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Vacuum ultraviolet photolysis of diclofenac and the effects of its treated aqueous solutions on the proliferation and migratory responses of *Tetrahymena pyriformis*



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HIGHLIGHTS

GRAPHICAL ABSTRACT

- The radical-scavenging effect of phosphates seems to be negligible.
- Only higher concentrations of HO₂ contribute to the degradation of diclofenac.
- Toxicity of VUV-treated samples decreases with increasing rate of mineralization.
- Dissolved O₂ enhances the mineralization of diclofenac by affecting the radical set.
- Treated samples retain the chemorepellent character of the parent compound.

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ABSTRACT

The effects of dissolved O₂, phosphate buffer and the initial concentration of diclofenac on the vacuum ultraviolet photolysis of this contaminant molecule were studied. Besides kinetic measurements, the irradiated, multicomponent samples were characterized via the proliferation and migratory responses (in sublethal concentrations) of the bioindicator eukaryotic ciliate *Tetrahymena pyriformis*. The results suggest that hydroxyl radicals, hydrogen atoms and hydroperoxyl radicals may all contribute to the degradation of diclofenac. The aromatic by-products of diclofenac were presumed to include a hydroxylated derivative, 1-(8-chlorocarbazolyl)acetic acid and 1-(8-hydroxycarbazolyl)acetic acid. The biological activity of photoexposed samples reflected the chemical

Abbreviations: AOP, advanced oxidation process; Chtx. Ind, Chemotaxis Index; DICL, diclofenac; k, reaction rate constant; k', apparent reaction rate constant; k_{DRCL} , second-order reaction rate constant between DICL and HO'; kHPO42—, second-order reaction rate constant between HPO₄²⁻ and HO'; kH2PO4—, second-order reaction rate constant between H₂PO₄⁻ and HO'; kH2PO4—, second-order reaction rate constant between H₂PO₄⁻ and HO'; kH2PO4—, second-order reaction rate between HO'; (HO')_{SS}, the steady-state concentration of hydroxyl radicals; PB, phosphate buffer; PhAC, pharmaceutically active compound; R', carbon-centered radical; r_{DICL} , reaction rate between DICL and HO'; rH0PO42—, reaction rate between H2PO4⁻ and HO'; ROO', peroxyl radical; SE, standard errors of the mean; WWTP, wastewater treatment plant.

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transformation of diclofenac and was also dependent on the level of dissolved O₂. The increase in toxicity of samples taken after different irradiation times did not exceed a factor of two. Our results suggest that the combination of vacuum ultraviolet photolysis with toxicity and chemotactic measurements can be a valuable method for the investigation of the elimination of micropollutants.

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1. Introduction

Diclofenac (DICL, 2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid) is an arylacetic acid nonsteroidal anti-inflammatory drug used for multiple indications in both human and veterinary medicine. Its annual consumption worldwide has been estimated to be 960 tons (Zhang et al., 2008). It was one of the first pharmaceutically active compounds (PhACs) reported to affect the wellbeing of living organisms as it was directly linked to the massive population decline of different vulture species on the Indian subcontinent (Oaks et al., 2004). Moreover, together with carbamazepine, it is the most frequently detected PhAC in natural waters (Zhang et al., 2008). The main route of its entry into the aquatic environment is via sewage: following human consumption of the drug, it is excreted either unchanged (2–15%) or in the form of hydrolysable conjugates (1–15%) (Khan and Ongerth, 2004; Ternes, 1998). The remaining ~70% is excreted renally in the form of inactive metabolites after its hepatic metabolism (Winker et al., 2008). At wastewater treatment plants (WWTPs), biodegradation has been demonstrated to be the major pathway for the elimination of DICL (Onesios et al., 2009). The rate and efficiency of its removal during conventional activated sludge treatment vary with the different operating conditions used, such as the solid retention time and the hydraulic retention time (Clara et al., 2005). Its reported removal efficiency of between 7% and 80% could be improved by advanced tertiary treatment options such as ozonation (Oulton et al., 2010).

Depending on the geographical location and the type of water, the environmental concentrations range from the low ng L^{-1} to 1000 ng L^{-1} . Environmental loads were recently reported for surface waters and wastewaters (Pal et al., 2010; Ratola et al., 2012; Santos et al., 2010), ground waters (Lapworth et al., 2012) and drinking waters (Daughton, 2010; Vulliet et al., 2011): 460–3300 ng L^{-1} , 21–40 ng L^{-1} , 11 ng L^{-1} and 0.2–1 ng L^{-1} , respectively.

As concerns its biological effects on ecosystems, a large number of studies have been carried out on diverse species, including bacteria, algae, ciliates, crustaceans or fish (Santos et al., 2010). These studies indicate that acute toxicity of DICL may occur at concentrations one or two orders of magnitude higher than typical levels in the aquatic environment (Farré et al., 2001). However, not enough is known regarding the toxicity of its metabolites and degradation products that may be formed in water bodies during its abiotic decomposition (e.g. photolysis) or biotic degradation (Zhang et al., 2008).

The above-mentioned data illustrating the loading of the environment with DICL clearly demonstrate the need for the improvement of waterpurifying techniques, which could be accomplished by the use of advanced oxidation processes (AOPs) (Kruithof et al., 2007; Legrini et al., 1993). The most significant of such processes are radiolysis (Homlok et al., 2011; Yu et al., 2013), photochemical processes (Boreen et al., 2003; Buser et al., 1998; Moore et al., 1990; Poiger et al., 2001), ozonebased methods (García-Araya et al., 2010; Sein et al., 2008; Vogna et al., 2004) and homogeneous (Pérez-Estrada et al., 2005a) or heterogeneous photocatalytic techniques (Calza et al., 2006; García-Araya et al., 2010; Martinez et al., 2011; Pérez-Estrada et al., 2005b).

Both ultraviolet (UV) lamps and the UV range of solar irradiation efficiently transform DICL, with quantum yields in the interval of 0.03–0.32 (Boreen et al., 2003; Buser et al., 1998; Moore et al., 1990; Poiger et al., 2001). However, the UV doses typically used during water disinfection in water treatment plants (400 J m⁻²) are usually

lower than those applied in the mentioned studies and are sufficient to eliminate only ~30% of the DICL (Canonica et al., 2008; Meunier et al., 2006).

AOPs are based on the generation of reactive radicals, which induce the degradation of pollutant molecules. Among the radicals formed, the hydroxyl radical (HO[•]) is the most reactive and least selective, reacting with organic and inorganic compounds with rate constants of $10^{7}-10^{10}$ mol⁻¹ L s⁻¹ (Anbar and Neta, 1967). The reaction between HO[•] and DICL follows second-order kinetics, with a reaction rate constant (k_{DICL}) of (0.6–2.4) × 10¹⁰ mol⁻¹ L s⁻¹ (Aruoma and Halliwell, 1988; Huber et al., 2003; Parij et al., 1995; Yu et al., 2013). Following the steady-state approximation for the concentration of HO[•] ([HO[•]]_{SS}), this value might be incorporated in the apparent reaction rate constant ($k' = k \times [HO[•]]_{SS}$) in homogeneous systems. Thus, the reaction is usually treated as a pseudo-first-order reaction.

The significance of reactive radicals in the degradation of emerging contaminants is supported by the high efficiency of ozonation and homogeneous or heterogeneous photocatalysis (Bernabeu et al., 2011; Huber et al., 2003; Klamerth et al., 2009; Pérez-Estrada et al., 2005b; Sein et al., 2008; Vogna et al., 2004).

HO[•] should be formed during the primary step of the method employed so as to ensure that this species is responsible for the degradation of DICL. Suitable techniques include radiolysis and vacuum ultraviolet (VUV) photolysis, where the generated radicals are known. Both methods excite the solvent molecules, but in radiolysis excited water molecules split to furnish hydrated electrons (e_{aq}^{-}), HO[•] and protons, whereas in VUV photolysis hydrogen atoms (H[•]) and HO[•] are formed as primary radicals (Gonzalez et al., 2004):

$$H_2O + hv_{172nm} \rightleftharpoons (H_2O)^* \longrightarrow H^{\bullet} + HO^{\bullet}.$$
(1)

Radiolytic experiments have revealed that both HO[•] and e_{aq}^- are effective in degrading DICL, e_{aq}^- making the lower contribution to the mineralization (Homlok et al., 2011; Yu et al., 2013). However, we are not aware of investigations of the VUV photolysis of DICL. This method might permit conclusions from the effects of the various parameters on the radicals and on the degradation of DICL, and the results could contribute to the optimization of other AOPs.

The use of AOPs as a post-treatment technique could enhance the efficiency of DICL elimination in WWTPs and thereby decrease the potential environmental risk of this compound. Although photolytic or photocatalytic treatment has been observed to lead to an enhanced toxic effect in some cases because of the formation of compounds more harmful than DICL itself (Calza et al., 2006; Schmitt-Jansen et al., 2007), prolonged treatment did result in the detoxification of the solutions (Calza et al., 2006; Homlok et al., 2011; Yu et al., 2013).

Our present aims were i) to describe the VUV photolysis of DICL; ii) to study the influence of the operating conditions on the kinetics and efficiency of DICL degradation and iii) to characterize the effects of samples taken after different periods of photolytic degradation on the proliferation and (in sublethal concentrations) the migratory responses of the bioindicator freshwater eukaryotic ciliate *Tetrahymena pyriformis*. The combination of VUV photolysis and investigations of the biological effects of treated multicomponent solutions would also be beneficial in the case of other PhACs: the effects of the radicals formed on the toxic and chemotactic character of such compounds could be established.

2. Material and methods

2.1. Chemicals and reagents

All the chemicals used were of analytical purity and were applied without further purification. From the sodium salt of DICL (Sigma, St. Louis, MO, USA), 1.0×10^{-5} mol L⁻¹, 4.0×10^{-5} mol L⁻¹, 7.0×10^{-5} mol L⁻¹ and 1.0×10^{-4} mol L⁻¹ solutions were prepared in ultrapure Milli-Q water (MILLIPORE Milli-Q Direct 8/16, Billerica, MA, USA) or in phosphate-buffered solution (PB) in toxicity and chemotaxis experiments. The permeate conductivity of the Milli-Q water was 13.3 µS cm⁻¹, its resistivity was 18.2 MΩ cm and its total organic carbon (TOC) content was 2 ppb. PB of pH = 7.4 contained 1.1×10^{-3} mol L⁻¹ NaH₂PO₄ (≥99%; Spektrum 3D, Debrecen, Hungary) and 1.9×10^{-3} mol L⁻¹ Na₂HPO₄ (≥99.0%; Fluka, Buchs, Germany) in Milli-Q water.

2.2. The photochemical apparatus

For the VUV measurements, a xenon excimer lamp (Radium XeradexTM, 20 W electric input power) emitting at 172 ± 14 nm was placed at the center of a water-cooled, triple-walled tubular reactor. The photon flux of the light source, determined by means of methanol actinometry (Oppenländer and Schwarzwalder, 2002), was found to be 3×10^{-6} mol_{photon} s⁻¹. The treated solution (250 mL) was circulated at 375 mL min⁻¹ in a 2-mm thick layer within the two inner walls of the reactor and the reservoir by a Heidolph Pumpdrive 5001 peristaltic pump (see Fig. SF1 in the Supplementary material). The reactor and the reservoir were thermostated at 25.0 \pm 0.5 °C. N_2 (>99.99% purity; Messer, Budapest, Hungary) or O₂ (>99.99% purity; Messer, Budapest, Hungary) was bubbled (600 mL min⁻¹) through the solution in the reservoir to attain deoxygenated or O₂-saturated conditions, respectively. The injection of N_2 and O_2 was started 30 or 15 min before each experiment, respectively, and was continued until the end of the irradiation.

The pH of the irradiated solutions was measured with an inoLab pH 730p instrument, the measuring electrode being introduced directly into the reservoir.

All the presented results are the averages of 2–5 experiments; the error bars show the standard deviation of the measured values.

2.3. High-performance liquid chromatography with mass spectrometry

Samples were analyzed on an Agilent 1100 series LCMSD VL system consisting of a binary pump, a micro vacuum degasser, a diode array detector, a thermostated column compartment, a 1956 MSD and ChemStation data managing software (Agilent Technologies, Palo Alto, CA, USA). As eluent, 1% aqueous acetic acid (HPLC grade; VWR, Fontenay-sous-Bois, France) and acetonitrile (ultra gradient HPLC grade; J.T. Baker, Deventer, The Netherlands) were used in 1:1 ratio, at a flow rate of 0.8 mL min⁻¹ on a C₁₈ (LiChroCART 4×125 mm, 5 µm) column. The quantification wavelengths for the UV detector were 240, 273 and 280 nm. For MS detection, a 1956 MSD with quadrupole analyzer and electrospray ionization was operated in the negative ion mode. N2 was used as drying gas (300 °C, 12 L min⁻¹), the fragmentor voltage was 70 V (for the measurement of by-products A and B) and 80 V (for the measurement of by-product C), the nebulizer pressure was 3.4 bar and the capillary voltage was 1000 V.

The aliphatic by-products were separated on a GROM-RESIN ZH (Herrenberg-Kayh, 250 \times 8 mm, 8 μ m) column, with detection at 206 nm. As eluent, 0.01 mol L⁻¹ sulfuric acid (AnalR NORMAPUR; VWR, Fontenay-sous-Bois, France) was used at a flow rate of 0.8 mL min⁻¹.

2.4. Adsorbable organic halogen content measurements

The adsorbable organic halogen (AOX) contents of the solutions were determined by using an APU2 sample preparation module (Analytik Jena AG, Jena, Germany) and a multi X 2500 instrument (Analytik Jena AG, Jena, Germany).

2.5. Total organic carbon content measurements

For determination of the TOC content of solutions, a multi N/C 3100 instrument (Analytik Jena AG, Jena, Germany) was used.

2.6. Kinetic modeling

The formal k' values of DICL degradation were determined by performing a nonlinear model fit on the concentrations measured during the HPLC analyses, with the help of Mathematica 8 (Wolfram) software. It should be mentioned that our system is very inhomogeneous. The VUV photons are absorbed in a very thin water layer (<0.1 mm) and therefore only a thin-walled hollow cylindrical volume of solution is irradiated, near the quartz/water interface. Further, the experimental setup consisted of a partly-irradiated reactor and a reservoir, the determined (apparent) k' values therefore referring to the overall transformation rate of DICL under the experimental conditions applied.

2.7. Cell culturing

The eukaryote ciliate *T. pyriformis* GL was maintained in a culture medium containing 0.1% (w/w) yeast extract (Difco, Michigan, USA) and 1% (w/w) Bactotriptone (Difco, Michigan, USA) in distilled water (pH = 7.4). Cells were grown under axenic conditions at 28 °C, and 24-h exponential growth phase cultures were used in the experiments.

2.8. Proliferation inhibition assays

Proliferation inhibition assays were carried out as previously described (Láng and Kőhidai, 2012). Briefly, 10³ cells well⁻¹ were placed in the core blocks of 60 wells in 96-well microtiter plates (Sarstedt AG, Nümbrecht, Germany) and incubated with the samples at 28 °C for 24 h. The cells were subsequently fixed with 4% formaldehyde (Reanal, Budapest, Hungary) containing PB and counted with an impedimetric CASY TT cell counter (Innovatis-Roche, Rotkreuz, Switzerland). Counting was achieved with the use of a 150um-diameter capillary and counted events in the diameter range of 10–100 µm were regarded as cells. The potential aggregation of cells was corrected for during the cell number evaluation by applying the aggregation factor, determined as the ratio of the peak cell diameter and the average cell diameter. The inhibitory effects of VUV-treated samples were determined by normalizing the numbers of cells in the treated sample wells to the cell numbers in the negative control wells. These wells contained cell culture medium with the appropriate volume proportion of PB. Measurements were performed in guintuplicate and repeated three times.

Samples from the VUV photolysis of 1.0×10^{-4} mol L⁻¹ DICL in PB were taken at 0 s, 10 s, 20 s, 40 s, 90 s, 150 s, 300 s, 450 s, 600 s, 900 s, 1200 s, 1500 s, 1800 s, 2100 s, 2400 s, 3000 s and 3600 s. They were then diluted to 1%, 5% and 25% (v/v) in the cell culture medium. The highest concentration was determined in preliminary experiments in order to avoid massive disintegration of the cells due to osmotic shock, which would disturb the objective evaluation of the toxic effects of the samples. In these experiments, cells were incubated with 1–90 v/v% of PB in culture medium for 24 h, and the number and morphology of the cells were then evaluated under a microscope (Zeiss Axio Observer, Göttingen, Germany).

2.9. Chemotaxis assay

Chemotaxis is the directed migratory response of motile cells to the gradient of a dissolved chemical. Chemotactic characterization of a substance includes the description of the elicited effect (positive, i.e. attractant, or negative, i.e. repellent) and the time and concentration dependences of the induced response. The chemotactic responses elicited by the VUV-treated samples were measured in a two-chamber multichannel capillary assay device (Kőhidai, 1995) for which the optimal incubation time was found to be 15 min (Sáfár et al., 2011). Samples were placed in the upper chamber of the device, whereas cells (10⁴) were loaded into the lower chamber. Following a 15-min incubation at 28 °C and fixation with 4% formaldehyde (Reanal, Budapest, Hungary) containing PB, the number of positive responder cells was determined with a CASY TT cell counter (Innovatis-Roche, Rotkreuz, Switzerland).

Samples were diluted to 0.1%, 0.01%, 0.001%, 0.0001%, 0.00001% and 0.0000001% (v/v) in cell culture medium. Control runs with pure culture medium in the upper chamber served for the normalization of cell numbers. The ratio obtained designated the Chemotaxis Index (Chtx. Ind.). Measurements were carried out in quadruplicate.

2.10. Statistical evaluation

Statistical evaluation of both bioassays was performed with Origin8Pro software. Significance was determined by one-way ANOVA. Normality of data was tested by the Shapiro–Wilkinson test, while the homogeneity of variances was checked by the Levene test and the Brown–Forsythe test, using the same software.

3. Results and discussion

3.1. Effects of the phosphate buffer

In unbuffered solutions, the pH of the samples ($[DICL]_0 = 1.0 \times 10^{-4} \text{ mol } L^{-1}$) irradiated for $\geq 300 \text{ s}$ was around the pK_a of DICL (4.2) (Huber et al., 2003); it was higher only in the first stage of VUV photolysis (Fig. 1). During the first 300 s of treatment, therefore, DICL was predominantly present in its dissociated form. Since the pH of the irradiated solutions prepared in Milli-Q water changed during the experiments, and the pH is known to influence the toxicity toward *T. pyriformis* (Schultz and Burgan, 2003), samples for toxicity and chemotaxis experiments were taken from buffered DICL solution. The test organism therefore indicated the formation and decomposition of toxic by-products, instead of the change in the solution pH. In this case the pH of the samples varied in the range 6.9–7.2, so that DICL was present as an anion in this case too.

Since both $H_2PO_4^-$ and HPO_4^{2-} are HO[•] scavengers (García-Araya et al., 2010), it is essential to investigate their effects on the DICL degradation kinetics during VUV photolysis. As revealed by Fig. 2, a significant



Fig. 1. Plots of the pH (y-axis) of DICL-containing solutions ([DICL]₀ = 1.0×10^{-4} mol L⁻¹) prepared in Milli-Q water as a function of the duration of irradiation (x-axis (s)) under oxygenated (\blacklozenge) or deoxygenated (\blacksquare) conditions.



Fig. 2. Plots of the concentration of DICL (y-axis (mol L⁻¹)) as a function of the duration of irradiation (x-axis (s)) under oxygenated (\diamond) or deoxygenated (\Box) conditions during the VUV photolysis of DICL ([DICL]₀ = 1.0×10^{-4} mol L⁻¹). Filled symbols denote data measured in Milli-Q water, and open symbols those in PB. Significance levels correspond to: x: p < 0.05.

difference between the decay of DICL dissolved in pure water or in buffered solutions ([DICL]₀ = 1.0×10^{-4} mol L⁻¹) was not observed either in O₂-saturated or in deoxygenated solutions. However, a slight increase was observed in the reaction rate in Milli-Q water in the presence of O₂ after 180 s of irradiation.

Although both HPO₄²⁻ and H₂PO₄⁻ react with HO[•], their reaction rate constants: $k_{\text{HPO}_4}^{2-}$ (Black and Hayon, 1970; García-Araya et al., 2010; Maruthamuthu and Neta, 1978) and $k_{\text{H}_2\text{PO}_4}^{--}$ (Anbar and Neta, 1967; Maruthamuthu and Neta, 1978) are 2–6 orders of magnitude lower than k_{DICL} :

$$HPO_{4}^{2-} + HO \rightarrow HPO_{4}^{-} + OH^{-}$$

$$k_{HPO_{4}^{2-}} - = 1.5 \times 10^{5} - 5 \times 10^{6} \text{ mol}^{-1} \text{ L s}^{-1}$$
(2)

$$\begin{aligned} &H_2 P O_4^{-} + H 0^{\bullet} \rightarrow H_2 P O_4^{\bullet} + O H^{-} \\ &k_{H_2 P O_4} - = 2 \times 10^4 - 1 \times 10^7 \text{ mol}^{-1} \text{ L s}^{-1}. \end{aligned}$$

From the reaction rate constants and the initial concentrations of DICL, HPO_4^2 and H_2PO_4^- (which at the beginning of the photolysis were roughly equal to their actual concentrations ([DICL], [$\text{HPO}_4^2^-$] and [H_2PO_4^-], respectively)), the reaction rates of DICL (r_{DICL}), $\text{HPO}_4^2^-$ ($r_{\text{HPO}_4}^2^-$) and H_2PO_4^- ($r_{\text{H}_2\text{PO}_4}^-$) may be calculated:

$$\frac{r_{\text{DICL}}}{r_{\text{HPO}_{4}^{2}}} = \frac{k_{\text{DICL}} \times [\text{HO}^{\bullet}] \times [\text{DICL}]}{k_{\text{HPO}_{4}^{2}} \times [\text{HO}^{\bullet}] \times [\text{HPO}_{4}^{2-}]}$$
(4)

$$63 < \frac{r_{\text{DICL}}}{r_{\text{HPO}_{2}^{-}}} < 8421$$
 (5)

$$\frac{r_{\text{DICL}}}{r_{\text{H}_2\text{PO}_4}} = \frac{k_{\text{DICL}} \times [\text{HO}^{-}] \times [\text{DICL}]}{k_{\text{H}_2\text{PO}_4} \times [\text{HO}^{-}] \times [\text{H}_2\text{PO}_4^{-}]}$$
(6)

$$55 < \frac{r_{\text{DICL}}}{r_{\text{H}_{2}\text{PO}_{a}}} < 109,091.$$
 (7)

Since both $r_{\rm HPO_4}^{2-}$ and $r_{\rm H_2PO_4}^{-}$ were found to be significantly lower than $r_{\rm DICL}$, the bulk of the HO[•] is likely to react with DICL rather than with HPO₄²⁻ or H₂PO₄⁻. The negligible difference found between the degradation rates of DICL in Milli-Q water and in PB (Fig. 2) may be attributed to the above findings.

During the VUV photolysis of DICL (with a chromatographic retention of 8.6 min), three aromatic by-products (A, B and C) were detected (their presumed chemical structures are presented in Section 3.4) with chromatographic retention times of 3.8, 6.2 and 2.7 min, respectively.



Fig. 3. The effects of PB on the formation and transformation of a) by-product A and b) by-product B (the y-axes reflect the areas under the chromatographic peaks (AU)) under oxygenated (\Diamond) or deoxygenated (\Box) conditions during the VUV photolysis of DICL ([DICL]₀ = 1.0×10^{-4} mol L⁻¹). Filled symbols denote data measured in Milli-Q water, and open symbols those in PB.

Their formation and transformation were influenced significantly by the medium. In solutions containing dissolved O_2 , the concentrations of the by-products were higher in the presence of PB, while in solutions purged with N_2 they were higher in the absence of phosphates (with the exception of by-product A, where no difference was observed) (Fig. 3).

Among the aliphatic by-products, oxalic and malonic acids were detected, but only in oxygenated solutions. This is in accord with the observation that the pH of the PB-free solutions was 0.4-0.7 units lower in the presence of O₂ (Fig. 1). Further, aliphatic by-products proved to be produced in higher concentrations in buffered solutions (Fig. SF2).

Although the *k* values of the reactions of the aromatic by-products with HO[•] are not known, in view of the structural similarities of these compounds and the nonselectivity of HO[•], they are most probably of the same order of magnitude as k_{DICL} . The differences between the rates of accumulation and decomposition of the by-products in the presence and absence of phosphates (Figs. 3 and SF2) may therefore be caused by the differences in the pH of the solutions.

In oxygenated solutions, the concentrations of by-products A–C reached their maxima between 90 and 150 s in the absence, and between 180 and 450 s in the presence of PB (Figs. 3a and 4), which corresponds to the observation that the rate of DICL degradation (in O_2 -saturated Milli-Q water) increased slightly after 180 s of irradiation (Fig. 2), when these by-products started to decompose. This may suggest that the increased concentrations of the by-products reduce the concentrations of reactive radicals and therefore the efficiency of DICL transformation.

In deoxygenated solutions, the presence of PB did not have a significant effect on the degradation of DICL (Fig. 2). Moreover, the concentrations of the aromatic by-products were lower in the presence of PB. In this case, the radicals formed from $H_2PO_4^-$ and HPO_4^{2-} ($H_2PO_4^-$ and HPO_4^{--}) might contribute to the transformation of the by-products, since the second-order reaction rates of these radicals with some organic compounds have been reported to be in the range 10^7 – 10^8 mol⁻¹ L s⁻¹ (Nakashima and Hayon, 1970).

The effects of PB in oxygenated solutions will be discussed in the next section.

3.2. The effects of dissolved O_2

Dissolved O_2 scavenges H^{*}, resulting in hydroperoxyl radicals (HO₂) and their conjugate base-pair, superoxide radical ions (O_2^{-}) (Gonzalez et al., 2004):

$$H^{+}+O_{2} \rightarrow HO_{2}^{*}$$
 $k_{8} = 1.2 \times 10^{10} \text{ mol}^{-1} \text{ Ls}^{-1}$ (8)

(Buxton et al., 1988)

$$HO_2 = H^+ + O_2 pK_a = 4.8$$
 (9)

(Bielski et al., 1985).

These oxygen-containing radicals may also contribute to the degradation of organic contaminants. Since the pH of the solutions in the absence of PB was below the pK_a of HO₂ after 300 s of irradiation (Fig. 1), the predominance of HO₂ over O₂⁻⁻ should be taken into consideration in oxygenated solutions on the use of longer reaction times. In buffered solutions, however, the pH was found to be above the pK_a of HO₂ (Fig. 1). This radical is therefore present in the form of its conjugate base-pair under these conditions.

The recombination of H^{*} and HO^{*} may be promoted by the surrounding water molecules, which can form a solvent cage (László, 2001). Dissolved O₂ might hinder this recombination reaction, resulting in an increase in the HO^{*} concentration ([HO^{*}]). However, no significant difference was found between the initial degradation rates of DICL in the presence or in the absence of O₂ either in PB solution or in Milli-Q water (Fig. 2). The only exception was the prolonged irradiation (t > 180 s) of solutions prepared in Milli-Q water, when the presence of O₂ slightly increased the degradation rate of DICL.

Previous investigations relating to the VUV photolysis of other nonsteroidal anti-inflammatory drugs confirmed that the concentrations



of H_2O_2 and HO_2'/O_2^- increase during the degradation of organic contaminants (Arany et al., 2012; Azrague et al., 2005; Robl et al., 2012). However, the reactivity of HO_2'/O_2^- is usually reported to be lower than that of H[•] (Gonzalez et al., 2004). The similarity between the degradation curves in oxygenated (the predominant radicals being HO_2'/O_2^- and $HO^•$) and deoxygenated solutions (the predominant species being H[•] and $HO^•$) suggests that the increased [HO[•]] under O_2 -saturated conditions is compensated by the difference in reactivity of H[•] and HO_2'/O_2^- . On the other hand, the slight increase in the DICL transformation rate after 180 s of irradiation in O_2 -saturated Milli-Q water indicates that the effect of HO_2^+ is considerable if its concentration is elevated. In contrast, the similarity of the DICL degradation curves in the presence and absence of O_2 in buffered solutions, even after longer irradiation times (Fig. 2), may be explained in terms of the low reactivity of O_2^- toward DICL, even at higher concentrations.

Dissolved O_2 significantly affected the formation and transformation of by-products. e.g. aliphatic acids could be detected only in oxygenated solutions (Fig. SF2). If HO[•] reacts with organic compounds (e.g. with DICL or its degradation by-products) through hydrogen abstraction or addition reactions, carbon-centered radicals (R[•]) form (Gonzalez et al., 2004; Legrini et al., 1993; Sosnin et al., 2006), which are scavenged by O_2 in oxygenated solutions, resulting in peroxyl radicals (ROO[•]). The formation of aliphatic acids has been interpreted in the reactions of ROO[•] resulting in cleavage of the aromatic rings (Getoff, 1996; Oppenländer, 2003). On the other hand, ROO[•] may also contribute to DICL degradation and could result in the increased transformation rate observed after 180 s of irradiation in Milli-Q water.

In Milli-Q water, the concentration of by-product A was higher in the presence of O_2 , while the concentrations of by-products B and C were higher in solutions purged with N_2 (Fig. 4a). It is likely that HO₂ contributed to the formation of the former and the transformation of the latter two compounds. The reactions of aromatic by-products with HO[•] and HO[•]_2/O⁻_2 (with unknown *k* values) might also contribute to the differences between the measured concentrations of by-products A–C.

In contrast, in PB-containing solutions the accumulation of aromatic by-products was more marked in the presence of O_2 (with the exception of by-product C, where no difference was seen) (Fig. 4b). In this case, the difference between the reactions of ROO[•] and R[•] (formed from the by-products in oxygenated and deoxygenated solutions, respectively) with $H_2PO_4^-$, $HPO_4^2^-$, $H_2PO_4^-$ and HPO_4^{--} might result in the difference between the accumulation of aromatic by-products in the presence and absence of O_2 (Fig. 4b).

Since the concentrations of aromatic by-products were higher in the presence of PB in oxygenated solutions (where HO_2^{\prime}/O_2^{-} is mainly present in the form of O_2^{-}) than in the samples prepared in Milli-Q water (where HO_2^{\prime}/O_2^{-} is mainly present in the form of HO_2^{\prime}) (Fig. 3a), the lower DICL degradation rate in the presence of phosphates may be



Fig. 5. The relative AOX content (y-axis) of DICL-containing solutions ($[DICL]_0 = 1.0 \times 10^{-4} \text{ mol L}^{-1}$) prepared in Milli-Q water as a function of the irradiation time (x-axis (s)), under oxygenated (\blacklozenge) or deoxygenated (\blacksquare) conditions.

explained by the probably lower reaction rates of DICL and its byproducts with O_2^{-} , relative to those of their reactions with HO₂.

During the degradation of DICL, various chlorine-containing (and therefore potentially toxic) by-products may form. Hence, the AOX contents of the solutions prepared in Milli-Q water ($[DICL]_0 = 1.0 \times 10^{-4} \text{ mol } L^{-1}$) were also measured. As demonstrated by Fig. 5, no significant difference was found between the rates of dehalogenation in the presence or the absence of O₂. This might be due to the similar initial degradation rates of DICL in oxygenated and deoxygenated solutions prepared in Milli-Q water (Fig. 2) and to the facts that by-product A was detected in higher concentration in the presence of O₂, while by-products B and C were more abundant in solutions purged with N₂ (Fig. 4a).

Since prolonged irradiation was needed to degrade the aromatic byproducts (1200–1500 s) as compared with the time (900 s) needed for the complete transformation of DICL (Figs. 2–4), it is not surprising that the TOC content of the solution was above 50% even after 900 s. Although no significant difference was observed at the beginning of the treatment between the rates of mineralization of DICL dissolved in Milli-Q water in the presence or in the absence of O₂, after 600 s (i.e. after almost complete transformation of DICL, Fig. 2), the essential role of O₂ became obvious (Fig. 6). After 2 h of VUV photolysis, virtually zero TOC content was observed in oxygenated DICL-containing solutions, which was in accordance with the almost complete degradation of the detected aliphatic acids within this treatment interval (Fig. SF2).

Although aliphatic acids could not be detected in solutions purged with N_2 , nearly 55% of the initial TOC content of the solution was detected even after 2 h of treatment. This would suggest that in deoxygenated solutions, some undetected recalcitrant by-products were formed. In the absence of O_2 , the recombination of the R^{*} formed in the reaction of DICL and HO^{*} is highly likely and may result in dimers and oligomers of DICL, analogously to the transformation of other organic contaminants (Gonzalez et al., 2004; Sosnin et al., 2006). The degradation of these compounds is much more difficult than that of the original molecule, which could explain the low efficiency of TOC loss in deoxygenated solutions (Fig. 6). The essential role of dissolved O_2 during the effective decontamination of DICL-containing solutions should therefore be underlined.

In the presence of dissolved O₂, ROO[•] may undergo recombination. The formation of tetroxides may be supported by the fact that the concentrations of the detected aliphatic acids reached their maxima after



Fig. 6. Time course of the mineralization and the proliferation-inhibiting ability during the VUV photolysis of DICL under O₂-saturated (\diamond) or deoxygenated (\Box) conditions. Filled symbols represent diminution of TOC compared to TOC₀ throughout the treatment (secondary y-axis (%)); open symbols correspond to the toxic potential of samples expressed as proliferation inhibition (%) on the primary y-axis. Significance levels correspond to: x: p < 0.05; y: p < 0.01; z: p < 0.001.

around 3000 s, although both DICL and the measured aromatic byproducts were completely transformed after 1500 s of irradiation (Figs. 2–4 and SF2). Thus, the source of malonic and oxalic acids should be other than DICL or by-products A–C, e.g. they could arise from tetroxides. The higher mineralization rate in this case (Fig. 6) might be explained by the different transformation pathways of tetroxides (e.g. via the Russell mechanism) (von Sonntag and Schuchmann, 1991).

3.3. The effects of the initial DICL concentration

If $[DICL]_0$ is fixed, the pseudo-first-order approach is suitable for a description of the degradation kinetics of the VUV photolysis of DICL. However, in oxygenated Milli-Q water, a decrease in k' was observed when $[DICL]_0$ was increased (Fig. 7). At higher $[DICL]_0$, more HO[•] is involved in reactions with DICL and $[HO^•]$ therefore decreases. Thus, our observation that $k' (= k \times [HO^•])$ decreases with the increase of $[DICL]_0$ can be explained by the decrease in $[HO^•]$ along with the constant value of k.

3.4. The possible chemical structures of the aromatic by-products

The HPLC–MS results permitted suggestions concerning the chemical structures of the aromatic by-products. In the negative ion mode, DICL was observed with an m/z value of 294, with two isotope peaks at 296 and 298, indicative of the replacement of one or two ³⁵Cl by ³⁷Cl (Fig. SF3). The m/z value of by-product A was found to be 310, with two isotope peaks at 312 and 314, suggesting that this compound also contains two Cl atoms (Fig. SF4). Since the difference between this m/z value and that of DICL is 16 and the UV absorbance spectrum of byproduct A is very similar to that of DICL (the maxima and minima in the absorbances of the two compounds are to be found at very similar wavelengths; Fig. 8), it is very likely that by-product A is a hydroxylated derivative of DICL.

Hydroxylation could occur on the aromatic rings, resulting in 5hydroxydiclofenac (A_1), 3-hydroxydiclofenac (A_2), 3'-hydroxydiclofenac (A_3) or 4'-hydroxydiclofenac (A_4) (Calza et al., 2006; Homlok et al., 2011; Landsdorp et al., 1990), on the second carbon atom of the acetic acid side-chain (A_5) (Calza et al., 2006) or on the nitrogen atom (A_6) (Huber et al., 2003) (Fig. 9). Although A_1 has been hypothesized to be the most probable structure during radiolysis and photo-Fenton treatment (Homlok et al., 2011; Pérez-Estrada et al., 2005a), the relative unselectivity of HO[•] (Sein et al., 2008) has been reported to lead to the formation of A_2 and A_4 together with A_1 during the H_2O_2/UV treatment and radiolysis of DICL (Vogna et al., 2004; Yu et al., 2013). Further



Fig. 7. Effects of the initial DICL concentration (x-axis (mol L^{-1})) on the apparent reaction rate constant (y-axis (s⁻¹)) of the VUV photolysis of oxygenated solutions prepared in Milli-Q water.



Fig. 8. UV absorbance of DICL, by-products A, B (primary y-axis (AU)) and by-product C (secondary y-axis, representing higher resolution (AU)) as a function of the wavelength (x-axis (nm)).

investigations are therefore needed to decide which structure corresponds to by-product A during the VUV photolysis of DICL.

The m/z value of by-product B (258) differed by 36 from that of DICL (294) and in this case only one isotope peak (m/z = 260) could be detected (Fig. SF5). These results and the obvious difference between the UV absorbance spectra of this compound and DICL (Fig. 8) suggested HCl elimination in this case and the formation of 1-(8-chlorocarbazolyl) acetic acid (Fig. 9; B), a well-known UV-photolytic and photocatalytic degradation product of DICL (Martinez et al., 2011; Moore et al., 1990; Petrovic and Barcelo, 2007).

The *m/z* value of by-product C (240) differed by 18 from that of byproduct B (258) (Fig. SF6). In this case, no isotope peaks were detected and the UV absorbance spectrum of this compound displayed marked similarities with that of by-product B (Fig. 8). It is likely therefore, that in this case the Cl atom in 1-(8-chlorocarbazolyl)acetic acid was substituted with an OH group to yield 1-(8-hydroxycarbazolyl)acetic acid, as proposed in the literature (Martinez et al., 2011; Moore et al., 1990; Petrovic and Barcelo, 2007) (Fig. 9; C).

3.5. The possible formation of aromatic by-products

Since HO[•] is an electrophilic radical, it usually attacks at the electrondense sites of aromatic rings, e.g. on carbon atoms 5, 3, 3' and 4' in DICL. Analogously to the mechanisms postulated for the formation of 5hydroxydiclofenac in HO[•]-initiated reactions (García-Araya et al., 2010; Homlok et al., 2011; Sein et al., 2008), Fig. 10 depicts HO[•] addition to 3 position in DICL, to result in a hydroxycyclohexadienyl-type radical. After the addition of an O₂ molecule and the elimination of a HO[•]₂, 3hydroxydiclofenac may be formed. The formation of hydroxylated byproducts is not likely in the absence of dissolved O₂. The fact that byproduct A was detected in significantly lower concentration both in the presence and in the absence of PB (Fig. 4b) in deoxygenated solutions as compared with the O₂-saturated conditions supports this assumption.

Our results suggest that O_2 addition and HCl elimination may be competitive processes as regards the transformation of the hydroxycyclohexadienyl-type radical. The latter process could result in ring closure and, after reaction with R^{*}, by-product B might be formed. A similar mechanism can be proposed for the formation of 1-(8-chlorocarbazolyl)acetic acid (B) as a result of the reaction of DICL with H^{*}, and by-product B might therefore also be formed in deoxygenated solutions (Fig. 10).

After the addition of HO[•] to by-product B in O₂-saturated solutions, a competition may again arise between [•]Cl elimination (to result in by-product C) and O₂ addition. Naturally, the latter process cannot occur in deoxygenated solutions (Fig. 10). This may be the reason for the higher concentration of by-product C in deoxygenated Milli-Q water than that under O₂ purged conditions (Fig. 4a).



Fig. 9. Possible chemical structures of by-products A, B and C.

3.6. Cell biological effects of VUV-treated samples on the freshwater ciliate Tetrahymena

Since both the direct phototransformations of PhACs and AOPs lead to the formation of complex mixtures of transformation products, these should be taken into account in assessments of the environmental risk of the parent compound or the efficiency of treatment technologies (Escher and Fenner, 2011; Fatta-Kassinos et al., 2011). With respect to this, in the present work the cell biological effects of whole VUV-treated samples were quantified by using a new, relatively simple and high-throughput, yet sensitive screening assay combination. Our aim was a rapid evaluation of the biological activity, taking into account the possible interactions occurring in these complex mixtures, rather than time-consuming and labor-intensive effect-directed sample analyses.

The choice of the freshwater ciliate *Tetrahymena* as a model is based on the fact that it is a member of the protozoon trophic level where the bioaccumulation of micropollutants is likely to take place (Gerhardt et al., 2010; Sanderson et al., 2003). Even if *Tetrahymena* assays have not yet been standardized, in studies focusing on freshwater or wastewater, this organism may be more relevant than the commonly used marine species *Vibrio fischeri* or *Artemia salina* (Fatta-Kassinos et al., 2011). Moreover, a study of the H₂O₂/UV photolysis of PhAC mixtures including DICL and quinolone antibiotics demonstrated that the treatment resulted in a reduced degree of toxicity toward algae, but not protozoa, which highlights the importance of taking the protozoon trophic level into account (Andreozzi et al., 2004).

The proliferation-inhibiting effect of the untreated sample $(2.5 \cdot 10^{-5} \text{ mol L}^{-1} \text{ DICL in PB})$ was ~13%, which was in accordance with our previous results (Láng and Kőhidai, 2012). Treated samples taken after definite periods of irradiation exerted slight, but significant proliferation-inhibiting effects that paralleled the chemical transformation of DICL, the formation of several by-products and the mineralization (Figs. 2–6 and SF2). Depending on the operating conditions applied (using O₂-saturated or deoxygenated solutions), the irradiation time vs. proliferation inhibition curves of 25% (v/v) diluted samples displayed different shapes, which permitted the distinction of three phases.

In Phase 1, on the irradiation of oxygenated samples for 10–300 s, the slight initial level of inhibition temporary increased to ~25% (90 s sample), and then returned to the starting value. Under deoxygenated conditions, however, the inhibitory effect decreased to almost 0% (after 90 s) before returning to the initial value. This may be related to the significantly higher amount of by-product A formed under O₂-saturated

conditions. These findings are in accordance with those of gamma radiolysis, indicating that the by-products formed under oxidative conditions are more toxic toward *V. fischeri* than those detected under reductive conditions (Yu et al., 2013).

In Phase 2, when the samples were treated for 600-1800 s, the level of inhibition stagnated at ~25–30% under both conditions, the oxygenated samples eliciting slightly stronger inhibition.

In Phase 3, when samples saturated with O_2 were irradiated for 2400-3600 s, the inhibitory potential exhibited a clear decreasing tendency, reaching only 8% at 3600 s. In contrast, in the case of the samples purged with N₂, no appreciable change was observed in the level of inhibition. These observations may be explained by the more efficient mineralization achieved under the oxygenated conditions. In this case, 70% mineralization was reached after 3000 s of treatment, in contrast with the ~25% under deoxygenated conditions. Further, the mineralization efficiency in O₂-saturated solutions increased to 75% after 3600 s, whereas in solutions purged with N₂ it did not exceed 45% after even 7000 s of irradiation. Moreover, this last phase of VUV treatment may be accompanied by the formation of di- and polymeric by-products that could not be detected with the applied analytical methods (Gonzalez-Rey and Bebianno, 2012; Sosnin et al., 2006), but which could contribute significantly to the mixture toxicity. A similar time course, but weaker effects were observed for the 5% (v/v) diluted samples, while the 1% (v/v) samples displayed significant toxicity in only 2 or 3 samples under oxygenated and deoxygenated conditions, respectively (Tables ST1 and ST2).

The maximal intermediate proliferation-inhibiting capacity under either condition (about 30%) was some 2 times higher than that of the parent compound, which is significantly lower than the other reported results. During the direct photolysis or photocatalytic degradation of DICL, for example (Rizzo et al., 2009; Schmitt-Jansen et al., 2007), the maximal toxic potential of the intermediate samples was 5 or 6-fold higher than that of the parent compound. The moderate toxicity enhancement encountered during VUV photolysis may also underline the adequacy of this technology.

Besides the proliferation-inhibiting effects of the treated samples, their impact in sublethal concentrations $(10^{-5\%} (v/v)-1\% (v/v))$ on the migratory response of *Tetrahymena* was also investigated. The use of such behavioral assays has certain advantages: behavioral changes, e.g. avoidance reactions, are in most cases 10–100 times more sensitive and less time-consuming indicators of the biological impact of a pollutant than are acute or chronic toxicity assays (Reinecke et al., 2002). The chemotaxis of *Tetrahymena* spp., i.e. the directed migratory response elicited by the gradient of a dissolved chemical, has been proposed by



Fig. 10. Possible pathway of formation of by-products A₂, B and C.

several authors as an indicator of water or soil contamination, because environmental pollutants often elicit a negative chemotactic reaction (i.e. they act as chemorepellents) (Gerhardt et al., 2010). We are not aware of any previous study in which a chemotactic response was utilized as an indicator for the biological assessment of samples generated by AOPs. Similarly as with toxicity profiles, differences in chemotactic character were observed, depending on the gas applied for purging.

Untreated samples in 1% (v/v) dilution exhibited a strong chemorepellent character (Chtx. Ind. = $50.0\% \pm 7.0\%$) and preserved this character throughout the whole concentration range studied ($47.6\% \pm 2.9\%$ < Chtx. Ind. < $79.0\% \pm 6.0\%$) (Tables ST3 and ST4). This was in agreement with previous findings when the chemorepellent effect of DICL was studied in a broad concentration range (Láng and Kőhidai, 2012). Similarly, treated samples acted predominantly as chemorepellents. Under both oxygenated and deoxygenated conditions, all samples at 1% (v/v) dilution induced a negative chemotactic response (Chtx. Ind. ranged from $48.0\% \pm 3.0\%$ to $84.0\% \pm 1.0\%$) with the exception of the neutral behavior of the O₂-saturated samples after irradiation for 150 s and 2400 s and the sample purged with N₂ and irradiated for 1500 s (Fig. 11). Although the very strong initial chemorepellent character decreased over time, even samples taken after irradiation for 3000 s or 3600 s elicited a marked negative chemotactic response under both O_2 and N_2 -purged conditions. However, there was no obvious trend in the evolution of the irradiation time vs. chemotactic effect curve for either condition: nonlinear multiphase curves were obtained. Under both oxygenated and deoxygenated conditions, the proportion of significantly chemorepellent samples decreased in parallel with the increase of the dilution factor (Tables ST3 and ST4).

In summary, the evaluation of the biological activity of photolysis samples suggests that O_2 -saturated conditions are more efficient in the elimination of the parent compound and the toxic degradation products generated. However, both cell proliferation and migratory responses may involve multiple potential biological signaling pathways (i.e. different membrane receptors and the respective downstream signaling cascades, mediated for example by cyclic adenosine monophosphate, cyclic guanosine monophosphate, phosphatidylinositol-3,4,5-triphosphate or Ca²⁺). Consequently,



Fig. 11. Migratory responses of *Tetrahymena* expressed in terms of the chemotaxis index (y-axis (%)) elicited by the VUV-treated samples (1 v/v %) after different periods of irradiation (x-axis (s)). Samples were generated under either O₂-saturated (\Diamond) or deoxygenated (\Box) conditions. Significance levels are: x < 0.05; y < 0.01; z < 0.001.

further investigations are needed to promote an understanding of the mechanisms of action of the individual transformation products and their possible interactions in their mixtures.

4. Conclusions

The similarities of the DICL degradation curves in the presence and the absence of dissolved O_2 suggest that the increased [HO[•]] under O_2 -saturated conditions is compensated by the difference in reactivity of H[•] and HO[•]_2/O⁻_2. The contribution of HO[•]_2 to the degradation of DICL or its by-products appears to be significant only if this radical accumulates, while the effects of O⁻_2 seem to be relatively less important.

Since the presence of phosphates affected mostly the formation and degradation of the by-products and influenced the transformation of DICL itself only slightly, it is very likely that their effects are due to the change in the solution pH. The radical-scavenging effect of PB is negligible both in oxygenated and in deoxygenated solutions.

During the VUV photolysis of DICL, some aliphatic acids and three major aromatic by-products (presumably a hydroxylated derivative of DICL, 1-(8-chlorocarbazolyl)acetic acid and 1-(8-hydroxycarbazolyl) acetic acid) were detected.

VUV photons are absorbed by a very thin water layer, and from a technological aspect are therefore not likely to be used in WWTPs; however, this AOP does have advantages. It is suitable for the preparation of ultrapure water (from pretreated water) and, as a "clean" and relatively simple method, it is useful for mechanistic investigations and for the study of the effects of various parameters (e.g. the concentration of dissolved O_2 or the initial concentration of the contaminant molecule). With this method the generated radical set is known, and suggestions may therefore be put forward concerning the effects of different parameters on the radical set and on the degradation of the target compound. These results could contribute to the optimization of other AOPs.

Our approach to the investigation of the biological effects of mixtures generated by the photoexposure of DICL for different periods allowed us to take into account the complex interactions that might occur in these multicomponent mixtures, containing numerous transformation products. With this rapid screening method, the impact of different operating conditions on the VUV photolysis of DICL and the efficiency of this technology were evaluated. The results suggest that the coupling of VUV photolysis with the rapid biological screening of treated samples will be valuable in elimination studies on other micropollutants from model waters.

Conflict of interest

Hereby, all authors of the manuscript entitled "Vacuum ultraviolet photolysis of diclofenac and the effect of the treated aqueous solutions on the proliferation and migratory responses of *Tetrahymena pyriformis*" disclose any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, their work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.scitotenv.2013.09.019.

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