bottle spiked with DCF-G, indicating that DCF-G is readily converted back into DCF in a sewage sludge treatment plant. Similar fast deconjugation of the acyl glucuronides of steroid estrogens has been observed (D'Ascenzo et al., 2003; Gomes et al., 2009).

3.3. Two-step enzymatic and microbial transformation assay

Fig. 4 shows the changes in concentrations of DCF and 4′-OH DCF in the combined enzymatic and microbial transformation assays. The concentration of DCF increased rapidly up to 0.6 μM in 5 d and the concentrations remained steady, although the values fluctuated. This confirms the fast deconjugation of DCF-G and negligible degradation of DCF. On the other hand, the concentration of 4′-OH DCF decreased gradually for 28 d. The changes in concentrations of DCF and 4′-OH DCF could be predicted well from independently measured kinetic parameters. This is indicated by the modeled concentrations of DCF (solid line, Fig. 4) and DCF-G (dotted line, Fig. 4) using the first-order deconjugation rate constant of DCF-G and the first-order decay of 4′-OH DCF (dashed line, Fig. 4) with the rate constant measured independently. The initial concentrations were estimated from V_{max} and K_m for both 4′-hydroxylation and acyl glucuronidation.

3.4. Implications on risk assessment of pharmaceuticals

As summarized in Table 2, many researchers have attempted to estimate the removal of diclofenac in STPs adopting various processes (Heberer, 2002; Clara et al., 2005; Lee et al., 2005; Lindqvist et al., 2005; Quintana et al., 2005; Bernhard et al., 2006; Lishman et al., 2006; Yu et al., 2006; Gómez et al., 2007; Kim et al., 2007; Kimura et al., 2007; Pedrouzo et al., 2007; Matamoros et al., 2008; Sim et al., 2010). Unlike many other pharmaceuticals readily degraded under STP conditions, the removal efficiencies of DCF were usually less than 50% and occasionally negative, meaning that the concentration of DCF in the effluent was higher than in the influent. Negative removal efficiencies in the literature may be attributable to the unaccounted effects of the deconjugation of DCF-G. As was the case in this study, DCF-G may not be easily sampled using solid-phase extraction. Thus, it is possible that missing the contribution of DCF-G in the influent of STP may explain the missing load of DCF to STPs and the resulting negative removal of DCF. Because the hydraulic retention time in STPs ranges from a few hours to 10 d (Metcalf and Eddy, Inc., 2003), and the density of microorganisms, particularly in the aeration tank, is approximately two orders of magnitude higher than that used in this study (approx. 30 mg L^{-1}), it is reasonable to expect that almost all DCF-G entering STPs is likely to be converted back to DCF. Therefore, it is important to include DCF-G in the mass balance analysis of DCF in STPs with the quantitative evaluation of sorption to sewage sludge.

Due to the lack of availability of many phase II metabolites and the difficulties in sampling them simultaneously with parent compounds, it is difficult to include deconjugation processes in the mass balance of pharmaceuticals in STPs. The simple two-step enzymatic and microbial transformation assay used in this study will be useful in screening for the potential contribution of deconjugation of phase II metabolites. Such screening tests will be useful for evaluating the fate of pharmaceuticals in STPs on a laboratory scale, without quantifying the parent compound and its metabolites under varying field conditions.

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