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Diuron biodegradation in activated sludge batch reactors under aerobic and anoxic conditions

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ARTICLE INFO

Article history:
Received 1 October 2008
Received in revised form
18 December 2008
Accepted 20 December 2008
Published online 3 January 2009

Keywords:
Pesticides
Removal
Fate
Runoff waters
Phenylureas

ABSTRACT

Diuron biodegradation was studied in activated sludge reactors and the impacts of aerobic and anoxic conditions, presence of supplemental substrate and biomass acclimatization on its removal were investigated. Diuron and three known metabolites, namely DCPMU (1-(3,4-dichlorophenyl)-3-methylurea), DCPU (1-3,4-dichlorophenylurea) and DCA (3,4-dichloroaniline), were extracted by solid-phase extraction (dissolved phase) or sonication (particulate phase) and determined using High Performance Liquid Chromatography–Diode Array Detector (HPLC–DAD). During the experiments only a minor part of these compounds was associated with the suspended solids. Under aerobic conditions, almost 60% of Diuron was biodegraded, while its major metabolite was DCA. The existence of anoxic conditions increased Diuron biodegradation to more than 95%, while the major metabolite was DCPU. Mass balance calculation showed that a significant fraction of Diuron is mineralized or biotransformed to other unknown metabolites. The presence of low concentrations of supplemental substrate did not affect Diuron biodegradation, whereas the acclimatization of biomass slightly accelerated its elimination under anoxic conditions. Calculation of half-lives showed that under aerobic conditions DCPMU, DCPU and DCA are biodegraded much faster than the parent compound. In the future, the sequential use of anoxic and aerobic conditions could provide sufficient removal of Diuron and its metabolites from runoff waters.

1. Introduction

The presence of pesticides in the environment is a matter of particular concern for the conservation of ecosystems and the protection of human health. Among the several classes of pesticides, Diuron, *N*-(3,4-dichlorophenyl)-*N*,*N*-dimethyl-urea, is extensively used on many agricultural crops and non-crop areas at application rates up to 3.0 kg ha^{-1} year^{-1} (Giacomazzi and Cochet, 2004). As a result, it is often detected in groundwater and surface water (Blanchoud et al., 2004; Lapworth and Gooddy, 2006). Recent studies indicated the role of urban and agricultural runoff in Diuron transport to the environment. Revitt et al. (2002) detected concentrations of this compound as high as 238.4 μg L^{-1} in urban runoff after a storm event. Determination of herbicides in highway runoff showed Diuron concentration up to 10.78 μg L^{-1} (Huang et al., 2004). Rupp et al. (2006), studying Diuron loss in surface runoff from two grass-seed fields, determined concentrations ranging from 120 to more than 1000 μg L^{-1} during the first large rainfall events. Diuron concentrations were significantly decreased over time, reaching levels of few μg L^{-1} during a period of 130 d after its application. Moreover, Stork et al.

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doi:10.1016/j.watres.2008.12.040

(2008) studied the fate of Diuron in a sugarcane farm and reported that during a storm event the average Diuron concentration in runoff waters was $93 \pm 23\ \mu g\ L^{-1}$.

Diuron has been listed as a priority substance in the Water Framework Directive (EU, 2001). Moreover, recent studies have shown that it is toxic to photosynthetic organisms at concentrations levels of a few $\mu g\ L^{-1}$ (Huang et al., 2005; Gatidou and Thomaidis, 2007). According to the above, the need for removing Diuron from agricultural and urban runoff is very intense and presents high importance. Previous studies have reported the aerobic biodegradation of Diuron in soil and the production of metabolites such as DCPMU (1-(3,4-dichlorophenyl)-3-methylurea), DCPU (1-3,4-dichlorophenylurea) and DCA (3,4-dichloroaniline) (Gooddy et al., 2002; Sorensen et al., 2003). Moreover, recent studies have indicated Diuron mineralization by pure cultures of microorganisms or two-member bacterial consortiums (Bazot et al., 2007; Bazot and Lebeau, 2008; Sorensen et al., 2008). Beside of the above, so far, there is a lack of data regarding the application of biological treatment processes, based on mixed cultures of microorganisms, for the removal of Diuron from contaminated water. Data available in the open literature have been mainly focused on the use of advanced oxidation processes (AOPs) solely (Maldonado et al., 2007) or coupled with biological processes (Lapertot et al., 2007) for Diuron removal from water. Despite the strong potential of AOPs for treating hazardous organic compounds, it is well known that operating costs for oxidation of xenobiotics remain relatively high compared to biological treatment processes. On the other hand, the activated sludge process has been extensively used for the biodegradation of pesticides (Kipopoulou et al., 2004; Celis et al., 2008). According to previous studies (Grady, 1985; Mangat and Elefsiniotis, 1999; Stasinakis et al., 2005), several parameters such as the existence or not of aerobic conditions, the presence of supplemental substrate, the acclimatization of biomass and the initial concentration of the toxic compound seem to affect biodegradation of synthetic organic compounds in activated sludge systems.

The main objective of this study was to investigate the use of biological treatment processes for Diuron elimination by contaminated water and to study the factors that affect its fate and enhance its possible biotransformation. For this reason, activated sludge batch biodegradation experiments were conducted at Diuron concentration levels similar to those reported in the literature for runoff waters. The effect of aerobic and anoxic conditions, biomass acclimatization and presence or absence of supplemental substrate on Diuron biodegradation potential was investigated. Biodegradation experiments were also performed under aerobic conditions for DCPMU, DCPU and DCA. Mass balances were calculated in batch reactors and half-lives were calculated for all the compounds. In the aforementioned experiments, Diuron and its metabolites were detected in the dissolved and particulate phase using High Performance Liquid Chromatography–Diode Array Detector (HPLC–DAD). Although several studies have reported the determination of Diuron in wastewater or sludge samples using Liquid Chromatography with either Mass Spectrometry (Ghanem et al., 2008) or UV detection (Maldonado et al., 2007), to the best of our knowledge, there is no analytical method available for the simultaneous determination of both Diuron and its main metabolites DCPMU, DCPU and DCA in wastewater and mixed liquor samples. For this reason full validation of the analytical methods was performed for dissolved and particulate phase samples.

2. Materials and methods

2.1. Chemicals

Analytical standards of Diuron (97.7%), DCPMU (97.5%), DCPU (99%) and DCA (99%) were supplied by Dr Ehrenstorfer-Schafers (Germany). The chemical structures of the target compounds are shown in Figure S1. Stock and working solutions of the studied compounds were prepared in methanol HPLC grade (Merck, Germany) and kept at −18 °C. Acetonitrile (ACN) was of HPLC grade (Merck, Germany), while HPLC grade water was prepared in the laboratory with a MillQ/MillRo system (MilliPore, USA). All the other chemicals were of analytical grade and were purchased from Merck (Germany).

2.2. Activated sludge cultivation

Four parallel laboratory-scale sequencing batch reactors (SBRs) were used to simulate activated sludge process and to provide biomass for the biodegradation experiments. Two of them were operated in aerobic mode (SBR_A and SBR_B), while the others in anoxic mode (SBR_C and SBR_D). All systems were operated in a 24-h fill and draw cycle; each cycle consisted of four stages: FILL (10 min), REACT (22.5 h with aeration), SETTLE (1 h) and DECANT (20 min), while their liquid volume was 2 L (Stasinakis et al., 2005).

Activated sludge from a municipal wastewater treatment plant (University of the Aegean, Lesvos) was used to seed the reactors, while municipal wastewater originating from the aforementioned plant was used as feed. The main characteristics of the influent wastewater were COD $590 \pm 167\ mg\ L^{-1}$, NH_4-N $31.3 \pm 4.7\ mg\ L^{-1}$ and pH 7.35 ± 0.32. For the supply of nitrates in anoxic SBRs, a solution of $NaNO_3$ was also used, providing concentrations of NO_3-N equal to $40.2 \pm 3.3\ mg\ L^{-1}$ to the influent wastewater. To acclimatize biomass to Diuron, a known amount of this compound was daily added to the influents of SBR_B and SBR_D, so as to achieve a concentration of $10\ \mu g\ L^{-1}$.

Throughout the study, all systems were maintained at 21.2 ± 1.8 °C, while sludge residence time (SRT) was kept constant at 8 days by wasting daily the appropriate amounts of biomass from each system. Aeration and efficient mixing in aerobic SBRs were provided using porous ceramic diffusers, while biomass remained in suspension in anoxic SBRs using a mechanical shaker. As a result, dissolved oxygen (DO) in aerobic reactors was kept above $4.0\ mg\ L^{-1}$, while DO in anoxic reactors was lower than $0.3\ mg\ L^{-1}$.

2.3. Batch experiments

Aerobic batch experiments were performed to investigate Diuron, DCPMU, DCPU and DCA biodegradation by activated

sludge (Table 1). After operating SBRs for a period of 32 days (4 SRT), activated sludge samples were introduced from SBR_A into four stoppered, dark, conical flasks (Table 1, Batch Experiments: A1, A4, A6, A8) to investigate target compounds' fate under aerobic conditions. All reactors were put on a shaker bath at 22.0 ± 2 °C and an aliquot of Diuron, DCPMU, DCPU or DCA solution in methanol was added to provide an initial concentration of 50–90 μg L^{-1}. The final working volume in batch reactors was 400 mL, while the concentration of mixed liquor volatile suspended solids (MLVSS) ranged between 1000 and 1200 mg L^{-1}. Aeration was supplied using porous ceramic diffusers. To quantify target compound biodegradation, homogenized samples of 10 mL of mixed liquor were collected periodically for a period of 8–13 days. The concentrations of the parent compounds as well as their metabolites were determined in the dissolved and particulate phase, using the analytical method described below. The above experiments were repeated in the absence of biomass to investigate the effect of abiotic conditions on target compounds removal (Table 1, Batch Experiments: A2, A5, A7, A9). In these cases, samples were collected at the beginning and at the end of the experiments. To investigate the effect of supplemental substrate on Diuron biotransformation, a similar experiment was performed in the presence of substrate (Table 1, Batch Experiment: A3). For this reason, a small amount of wastewater (5–10 ml) was daily added to the batch reactor to achieve a final concentration of 12.5 mg L^{-1} COD. The role of biomass acclimatization on Diuron fate was studied in a batch experiment with acclimatized biomass, originating from SBR_B (Table 1, Batch Experiment: B1).

Anoxic batch experiments were also performed to investigate Diuron biotransformation by non-acclimatized biomass, acclimatized biomass and in the presence of supplemental substrate (Table 1, Batch Experiments: C1, D1 and C3, respectively). The reactors were purged with N_2 gas to ensure anoxic conditions. For the addition of supplemental substrate, wastewater and a solution of $NaNO_3$ were daily added to the batch reactor C3 (Table 1) to achieve a final concentration of 12.5 mg L^{-1} COD and 30 mg L^{-1} NO_3-N.

Table 1 – Experimental protocol used in batch experiments (the initial concentration of the target compounds ranged between 50 and 90 μg L^{-1}, the pH was 7.4 ± 0.3 and the concentration of mixed liquor volatile suspended solids (MLVSS) in biotic experiments ranged between 1000 and 1200 mg L^{-1}).

SBR	Batch Experiment	Constituents	Conditions
SBR_A	A1	Biomass + Diuron	Aerobic
	A2	H_2O + Diuron	Aerobic
	A3	Biomass + WW + Diuron	Aerobic/ Substrate
	A4	Biomass + DCPMU	Aerobic
	A5	H_2O + DCPMU	Aerobic
	A6	Biomass + DCPU	Aerobic
	A7	H_2O + DCPU	Aerobic
	A8	Biomass + DCA	Aerobic
	A9	H_2O + DCA	Aerobic
SBR_B	B1	Biomass + Diuron	Aerobic/ Acclimatization
SBR_C	C1	Biomass + Diuron	Anoxic
	C2	H_2O + Diuron	Anoxic
	C3	Biomass + WW + Diuron	Anoxic/ Substrate
SBR_D	D1	Biomass + Diuron	Anoxic/ Acclimatization

WW: wastewater.

2.4. *Analytical methods*

To control the operation of SBRs, analyses of influent and effluent COD, NH_4-N, NO_3-N, suspended solids in the effluents (SS_{out}) and MLVSS were periodically performed, according to Standard Methods (APHA, 1998). Moreover, temperature, DO and pH values were measured daily in all systems using portable instruments.

For the investigation of target compounds fate in SBRs and batch experiments, samples were filtered through pre-ashed glass fiber filters (GF/F, pore size 0.7 μm, Whatman, England). Filtrates were stored in the dark at 4 °C overnight, while for the determination of the target compounds in the particulate phase, filters were oven dried until constant weight and stored at −18 °C. Samples extraction and analysis were always performed the next day.

Isolation of the target compounds from the dissolved phase was performed using a solid-phase extraction (SPE) procedure. Analysis was based on a method developed by Gatidou et al. (2005) for the determination of Diuron and its metabolites in seawater after optimization and full validation. OASIS HLB cartridges were conditioned by 2 × 5 mL of methanol and 2 × 5 mL of MilliQ grade water. The samples (100 mL) were passed through the cartridges with a flow rate of 10 mL min^{-1}. In order to remove any impurities, the cartridges were washed with 4 × 2.5 mL MilliQ grade water and then air-dried for 10 min. The compounds were eluted with 3 × 2 mL of methanol. The eluates were reduced in volume to 500 μL under a gentle stream of nitrogen (40 °C) and reconstituted to 1 mL by adding 500 μL of the initial mobile phase (20% ACN – 80% MilliQ grade water).

For the determination of the target compounds in the particulate phase a previously developed method for analysis of endocrine disrupting compounds in sludge samples was used (Gatidou et al., 2007). Filters (20 mg dw) were ultrasonicated at 50 °C for 45 min using 8 mL mixture of methanol (5 mL) and MilliQ grade water (3 mL) as the extraction solvent. The supernatant was collected, diluted to a final volume of 100 mL with MilliQ grade water and extracted according to the aforementioned SPE procedure.

Chromatographic analysis was performed by a Shimatzu (Japan) LC-20AD prominence liquid chromatographer associated with a SPD-M20A prominence diode array detector and an SIL-20AC auto sampler. The column was a Zorbax SB–C18 4.6 mm × 15 cm (5 μm) connected with a Zorbax SB–C18 pre-column (Agilent, USA). The column and pre-column were heated at 40 °C with a CTO-20AC column oven (Shimatzu, Japan). The mobile phase consisted of ACN (solvent A) and MilliQ grade water (solvent B). Gradient elution was performed as follows: from 20% ACN to 100% ACN in 23 min. Flow rate was 1.7 mL min^{-1}. The diode array detector (DAD) was set at 244 nm for Diuron and its metabolites. The identification of the four substances in the samples was accomplished on the

Table 2 – Analytical characteristics of the optimized HPLC–DAD methods for the determination of target compounds in wastewater (dissolved and particulate phase): calibration equations of the analytical procedure and correlation coefficients (R^2) for concentration ranged from 50 to 1000 $\mu g\,L^{-1}$ and 50 to 1000 $ng\,g^{-1}$ for each analyte; methods limits of detection (LODs).

Compound	Calibration Equation (dissolved phase)	R^2	LOD ($\mu g\,L^{-1}$)	Calibration Equation (particulate phase)	R^2	LOD ($\mu g\,g^{-1}$)
Diuron	$y = 244.24x - 1041.8$	0.999	0.07	$y = 221.62x - 349.18$	0.990	0.36
DCPMU	$y = 261.46x - 2147$	0.999	0.08	$y = 234.36x - 568.47$	0.990	0.67
DCPU	$y = 261.63x - 3182.5$	1.000	0.10	$y = 231.66x - 1779$	0.990	0.53
DCA	$y = 85.35x + 703.8$	0.993	0.77	$y = 34.473x + 2774.9$	0.982	4.11

basis of their retention times and by comparison between the UV spectrum of the compounds in the standard solutions and the UV spectrum of the detected peaks in the samples. A match equal or higher than 99% was fixed to confirm identification between both spectra for all the compounds.

2.5. *Validation data*

Validation of the analytical methods included analytical methods calibration, determination of limits of detection (LODs), assessment of precision and evaluation of trueness for both dissolved and particulate phase samples.

Analytical methods calibration was carried out for concentrations ranging from 50 to 1000 $\mu g\,L^{-1}$ and from 50 to 1000 $ng\,g^{-1}$ for the dissolved and particulate phase, respectively. Three replicates were performed per concentration and analyte. As shown in Table 2, the response of diode array detector was linear for all the target compounds with coefficients of correlation, $R^2 > 0.99$. For the determination of methods LODs, 100 mL of wastewater and about 20 mg of particulate matter were extracted. The LOD of each compound for the two types of samples was determined as three times the standard deviation of eight independent replicate analyses (Table 2). For dissolved samples, the obtained LODs ranged from 0.07 (Diuron) to 0.77 $\mu g\,L^{-1}$ (DCA), whereas for particulate samples the LODs varied between 0.36 (Diuron) and 4.11 $\mu g\,g^{-1}$ dw (DCA). The achieved LODs were adequate for environmental monitoring of the target compounds and low enough, taking into account the complexity of the samples and the low sample amounts used especially for particulate samples.

For both type of samples, precision was assessed by performing repeatability and reproducibility experiments. For repeatability experiments, six replicates of a sample were spiked at a level of 100 $\mu g\,L^{-1}$ (dissolved phase) and 0.5 $\mu g\,g^{-1}$ (particulate phase) of the target compounds and were analyzed during 1 day ($n = 6$, intra-day precision). For reproducibility experiments, three replicates of dissolved and particulate samples were spiked at the same level as above and were analyzed at three different days ($k = 3$) over a period of 1-week (inter-day precision). According to the results (Table S1), satisfactory precision of the analytical procedures was achieved in both substrates, while relative standard deviations' (RSDs) values were less than 13% for all the compounds.

In order to evaluate the trueness of the methods, recovery experiments were performed at three fortification levels for each compound. Satisfactory recoveries were achieved ranging from 94% (Diuron) to 112% (DCA) and from 73% (DCPU) to 108% (DCA) for dissolved and particulate samples (Table 3), respectively.

2.6. *Calculations of half-lives*

The half-lives of the target compounds in aerobic and anoxic batch experiments were estimated using first-order (Eqs. (1) and (2)) and zero-order kinetics (Eqs. (3) and (4)), respectively. The correlation coefficients (R^2) for the regression lines ranged between 0.80 and 0.99 for the different experiments.

$$C_t = C_0 e^{-kt} \quad (1)$$

$$t_{1/2} = \frac{\ln 2}{k} \quad (2)$$

$$C_t = C_0 - kt \quad (3)$$

$$t_{1/2} = \frac{C_0}{2k} \quad (4)$$

where C_t and C_0 are the total (dissolved + particulate) target compound concentration in the reactor at time t and $t = 0$, respectively, ($\mu g\,L^{-1}$), k is the degradation coefficient (d^{-1}) and $t_{1/2}$ is the half-life (h).

Table 3 – Mean recoveries (%) and standard deviations ($n = 3$) of the target compounds in spiked samples at different fortification levels.

Compound	Dissolved Phase			Particulate Phase		
	1 $\mu g\,L^{-1}$ Recovery (%)	10 $\mu g\,L^{-1}$ Recovery (%)	20 $\mu g\,L^{-1}$ Recovery (%)	0.1 μg Recovery (%)	0.5 μg Recovery (%)	1 μg Recovery (%)
Diuron	93.7 ± 3.2	98.3 ± 1.7	95.3 ± 5.6	85.8 ± 8.6	79.6 ± 6.3	76.5 ± 8.7
DCPMU	97.8 ± 2.5	101.5 ± 1.0	95.6 ± 5.7	86.7 ± 5.3	75.3 ± 6.3	76.7 ± 8.7
DCPU	95.9 ± 10.4	99.0 ± 1.4	94.7 ± 5.3	81.1 ± 8.7	72.9 ± 7.6	75.1 ± 8.5
DCA	99.7 ± 5.4	112.0 ± 2.2	102.8 ± 7.5	108.0 ± 9.7	92.5 ± 6.9	83.6 ± 10.8

3. Results and discussion

3.1. Biomass cultivation

During biomass cultivation, significant removal of dissolved COD (COD_{dis}) and efficient suspended solids clarification were achieved in all SBRs (Table S2). Specifically, the reduction of COD_{dis} was greater than 91%, while the concentration of SS_{out} was lower than 25 mg L^{-1}. In aerobic reactors, more than 88% of the NH_4-N was removed, while in anoxic SBRs, NO_3-N removal was greater than 74% (Table S2). No deterioration of SBR_B and SBR_D performance was noticed in the presence of 10 μg L^{-1} Diuron (Table S2). So far, there are no data available on the potential of Diuron toxicity to affect/disrupt the activated sludge process. However, Sumpono et al. (2003), investigating Diuron effects on wastewater treatment ponds, reported that in the presence of much higher Diuron concentration (10 mg L^{-1}), COD and NH_4-N removal decreased from 73.3 to 63.7% and from 83.5 to 59.5%, respectively. Determination of Diuron and its metabolites in treated wastewater originating from SBR_B and SBR_D showed a slight decrease of Diuron (<15%) as well as the existence of DCPMU, DCPU and DCA in concentrations ranging from 0.5 to 3.5 μg L^{-1} (data not shown).

3.2. Aerobic biodegradation experiments

The behavior of Diuron was studied in parallel batch reactors, containing a known concentration of the target compound. Neither transformation of Diuron to other chemical species due to abiotic causes, nor adsorption on the conical flask walls was observed in the experiment conducted in the absence of biomass. According to the literature, Diuron has a very slow rate of abiotic hydrolysis in neutral conditions and temperature of 25 °C (Giacomazzi and Cochet, 2004), suggesting that chemical degradation is of minor importance.

Determination of Diuron in batch experiment performed in the presence of biomass showed that twenty-four hours after the start of the experiment, the greatest amount of Diuron remained in the dissolved phase, while a small part (<10%) was accumulated on the suspended solids (Fig. 1). This observation is consistent with previous data regarding Diuron hydrophobicity. According to the literature, Diuron has a low to moderate octanol–water partition coefficient ($\log K_{ow} = 2.6$) (Giacomazzi and Cochet, 2004). Moreover, Voulvoulis et al. (2002), investigating Diuron partitioning in seawater, reported that after 24 h contact time Diuron removed from the dissolved phase to a percentage ranging between 4 and 10%.

During the following hours, Diuron concentration in the particulate phase remained almost constant, while its concentration in the dissolved phase was gradually decreased (Fig. 1). As a result, up to the end of the experiment (312 h), almost 58% of initial Diuron had been eliminated. Based on the fact that Diuron is not volatile (Giacomazzi and Cochet, 2004) and photodegradation was prohibited by the experimental conditions used, biodegradation was the major process governing its loss. The ability of activated sludge to biodegrade Diuron was also confirmed in aerobic experiments performed in the presence of supplemental substrate or using

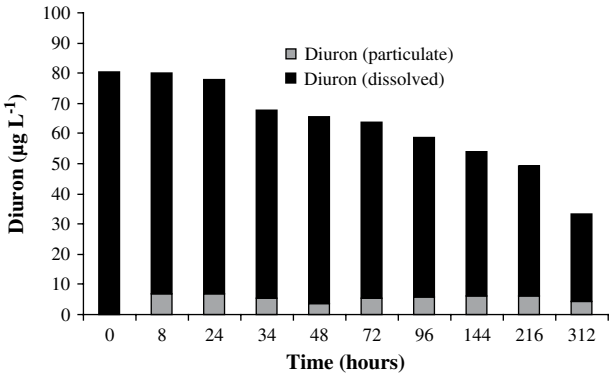

Fig. 1 – Fate of Diuron in aerobic batch reactor in the absence of supplemental substrate and using non-acclimatized biomass (Batch Experiment: A1).

acclimatized biomass. Specifically, determination of total (dissolved + particulate) Diuron concentration showed that 63% of this compound was biodegraded in the presence of supplemental substrate (Figure S2a), while 53% was biodegraded by acclimatized activated sludge (Figure S2b). Investigation for the existence of Diuron metabolites revealed the presence of low concentrations of DCPMU, DCPU and DCA in all aerobic experiments (Figure S2a–c).

To the best of our knowledge, so far there is only one study regarding Diuron biodegradation by activated sludge. Specifically, Lapertot and Pulgarin (2006) used Zahn–Wellens method to estimate biodegradability of Diuron and they reported that no DOC was removed during the test (28 days). However, in that test a significant higher initial concentration of the target compound was used (50 mg L^{-1}). Moreover, cellular lysis was observed in almost 60% of the bacterial population due to the presence of Diuron. According to the literature (Grady, 1985; Kawai et al., 1998), the biodegradation potential of xenobiotics compounds is affected by the initial concentration used in biodegradation experiments. In the presence of high initial concentrations, toxic effects of the parent compound or its metabolites can be observed, preventing the growth of microorganisms required for their degradation. In spite of the lack of data for the biodegradation of Diuron in activated sludge systems, previous studies in soil or using specific bacteria and fungi have also shown that Diuron can be biodegraded under aerobic conditions (Giacomazzi and Cochet, 2004; Cederlund et al., 2007). This is believed to occur by successive demethylation of the urea groups, followed by hydrolysis to give DCA and via direct formation of DCA (Widehem et al., 2002; Sorensen et al., 2003).

To investigate the ability of activated sludge for Diuron metabolites biodegradation, similar experiments were performed using three batch reactors initially fed on DCPMU, DCPU and DCA, respectively. The objective of these experiments was to provide a first insight on the fate of Diuron metabolites in completely aerated activated sludge systems. Similarly to Diuron, determination of these compounds in the dissolved and particulate phase indicated that during the experiments only a small part (<15%) was accumulated on the suspended solids, while their major part was detected on the dissolved phase. As it can be seen in Fig. 2a–c, almost

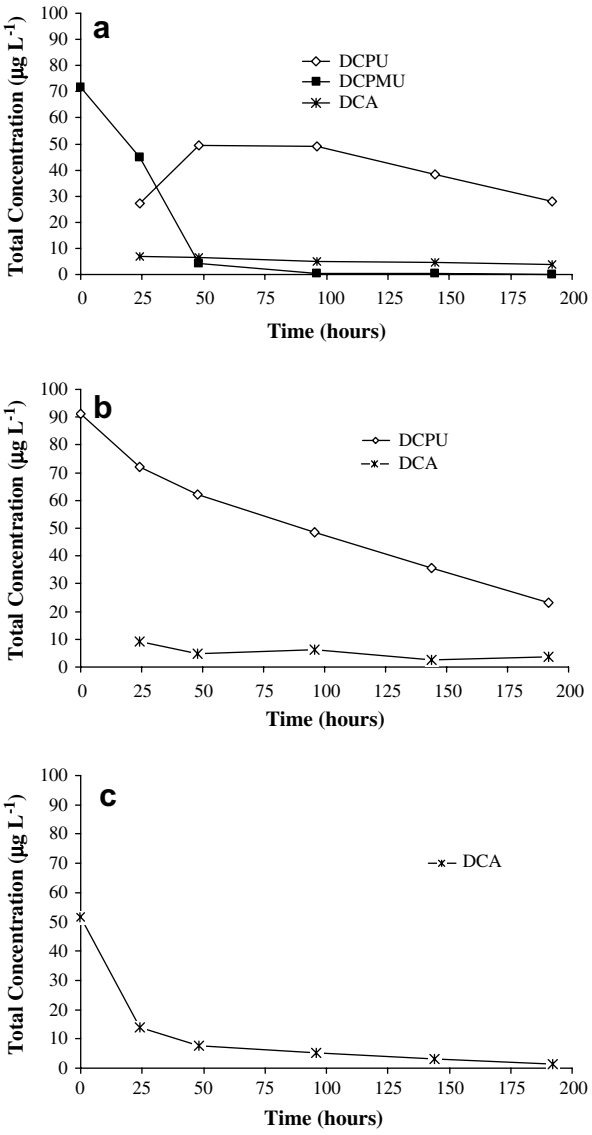

Fig. 2 – (a) DCPMU biodegradation in aerobic batch reactor containing non-acclimatized biomass (Batch Experiment: A4), (b) DCPU biodegradation in aerobic batch reactor containing non-acclimatized biomass (Batch Experiment: A6), (c) DCA biodegradation in aerobic batch reactor containing non-acclimatized biomass (Batch Experiment: A8).

99%, 75% and 96% of DCPMU, DCPU and DCA was biodegraded up to the end of the experiments (192 h). The decrease of DCPMU was rapid and it was accompanied by simultaneous increase of DCPU (Fig. 2a). This observation is consistent with the literature as the demethylation of DCPMU to DCPU under aerobic conditions has already been reported (Giacomazzi and Cochet, 2004). Regarding DCPU, it has been reported that this compound can be biotransformed to DCA via hydrolysis (Giacomazzi and Cochet, 2004). However, in batch reactors A4 and A6, concentration of DCA was almost constant from the start of the experiments and it was not increased with DCPU removal (Fig. 2a, b). This observation indicated that possibly another metabolite of DCPU was formed under aerobic conditions. Moreover, according to the results of these experiments, it is possible that microorganisms cannot degrade DCA at concentrations lower than those recorded in biodegradation tests. Regarding DCA, its biodegradation under aerobic conditions has been reported leading to mineralization or formation of several metabolites (e.g. dichlorocatechol, 3,4-dichloroacetanilide, *N*-(3,4-dichlorophenyl)-β-ketoglutanyl-δ-amide and dimers) (Sandermann et al., 1998; Giacomazzi and Cochet, 2004).

The half-lives obtained for Diuron in aerobic activated sludge experiments (Table 4) were significantly lower than those previously reported for soil (Barra Caracciolo et al., 2005; Cederlund et al., 2007), marine sediment (Thomas et al., 2003) and constructed wetlands (Rose et al., 2006), indicating the potential of activated sludge to biodegrade Diuron. Regarding the other target compounds, a similar half-life value has been reported for DCA in soil experiments (Barra Caracciolo et al., 2005), while half-lives of 1 day and 3 days have been reported for DCPMU and DCPU in marine sediment, respectively (Thomas et al., 2003).

3.3. *Anoxic biodegradation experiments*

Investigation of Diuron biodegradation under anoxic conditions showed that only 2% of the parent compound remained at the end of the experiment (312 h), while the rest was biodegraded (Fig. 3). Determination of Diuron possible metabolites indicated the rapid formation of DCPU, DCPMU and DCA. Amongst them, DCPU was the major metabolite, presenting an increasing trend during the last hours of the experiment (Fig. 3). Similar results were also obtained in anoxic experiment performed in the presence of supplemental substrate (Figure S3a). The use of acclimatized biomass seems to slightly accelerate Diuron removal and DCPU formation. As a result, 264 h after the start of the experiment, almost 96% of Diuron was removed, while high concentration of DCPU was determined (Figure S3b). Calculation of Diuron half-lives showed

Table 4 – Estimated time required for 50% degradation of the target compounds in activated sludge batch experiments.

Target Compounds	Time required for 50% degradation of target compound (hours)	
	Aerobic conditions[d]	Anoxic conditions[e]
Diuron[a]	277	165
Diuron[b]	286	178
Diuron[c]	290	140
DCPMU[a]	19	Not determined
DCPU[a]	103	Not determined
DCA[a]	44	Not determined

a Experiments performed using non-acclimatized biomass.
b Experiments performed using non-acclimatized biomass in the presence of supplemental substrate.
c Experiments performed using acclimatized biomass.
d First-order kinetics was used.
e Zero-order kinetics was used.

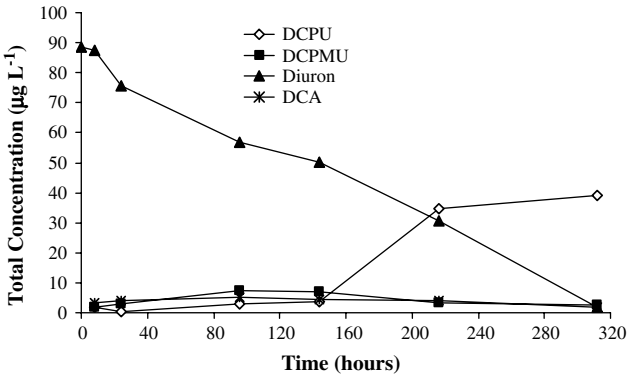

Fig. 3 – Diuron biodegradation in anoxic batch reactor containing non-acclimatized biomass (Batch Experiment: B1; absence of supplemental substrate).

that its degradation under anoxic conditions was faster comparing to aerobic conditions (Table 4). To the best of our knowledge, so far there are no data for the role of anoxic conditions on Diuron removal.

So far, microorganisms capable of degrading Diuron through metabolic as well as co-metabolic pathways have been isolated (Widehem et al., 2002; Giacomazzi and Cochet, 2004). Co-metabolism exists when a chemical can be biodegraded only in the presence of other organic compounds acting as growth substrates, while catabolic metabolism prevails when the existing organic compound act as source of carbon and energy for microorganisms (Grady, 1985). In cases that degradation is growth-linked, an accelerated degradation rate with time is expected due to increase of the number or/and activity of specific microorganisms responsible for toxic compounds biodegradation (Alexander, 1999). In the present study, under anoxic conditions, increasing rates of Diuron degradation with time were observed (Figure S3a), whereas, the use of acclimatized biomass stimulated Diuron degradation rates (Figure S3b). These data indicate that catabolic metabolism seems to be the main mechanism for Diuron biodegradation under anoxic conditions. However it has to be underlined that due to the low Diuron concentrations initially added (50–90 μg/L), a small increase in bacteria concentration is expected within the duration of the batch experiments and therefore these results cannot be conclusive. Further research should be performed in order to investigate how the addition of wastewater affects the subset of the microbial community which is responsible for Diuron degradation in activated sludge systems.

Regarding the effect of acclimatization, previous studies have shown that biodegradation of xenobiotics compounds is enhanced in the presence of acclimatized biomass (Mangat and Elefsiniotis, 1999; Stasinakis et al., 2005). In the present study, a slightly positive effect was noticed in anoxic experiments, whereas no effect was observed in aerobic experiments (Table 4). Moreover, in all biodegradation experiments a rapid decrease of Diuron was noticed and a very short lag phase (<24 h). During lag phase, microorganisms are acclimatized to the toxic compound or/and the number of specific microorganisms is increased until it reaches a threshold value which is appropriate for toxic compound biodegradation (Alexander, 1999). The short lag phase observed in this study could also be due to relatively low concentrations of Diuron initially added. Previous researchers have reported that the lag time observed prior to the start of pesticides degradation is affected by the initial concentration of the target compound and higher initial concentrations result to longer lag phases (Greer et al., 1990).

3.4. *Calculation of mass balances*

Mass balances on the basis of molarity were calculated at the end of the experiments for aerobic and anoxic batch reactors. Under aerobic conditions, the major metabolite of Diuron was DCA (Fig. 4a), while under anoxic conditions the major metabolite was DCPU (Fig. 4b). With the exception of the anoxic batch experiment with acclimatized biomass (Batch Experiment: D1), significant fractions of Diuron ranging from 30 to 49% could not be accounted of in all Diuron biodegradation experiments (Fig. 4; Table S3). Similar observations for partially loss of initially added Diuron have already been reported in previous studies in lysimeters or using fungal strains (Tixier et al., 2000; Guzzella et al., 2006). This loss of Diuron could be explained by mineralization to CO_2 or/and formation of metabolites that were not analyzed in the present study. So far, the degradation processes leading to Diuron mineralization have not been completely clarified; however, recent studies suggested the involvement of

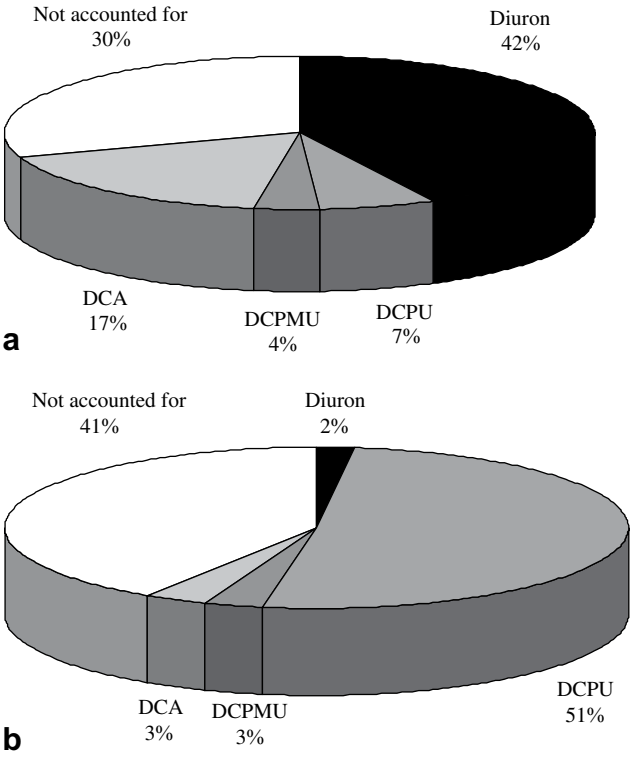

Fig. 4 – (a) Mass balance in aerobic batch reactor in the absence of supplemental substrate and using non-acclimatized biomass (Batch Experiment: A1), (b) Mass balance in anoxic batch reactor in the absence of supplemental substrate and using non-acclimatized biomass (Batch Experiment: C1).

a bacterial consortium, rather than single strains (Bazot et al., 2007; Bazot and Lebeau, 2008; Sorensen et al., 2003, 2008). Based on this fact, a mixed culture of microorganisms such as activated sludge could possibly enhance Diuron mineralization. However, further experiments should be performed to confirm this hypothesis.

So far, there are few studies comparing toxicity of Diuron and its metabolites and in most cases the published results are contradictory. Gatidou and Thomaidis (2007) reported that Diuron was much more toxic than DCPMU, DCPU and DCA on phytoplanktonic microorganisms. On the other hand, Tixier et al. (2001), using marine bacterium *Vibrio fischeri*, reported that Diuron metabolites presented higher toxicity than the parent compound. According to the results of this study and independently of the toxicity of the studied compounds, it seems that the best biological process for the satisfactory treatment of runoff water containing Diuron could be the sequential use of anoxic and aerobic conditions. Under anoxic conditions, Diuron could be rapidly eliminated and biotransformed mainly to DCPU, while afterwards the existence of aerobic conditions could enhance biodegradation of produced DCPU. Having in mind that runoff waters are usually highly contaminated with nitrogen, mainly in the form of nitrates and secondarily as ammonium, the aforementioned treatment processes could also offer satisfactory nitrogen removal via the mechanisms of nitrification and denitrification.

4. Conclusions

The present study demonstrated that, for the concentrations tested, Diuron could be biodegraded in activated sludge reactors operating on aerobic and anoxic mode. In all experiments, the role of sorption to biomass was not significant, while under anoxic conditions the main mechanism of Diuron biodegradation seems to be catabolic metabolism. The use of acclimatized biomass under anoxic conditions enhanced Diuron biodegradation and as a result, almost 50% of Diuron was degraded in a period of 140 h. Calculation of mass balances showed that at the end of the experiments Diuron was detected as DCPMU, DCPU and DCA, while almost 30–49% had been mineralized or biotransformed to other unknown metabolites. Under aerobic conditions, Diuron metabolites were biodegraded faster than the parent compound. Half-lives equal to 19, 103 and 44 h were calculated for DCPMU, DCPU and DCA, respectively. Further experiments should be performed in order to investigate the biodegradation potential of these compounds under anoxic conditions and to study the role of acclimatization and presence of supplemental substrate on their fate in activated sludge systems. Based on the results of this study, the sequential use of anoxic and aerobic conditions could offer in the future satisfactory removal of Diuron and its metabolites from runoff water.

Acknowledgments

The authors would like to thank Mrs Eva Iatrou and Mrs Elena Koumaki for their valuable help during the experiments.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.watres.2008.12.040.

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