Biodegradation (2011) 22:751–761
DOI 10.1007/s10532-010-9448-7

ORIGINAL PAPER

Comparison of experimental methods for determination of toxicity and biodegradability of xenobiotic compounds

A. M. Polo · M. Tobajas · S. Sanchis · A. F. Mohedano · J. J. Rodríguez

Received: 20 April 2010 / Accepted: 21 December 2010 / Published online: 9 January 2011

Abstract Different methods for determining the toxicity and biodegradability of hazardous compounds evaluating their susceptibility to biological treatment were studied. Several compounds including chlorophenols and herbicides have been evaluated. Toxicity was analyzed in terms of EC_{50} and by a simple respirometric procedure based on the OECD Method 209 and by the Microtox® bioassay. The values of EC_{50} obtained from respirometry were in all the cases higher than those from the Microtox® test. The respirometric inhibition values of chlorophenols were related well with the number of chlorine atoms and their position in the aromatic ring. In general, herbicides showed lower inhibition, being alachlor the less toxic from this criterion. For determination of biodegradability an easier and faster alternative to the OECD Method 301, with a higher biomass to substrate ratio is proposed. When this test was negative, the Zahn-Wellens one was performed in order to evaluate the inherent biodegradability. In the fast test of biodegradability, 4-chlorocatechol and 4-chlorophenol showed a complete biodegradation by an unacclimated sludge upon 48 h. These results together with their low respirometric inhibition, allow concluding that these compounds could be conveniently removed in a WWTP. Alachlor, 2,4-dichlorophenol, 2,4,6-trichlorophenol and MCPA showed a partial biodegradation upon 28 days by the Zahn-Wellens inherent biodegradability test.

Keywords Chlorinated compounds · Herbicides · Toxicity · Biodegradability · Respirometry

A. M. Polo (✉) · M. Tobajas · S. Sanchis · A. F. Mohedano · J. J. Rodríguez
Sección de Ingeniería Química, Facultad de Ciencias, Universidad Autónoma de Madrid, C/Francisco Tomás y Valiente 7, 28049 Madrid, Spain
e-mail: alicia.polo@uam.es

Introduction

Increasing industrial activity, along with more stringent discharge limits enforces the need for the development of new or improved methods for a cost-effective treatment of industrial wastewaters (Celis et al. 2008; Ballesteros Martín et al. 2008). Biological treatments are environmentally friendly and in general less expensive than physicochemical methods for wastewater treatment (Kargi and Eker 2006), but they can be excessively slow or even unfeasible when the microbial activity is affected by the toxicity of xenobiotic compounds (Meric et al. 2003; Tamer et al. 2006). Thus, before performing the biological treatment of a specific wastewater, it is necessary to assess its toxicity and biodegradability (Gutiérrez et al. 2002).

A number of bioassays have been developed for assessing the toxicity of chemical compounds to microorganisms, based on the evaluation of different

parameters linked to bacterial activity (Farré and Barceló 2003). This includes growth inhibition tests on *Pseudomonas* (ISO 10712 1995) or activated sludge (ISO 15522 1999), enzyme inhibition (McNicholl et al. 2007), ATP luminescence (Dalzell and Christofi 2002), acute toxicity test (*Daphnia magna* and *Vibrio fischeri*) or activated sludge oxygen uptake inhibition test (OECD 1993a, ISO 8192 1986). One of the most commonly used toxicity tests is based on the inhibition of the metabolic activity of the marine bacteria *Vibrio fischeri*, due to its high reproducibility and sensitivity. Among the available commercial systems the Microtox® test is the most widely used (Jennings et al. 2001). However, several authors have argued that the results of this assay cannot be easily related with the effect of the toxicant on the microbial community of a biological reactor (Bonnet et al. 2007; Santos-Juanes et al. 2008). The Microtox® test allows only an estimate of the potential toxicity of a given substance relative to a specific reference and a defined process but underestimates the capacity of a biological sludge for maintaining the degradative activity in the presence of toxicants (Dalzell et al. 2002; Ricco et al. 2004). Therefore, the use of activated sludge would provide a more realistic evaluation of the potential toxicity of a given compound on a conventional biological treatment. Among the methods commonly used to evaluate wastewater toxicity with activated sludge, respirometry is one of the most widely accepted, because it is a direct method for assessing sludge activity (Strotmann et al. 1995). The basis is that respiration rate can be reduced in the presence of toxicants. Although some assays use other methods for quantifying respiration rate, the most common criterion is the oxygen uptake rate (Guisasola et al. 2003; Ren 2004; Insel et al. 2006).

Assessing the potential impact of a chemical in a bioreactor should include not only studying the inhibition of the activity of the sludge but also evaluation of the biodegradability, since low toxicity and high biodegradability are not necessarily linked (Oller et al. 2007; Lapertot et al. 2008) and the accumulation of non-biodegradable compounds can lead to destabilization of the biological reactor. However, toxicity, biodegradability and inhibition, are terms sometimes interchangeably considered in the literature. Thus, in many studies focused on wastewater chemical treatments, the quality of the treated effluent is assessed only by measuring its acute toxicity.

The Organization for Economic Co-operation and Development (OECD) has published a series of standardized tests for determining the biodegradability of a given compound, based on the evaluation of overall parameters (such as COD, TOC and BOD): ready biodegradability tests (OECD 1993b), inherent biodegradability (OECD 1993c) and simulation test (OECD 1993d). A comparative study, analysing the range of applicability of these and equivalent tests by ISO protocols was reported by Pagga (1997). Both ready and inherent biodegradability tests last for 28 days and differ in the inoculum concentration used. Ready biodegradability tests are fairly restrictive because of the low inoculum concentration employed (Reuschenbach et al. 2003) and are normally used to predict the potential risk of a pure chemical compound in a natural environment. Inherent biodegradability test (Zahn-Wellens test) has less restrictions but is not useful in practice for predicting the behavior in a treatment plant, since it is quite time-consuming (Lapertot et al. 2008). Simulation tests are carried out in continuous regime commonly upon a testing time of 12 weeks, in order to study the biodegradation capacity of acclimated biomass. The choice of an adequate test must consider the testing purpose and thus, when trying to predict the behaviour in a WWTP, a much higher biomass/substrate concentration ratio and a much shorter hydraulic retention time must be used, in order to approach to real conditions.

The aim of this work is to evaluate different methods for determining the toxicity and biodegradability of hazardous compounds for the sake of assessing the suitability of biological treatment for these substances. In this context, a simple respirometric test (using standard laboratory equipment available in any WWTP) is proposed capable of providing a rapid evaluation of the potential detrimental effect of a waste stream entering the bioreactor. As target compounds for this study, a set of chlorophenols and chlorinated herbicides, some of them included in the list of priority hazardous substances of the European Union (Directive 2008/105/EC) have been selected. Chloroaromatics are known as persistent toxic compounds for which the environment has little assimilative capacity. Among them, chlorophenols, have been widely used as pesticides, disinfectants, wood preservatives, resins and lubricants, as well as byproducts of several industrial processes. Herbicides are frequently

used in agriculture and in railway lines and roads for the control of weeds. They can reach the aquatic environment through many different pathways which include direct run-off from crops, leaching, careless disposal of empty containers or equipment washing. Some chemical and photochemical treatments have shown a high efficiency for the removal of both, chlorophenols (Calvo et al. 2004, 2006; Diaz et al. 2008) and pesticides (Hincapie et al. 2006; Maldonado et al. 2006; Calvo et al. 2008), but presumably at a high cost. Therefore, despite the recalcitrant nature of these compounds, research efforts are being addressed on the potential application of biological processes, like using acclimated biomass bioreactors (González et al. 2006; Celis et al. 2008, Monsalvo et al. 2009) or bioaugmentation (Smejkal et al. 2003).

Materials and methods

Inoculum and culture medium

Unacclimated activated sludge, obtained from a domestic sewage plant was used in the toxicity and biodegradability tests. The sludge was maintained with sodium acetate (150 mg COD/l) and glucose (150 mg COD/l) as carbon sources in a sequencing batch reactor (SBR) operated at 25°C. Ammonium sulphate and phosphoric acid were used as nitrogen and phosphorous sources, respectively. A COD:N:P of 100:5:1 (w/w) was fixed and mineral salts were also added as micronutrients supply in a COD:micronutrients (Fe, Ca, K and Mg) ratio of 1:0.05. The mineral solution consisted in $FeCl_3$, $CaCl_2$, KCl and $MgCl_2$.

Chemicals

The following chemicals were selected for this study: 4-chlorophenol (4 CP), 4-chlorocatechol (4 CC), 2,4-dichlorophenol (2,4 DCP), 3,5-dichlorophenol (3,5 DCP), 2,4,6-trichlorophenol (2,4,6 TCP) and pentachlorophenol (PCP) as representative of chlorinated compounds and 2-(4-Chloro-2-methylphenoxy) acetic acid (MCPA), 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (diuron), (2,4-Dichlorophenyloxy) acetic acid (2,4-D), 1-Chloro-3-(ethylamino)-5-(isopropylamino)-s-triazine (atrazine) and 2-chloro-N-(methoxymethyl)-N-(2',6'-diethyl-phenyl)-acetamide (alachlor) as representive of herbicides. All the reagents were supplied by Sigma-Aldrich, chlorophenols, 2,4-D and diuron in analytical grade (97–99%) and the rest of pesticides in HPLC-grade.

Respirometer

Batch assays were carried out in a Liquid-Static-Static (LSS) respirometer (Chica et al. 2007), measuring the dissolved oxygen concentration decay between two set values sufficiently high to avoid oxygen limitation in the system. Aeration and oxygen probes were controlled by an electronic interface. The respirometer operated with two independent reactors simultaneously to guarantee reproducibility. The reaction vessels had a very small headspace so that oxygen transfer from the gas to the liquid can be neglected. The reaction vessels were placed in a thermostatic bath and continuously stirred by magnetic bars.

Analytical methods

Chlorophenols were analyzed by means of HPLC (Varian Pro-Start 240) with a diode array detector (330 PDA). A Microsorb C18 5 mm column (MV 100, 15 cm long, 4.6 mm diameter) was used as stationary phase and a mixture of acetonitrile and water (1:1, v/v) at 1.0 ml/min as mobile phase. All compounds were detected at a wavelength of 280 nm. Herbicides were quantified by HPLC/UV (Prostar, Varian) using a Teknokroma (mediterranea seal 8 5 μm 15 × 0.46 cm) column as stationary phase and a mixture of acetonitrile/H_2O (80/20–65/35 (0–15 min) and 65/35–25/75 (15–30 min)) with a constant flow of 0.60 ml/min as mobile phase. A wavelength of 280 nm was used for all compounds except for alachlor (220 nm). Samples were filtered through 0.45 μm syringe filters (OlimPeak PTFE, 25 mm, Teknokroma) before HPLC injection. The detection limit of HPLC was in all cases better than 1 mg/l. Total organic carbon (TOC) was measured using an OI Analytical Model 1010 TOC apparatus. All samples were analyzed after filtration through fiber glass filters (Albet FV-C). Biomass was determined gravimetrically by measuring volatile suspended solids (VSS).

Toxicity assays

Toxicity was determined by the Microtox® Acute Toxicity Test (SCI 500 Analyzer) following the ISO 11348-3 (1998) standard. Toxic effects were monitored as a percent decrease of the light emission of *Vibrio fischeri* after 5 min and 15 min of incubation at 15°C. The toxicity results were expressed as EC_{50}, defined as the effective concentration of a sample that causes 50% reduction of bioluminescence.

Toxicity was also evaluated by using a modification of the method proposed by Ricco et al. (2004), based on the OECD 209 (OECD 1993a) respiration inhibition test for activated sludge. The procedure consisted in short-term respirometric measurements using unacclimated sludge in presence of an easily biodegradable substrate (sodium acetate) alone or together with different concentrations of the target compound. Nitrification was inhibited by using N-allyltiourea.

In each run, reactors, with 350 mg/l of volatile suspended solids, were aerated up to oxygen saturation. Then aeration was stopped and oxygen concentration decay was recorded for 15 min in order to obtain the endogenous respiration rate. The reference compound was then added and the Oxygen Uptake Rate (OUR) was obtained from the slope of the oxygen concentration vs time plot. The test was repeated adding increasing concentrations of each toxicant. Fresh activated sludge was used in each test to avoid partial acclimation of the microorganisms to the tested chemicals leading to possible underestimation of the toxicity effects. The biomass activity was measured in terms of specific exogenous oxygen uptake rate ($SOUR_{ex}$), calculated from dissolved oxygen and volatile suspended solids (VSS) concentration values. Inhibition percentage for each concentration of toxicant was determined as described by Tomei et al. (2004):

$$\text{Inhibition}(\%) = \left(1 - \frac{\text{SOUR}_{\text{exT}}}{\text{SOUR}_{\text{exR}}}\right)100 \quad (1)$$

where $SOUR_{exT}$ represent the specific exogenous oxygen uptake rate for the reference substrate (sodium acetate) in the presence of a given concentration of toxicant and $SOUR_{exR}$ is the specific exogenous oxygen uptake rate measured for the reference substrate, both of them corrected by the previously measured endogenous SOUR.

Inhibition was also estimated in terms of EC_{50} defined as the concentration of the toxicant causing a 50% reduction of the $SOUR_{exR}$. Two procedures were used to calculate EC_{50}. In the first one, a logistic model was used to fit the inhibition percentage data as a function of the compound concentration:

$$\text{Inhibition}(\%) = \frac{100}{1 + e^{-k(\ln C - \ln \text{EC}_{50})}} \quad (2)$$

The second is based on the approach used in the Microtox® assays, defining γ parameter as:

$$\gamma = \frac{\text{SOUR}_{\text{exR}}}{\text{SOUR}_{\text{exT}}} - 1 \quad (3)$$

The relationship between γ and the toxicant concentration, C, can be expressed as:

$$\gamma = KC^P \quad (4)$$

Thus, when the toxicant causes a 50% reduction of the $SOUR_{exT}$, γ takes the value of 1 and EC_{50} can be obtained as:

$$\text{EC}_{50} = e^{-\left(\frac{\ln K}{P}\right)} \quad (5)$$

Biodegradability tests

A new biodegradability test is proposed in order to check in an easy and rapid way the behaviour of the target compounds in a biological reactor. The method was designed on the basis of the typical operation conditions of an activated sludge process, measuring both the activity of the microorganisms and toxicant removal. Activated sludge was maintained in starvation and continuous aeration overnight in the reactors in order to eliminate any residual COD. Subsequently, mineral medium (APHA 1992) and the target compound were incorporated. The reactors were closed to avoid evaporation and continuously aerated during the whole reaction time so that oxygen is near saturation, avoiding oxygen limitation and oxygen transfer from air. The SOUR profile was obtained by interrupting the air supply and registering the DO decay within a range of 0.2 mg/l. A concentration lower than its EC_{50} value was selected for each target compound and the biomass concentration was set at 350 mg VSS/l. This concentration was selected by preliminary tests in order to ensure a significant oxygen consumption detectability at the

lowest respiration rate and to prevent that the maximum OUR was greater than the oxygen transfer rate during the aeration period. Tests were carried out for 24–48 h. The biodegradation of each toxicant was followed from the evolution of its concentration and the SOUR profile. The initial and final TOC were also analyzed to learn on the mineralization of each compound. All the tests were performed at 20°C.

The Zahn-Wellens test for determination of inherent biodegradability was carried out according to the OECD guidelines (OECD 1993c). In this test, a mixture containing the target substance and a certain amount of activated sludge in aqueous medium is agitated and aerated at 20–25°C in diffuse light for up 28 days. The ratio between the carbon content of the sample and the dry-weight of the inoculum must range between 1 and 4. Degradation of the compound is evaluated in terms of TOC or COD. In this work samples were taken periodically for measuring TOC and the concentration of target substance. Each compound was tested in parallel in two different bottles to guarantee reproducibility. In order to check the activity of the sludge, a test using ethylene glycol as reference compound was also carried out.

Results and discussion

Inhibition study

A series of respirometric tests were carried out for each target compound over a wide range of concentrations, depending on the corresponding water solubility. At least two replicates were always carried out and the inhibition was calculated as the mean value. Endogenous SOUR was in the range of 2.0–6.0 mg O_2/g VSS.h and the $SOUR_{ex}$ of the reference substrate (sodium acetate) varied between 30 and 80 mg O_2/g VSS.h depending on the state of the activated sludge during the experimental period.

Figure 1 shows the dissolved oxygen concentration profile observed in the respirometric test for 3,5 DCP. This compound was selected for control as recommended by the OECD 209 protocol. As can be observed, dissolved oxygen decays in the first 15 min due to endogenous respiration, then the test mixture is added and the slope corresponds to the total SOUR. Comparing the reference curve with those in presence of this toxicant, a progressive decrease of SOUR can

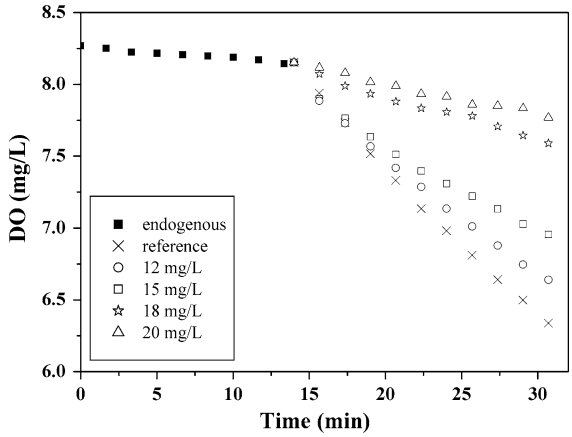

Fig. 1 Measured oxygen concentration profile for 3,5 DCP

be observed at increasing 3,5 DCP concentration. A lag stage did not occur upon the addition of the mixture of substrate and toxicant and the registered oxygen concentration decay maintained a linear trend. This is important since it indicates that there is no need for waiting 30 min (as established in the OECD 209 protocol) to obtain the respiration rate and, therefore, the time required for the experiments can be drastically reduced.

EC_{50} was calculated by using the logistic model and the gamma method. Figure 2 shows the inhibition percentage curve (S shaped) and the corresponding linear plot of Ln γ vs. Ln C. The resulting EC_{50} values (14.6 and 14.0 mg/l, respectively) are within the range of the protocol (5–30 mg/l). The calculated EC_{50} values for the chlorophenols and herbicides tested are shown in Table 1. As can be seen, there is a good agreement between the EC_{50} values obtained from the logistic and linear methods. Whereas logistic method requires a compound concentration which produces 100% inhibition as the upper limit, in the linear method a maximum limit is not needed for EC_{50} calculation. This fact could be useful in EC_{50} determination of poorly soluble or low toxic compounds. Moreover, the amount of data required is lower for the linear method. The logistic model for EC_{50} determination could not be used for alachlor, atrazine and diuron since the concentration corresponding to their water solubility does not cause the complete inhibition of the microbial activity (Table 1).

Only limited data is available in the literature relative to the inhibitory effect of these compounds on activated sludge, all of them corresponding to

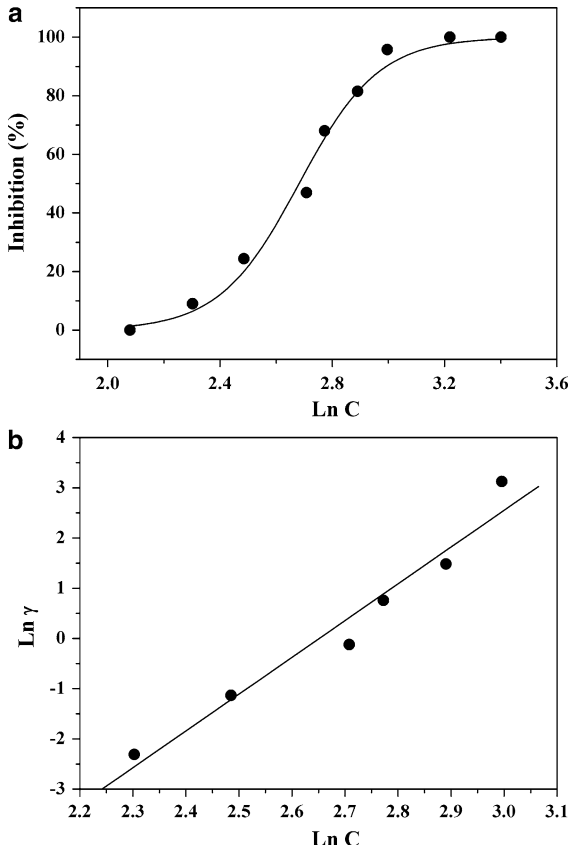

Fig. 2 Respirometric inhibition measurements for 3,5 DCP analysed by both logistic and γ-lineal method

chlorophenols. EC_{50} values reported for 3,5 DCP, one of the most studied compounds, ranged from 5 to 13.25 mg/l (Beltrame et al. 1984; Yoshioka et al. 1986; Gutiérrez et al. 2002; Gendig et al. 2003; Ricco et al. 2004), in good agreement with the obtained in this work (Table 1). The EC_{50} value obtained for 4 CP was also comparable to that reported by Beltrame et al. (1984) (71 mg/l), whereas the toxicity of PCP was much lower than that reported by Gutiérrez et al. (2002) using OECD 209 protocol (EC_{50} = 0.82 mg/l).

The EC_{50} values obtained for chlorophenols show that a higher chlorine content is associated with a higher toxicity, with the exception of 3,5 DCP which does not fit that trend. It has been reported that the toxicity of chlorophenols is reduced when chlorine occupies the ortho positions (Liu et al. 1982; Briens et al. 1999). Thus, 3,5-DCP should be more toxic than 2,4,6-TCP (Beltrame et al. 1984) and 2,4-DCP. Therefore, our results confirm that toxicity of chlorophenols is affected by the number and position of chlorine atoms present in the molecule.

Except diuron, the tested herbicides showed significantly lower inhibition effects on the biological activity than the chlorophenols according to the EC_{50} obtained by respirometry. Hence, herbicides could be classified as less toxic than chlorophenols taking into account the respirometric measurements. However, biodegradability must be also determined.

Table 2 shows the results obtained from the Microtox® test at 5 and 15 min, as well as the range of values reported by other authors. Except for MCPA and 2,4 D, our experimental EC_{50} values are in reasonable agreement with those reported in the literature, although it is also true that the literature

Table 1 EC_{50} values from respirometric measurements

Compound	No tests [conc. range] (mg/l)	Logistic fit		Linear fit	
		EC_{50} (mg/l)	R^2	EC_{50} (mg/l)	R^2
4 CC	8 [50–400]	117.9	0.957	111.2	0.999
4 CP	8 [25–500]	95.0	0.998	97.4	0.998
2,4 DCP	10 [5–330]	69.5	0.983	67.1	0.985
3,5 DCP	9 [8–30]	14.6	0.990	14.2	0.978
2,4,6 TCP	9 [5–90]	27.9	0.828	30.5	0.984
PCP	7 [10–75]	21.1	0.956	21.3	0.982
Alachlor	5 [15–200]	–	–	217.0	0.922
Atrazine	3 [5–30]	–	–	No toxic[a]	–
Diuron	3 [10–35]	–	–	37.0	0.999
MCPA	7 [50–275]	136.4	0.957	144.2	0.996
2,4 D	8 [100–350]	208.3	0.969	213.4	0.982

[a] No toxic effect at the maximum concentration tested corresponding to the water solubility

Table 2 EC_{50} values obtained from the Microtox® test and reported in literature

Compound	Solubility (mg/l)	This work				Other authors	
		EC_{50}, 5 min (mg/l)	R^2	EC_{50}, 15 min (mg/l)	R^2	EC_{50} (mg/l) range	References
4 CC	NA	19.5	0.990	16.1	0.974	NA	
4 CP	27100	2.1	0.990	1.9	0.983	1.1–8.3	Kaiser and Palabrica (1991) Jenning et al. (2001)
2,4 DCP	4500	4.2	0.996	3.7	0.993	1.2–6.1	Kaiser and Palabrica (1991) Drzewicz et al. (2004)
3,5 DCP	2600	6.8	0.993	6.0	0.999	2.8–4.1	Kaiser and Palabrica (1991) Jenning et al. (2001)
2,4,6 TCP	800	12.0	0.974	10.7	0.930	6.0–8.2	Kaiser and Palabrica (1991) Svenson and Zhang (1995)
PCP	14	0.6	0.973	0.6	0.981	0.7–1.1	Bogaerts et al. (2001) Kahru et al. (1996)
Alachlor	200	114.3	0.966	105.4	0.926	105–206	Lapertot et al. (2008) Bonnet et al. (2007)
Atrazine	35	103.3	0.999	87.5	0.884	89–150	Lapertot et al. (2008) Bogaerts et al. (2001)
Diuron	42	54.1	0.997	42.7	0.973	58–86	Bogaerts et al. (2001) Lapertot et al. (2008)
MCPA	300	12.0	0.997	11.6	0.994	75.6–121	Bojanowska-Czajka et al. (2006) Vismara and Garavaglia (1997)
2,4 D	350	20.5	0.995	21.1	0.999	59	Zona and Solar (2003)

NA not available

range is fairly wide in some cases. Comparing the results from Tables 1 and 2, it can be concluded that the Microtox® test clearly overestimates the potential negative effect on the activated sludge of a wastewater treatment plant. Moreover, the EC_{50} values from that test do not follow the same trends than those from respirometry. Although several authors have found a good agreement between the response to different chemicals of *V. fischeri* and other microorganisms living in freshwater, such as *Ceriodaphnia dubia* (Doherty et al. 1999), *Daphnia magna* (Vasseur et al. 1984) and *Tetrahymena pyriformi* (Cronin et al. 1991), some of those authors have concluded that for certain groups of compounds the behaviour of *V. fischeri* does not serve as a good model for toxicity (Dalzell et al. 2002).

Biodegradability study

In order to assess the impact of a possible release of these compounds in the influent of a WWTP, the proposed fast biodegradability test described in the Materials and Methods section was carried out. An initial concentration of 10 mg/l was chosen for all the compounds tested. This concentration was below the EC_{50} value obtained with the activated sludge in all cases. Table 3 shows the values of the VSS to theoretical COD ratio used in each case.

Figure 3 shows two examples of the results obtained from this test. As can be seen, after a lag of 5 h, 4 CP concentration starts decaying and this compound is completely degraded in 30 h. The respirometric profile shows an initial decay of microbial activity due to the inhibition caused by the toxicant. After some adaptation time, there is an increase of sludge respiration which is maintained during the next 20 h reaching a maximum at 28 h before lowering to endogenous respiration. In the case of alachlor, there was no degradation of the chemical upon 25 h and the microbial activity decreased continuously throughout the testing period. Fall of endogenous respiration was observed in all the

Table 3 Biomass:compound concentration ratio used in the biodegradability experiments

Compound	Concentration (COD, mg/l)[a]	VSS:COD	Compound	Concentration (COD, mg/l)	VSS:COD
4CC	13.3	26	Alachlor	21.9	16
4CP	16.2	22	Atrazine	11.1	31
2,4 DCP	11.8	30	Diuron	12.4	28
3,5 DCP	11.8	30	MCPA	15.2	23
2,4,6 DCP	8.9	39	2,4 D	10.9	32
PCP	5.4	65			

[a] COD calculated as theoretical oxygen demand without nitrification ($ThOD_{NH3}$) as defined in OECD 301 guideline, annex IV (OECD 1993)

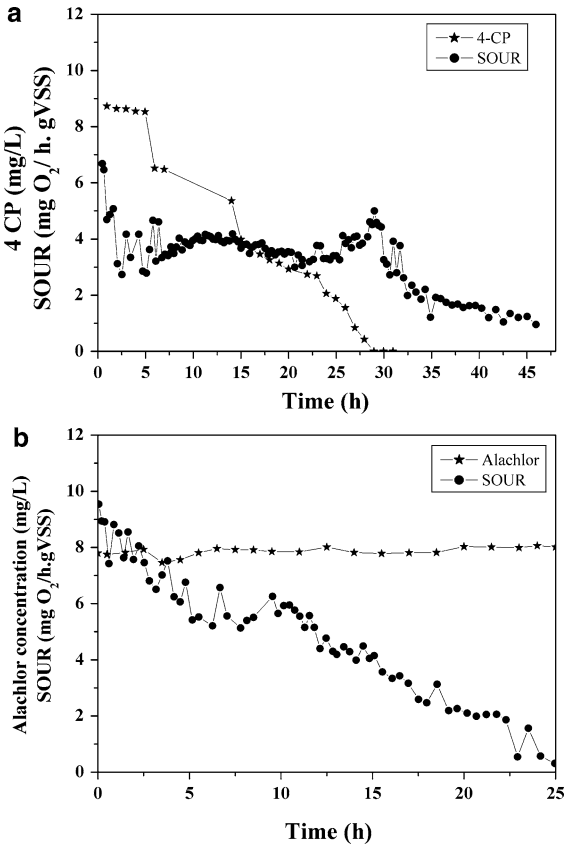

Fig. 3 Evolution of concentration and respirometric profiles obtained in the fast biodegradability test for **a** 4 CP and **b** alachlor

experiments, including those with 4 CP and 4 CC. This suggests that part of the biomass has been irreversibly affected by the presence of toxicants as well as a long-term toxicity effect.

The biodegradability test was positive only for 4 CP and 4 CC (figure not shown), whereas no degradation was observed for any of the pesticides or the rest of chlorophenols. This suggests that these compounds cannot be degraded in a WWTP, even in small quantities, since they would go out with the treated water in the case of a continuous reactor or be accumulated in a batch bioreactor (like an SBR) thus reaching the EC_{50} value, and destabilization of the system would occur. Therefore, the only possibility for biological treatment would be using acclimated sludge.

Inherent biodegradability was checked by the Zahn-Wellens test for all the compounds showing a negative response in the fast test of biodegradability. The objective in this case was evaluating the ability of microorganisms to adapt to the presence of chlorophenols and herbicides in order to use acclimated biomass for the elimination of such compounds. Preliminary tests were performed to check that neither volatilization nor adsorption occurred during the testing period. Both TOC and the concentration of each chemical were measured every three days and the percentages of degradation were calculated. Ethylene glycol, used as a reference compound to check the experimental procedure, was degraded up to 80% in 5 days. Figure 4 shows the results obtained in terms of percentage of TOC degradation for all the compounds showing a positive response to this test. Only two clorophenols showed a partial biodegradability. In the case of 2,4 DCP a moderately fast disappearance was observed, and a final COT removal of 38% was achieved. For the other compounds the degradation curves show a clear trend of slow adaptation of microorganisms to reach a partial degradation of 35%, in terms of TOC, in the case of 2,4,6 TCP and 20% and 25% for MCPA and alachlor respectively. The other chlorophenols

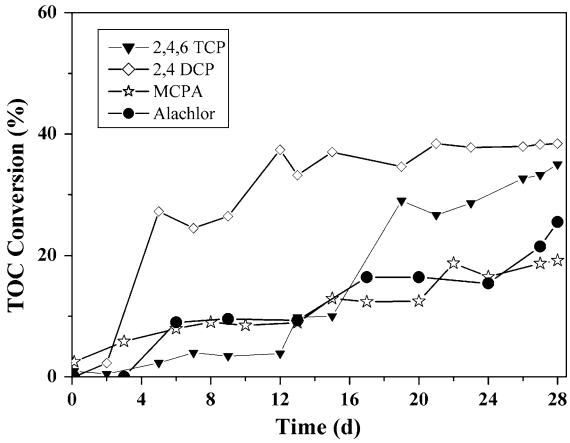

Fig. 4 Zahn-Wellens biodegradability test evolution for 2,4,6 TCP ($TOC_i = 4.1$ mg/l) (*inverted filled triangle*), 2,4 DCP ($TOC_i = 4.9$ mg/l) (*open diamond*), MCPA ($TOC_i = 10.5$ mg/l) (*open star*) and alachlor ($TOC_i = 19.2$ mg/l) (*filled circle*). *Curves* represent percentage of TOC degradation

(3,5 DCP and PCP) and herbicides (diuron, atrazine and 2,4 D) seem to be refractory to a direct biotreatment. In order to optimize their elimination, a chemical pretreatment leading to more biodegradable intermediates (i.e. dechlorination, partial oxidation) should be investigated.

Conclusions

The analysis of the potential risk of discharging a given chemical in the biological reactor of a WWTP must consider not only the inhibition caused in the microbial activity but also the biodegradability of that compound. Low inhibitory compounds but with a poor or almost inexistent biodegradability could be accumulated and reach quite rapidly its inhibitory level. In these terms, the characterization of a substance as toxic or hazardous for a biological treatment system includes the combination of low EC_{50} values and low or no biodegradability within a reasonable period of time. In this work, the tested herbicides have shown lower inhibitory effects on the microbial activity than chlorophenols or even 4 CC. Nevertheless, further biodegradability studies proved that 4 CC and 4 CP are biodegradable by a non acclimated activated sludge within a lapse of time comparable to typical hydraulic residence time in a WWTP, while apparently less toxic compounds such as alachlor and 2,4-D could only be treated using acclimated sludge. For realistic knowledge of the behaviour of a chemical upon biological treatment in a wastewater plant, experiments should be carried out with its own activated sludge and in similar operating conditions since, otherwise, the standard tests could provide pessimistic (case of the Microtox®) or optimistic (case of Zahn-Wellens test) predictions. The proposed fast test of biodegradability can be useful both for plant and laboratory purposes. It can be used to have a rapid screening for optimizing conditions of coupled chemical–biological processes.

Acknowledgments This work was financially supported by Comunidad de Madrid and Universidad Autónoma de Madrid through the project CCG08-UAM/AMB-4436 and by the Spanish Ministerio de Ciencia e Innovación through the projects CTM2007-60959 and CDS2006-0044.

References

APHA-AWWA-WPFC (1992) Standard methods for the examination of water and wastewater, section 5210, 18th edn. American Public Health Association, Washington DC, pp 5.1–5.6

Ballesteros Martín MM, Sánchez Pérez JA, García Sánchez JL, Montes de Oca L, Casas López JL, Oller I, Malato Rodríguez S (2008) Degradation of alachlor and pyrimethanil by combined photo-Fenton and biological oxidation. J Hazard Mater 155:342–349

Beltrame P, Beltrame PL, Carniti P (1984) Inhibiting action of chloro-phenols and nitro-phenols on biodegradation of phenol—a structure-toxicity relationship. Chemosphere 13:3–9

Bogaerts P, Bohatier J, Bonnemoy F (2001) Use of the ciliated protozoan *Tetrahymena pyriformis* for the assessment of toxicity and quantitative structure–activity relationships of xenobiotics: comparison with the Microtox test. Ecotoxicol Environ Saf 49:293–301

Bojanowska-Czajka A, Drzewicz P, Kozyra C, Nalecz-Jawecki G, Sawicki J, Szostek B, Trojanowicz M (2006) Radiolytic degradation of herbicide 4-chloro-2-methyl phenoxyacetic acid (MCPA) by gamma-radiation for environmental protection. Ecotoxicol Environ Saf 65:265–277

Bonnet JL, Bonnemoy F, Dusser M, Bohatier J (2007) Assessment of the potential toxicity of herbicides and their degradation products to nontarget cells using two microorganisms, the bacteria *Vibrio fischeri* and the ciliate *Tetrahymena pyriformis*. Environ Toxicol 22(1):78–91

Briens F, Bureau R, Rault S (1999) Applicability of CATALYST in ecotoxicology, a new promising tool for 3D-QSAR: study of chlorophenols. Ecotoxicol Environ Saf 43:241–251

Calvo L, Mohedano AF, Casas JA, Gilarranz MA, Rodriguez JJ (2004) Treatment of chlorophenols-bearing wastewaters through hydrodechlorination using Pd. Carbon 42: 1377–1381

Calvo L, Gilarranz MA, Casas JA, Mohedano AF, Rodríguez JJ (2006) Hydrodechlorination of 4-chlorophenol in aqueous phase using Pd/AC catalysts prepared with modified active carbon supports. Appl Catal B: Environ 67:68–76

Calvo L, Gilarranz MA, Casas JA, Mohedano AF, Rodríguez JJ (2008) Hydrodechlorination of alachlor in water using Pd, Ni and Cu catalysts supported on activated carbon. Appl Catal B: Environ 78:259–266

Celis E, Elefsiniotis P, Singhal N (2008) Biodegradation of agricultural herbicides in sequencing batch reactors under aerobic or anaerobic conditions. Water Res 42:3218–3224

Chica A, Martin A, Vazquez FJ, Carmona FJ, Mohedo JJ (2007) Respirometer to analyze measure dissolved oxygen and oxygen demand of microbes in leachate from municipal waste. Patent ES 2283171

Cronin MTD, Dearden JC, Dobbs AJ (1991) QSAR studies of comparative toxicity in aquatic organisms. Sci Total Environ 109–110:431–439

Dalzell DJB, Christofi N (2002) An ATP luminescence method for direct toxicity assessment of pollutants impacting on the activated sewage sludge process. Water Res 36: 1493–1502

Dalzell DJB, Alte S, Aspichueta E, de la Sota A, Etxebarria J, Gutierrez M, Hoffmann CC, Sales D, Obst U, Christofi N (2002) A comparison of five rapid direct toxicity assessment methods to determine toxicity of pollutants to activated sludge. Chemosphere 47:535–545

Diaz E, Casas JA, Mohedano AF, Calvo L, Gilarranz MA, Rodriguez JJ (2008) Kinetics of the hydrodechlorination of 4-chlorophenol in water using Pd, Pt, and Rh/Al_2O_3 catalysts. Ind Eng Chem Res 47:3840–3846

Doherty FG, Qureshi AA, Razza JB (1999) Comparison of the *Ceriodaphnia dubia* and Microtox® inhibition tests for toxicity assessment of industrial and municipal wastewaters. Environ Toxicol 14:375–382

Drzewicz P, Nalecz-Jawecki G, Gryz M, Sawicki J, Bojanowska-Czajka A, Gluszewski W, Kulisa K, Wolkowicz S, Trojanowicz M (2004) Monitoring of toxicity during degradation of selected pesticidas using ionizing radiation. Chemosphere 57:135–145

Farré M, Barceló D (2003) Toxicity testing of wastewater and sewage sludge by biosensors, bioassays and chemical analysis. TrAC Trend Anal Chem 22:299–310

Gendig C, Domogala G, Agnoli F, Pagga U, Strotmann UJ (2003) Evaluation and further development of the activated sludge respiration inhibition test. Chemosphere 52:143–149

González S, Müller J, Petrovic M, Barceló D, Knepper TP (2006) Biodegradation studies of selected priority acidic pesticides and diclofenac in different bioreactors. Environ Pollut 144:926–932

Guisasola A, Baeza JA, Carrera J, Casas C, Lafuente J (2003) An off-line respirometric procedure to determine inhibition and toxicity of biodegradable compounds in biomass from an industrial WWTP. Water Sci Technol 48:267–275

Gutiérrez M, Etxebarria J, de las Fuentes L (2002) Evaluation of wastewater toxicity: comparative study between Microtox® and activated sludge oxygen uptake inhibition. Water Res 36:919–924

Hincapie M, Peñuela G, Maldonado MI, Malato O, Fernández-Ibáñez P, Oller I, Gernjak W, Malato S (2006) Degradation of pesticides in water using solar advanced oxidation processes. Appl Catal B: Environ 64:272–281

Insel G, Karahan O, Ozdemir S, Pala L, Katipoglu T, Cokgor EU, Orhon D (2006) Unified basis for the respirometric evaluation of inhibition for activated sludge. J Environ Sci Health, Pt A Toxic/Hazard Subst Environ Eng 41:1763–1780

ISO (1986) ISO 8192 Water quality: test for inhibition of oxygen consumption by activated sludge. International Standardization Organization, Geneva

ISO (1995) ISO 10712 Water quality: *Pseudomonas putida* growth inhibition test (Pseudomonas cell multiplication inhibition test). International Standardization Organization, Geneva

ISO (1998) ISO 11348-3 Water quality: determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)—part 3: method using freeze-dried bacteria. International Standardization Organization, Geneva

ISO (1999) ISO 15522 Water quality: determination on the inhibitory effect of the water constituents on the growth of activated sludge micro-organism. International Standardization Organization, Geneva

Jennings VLK, Rayner-Brandes MH, Bird DJ (2001) Assessing chemical toxicity with the bioluminescent photobacterium (*vibrio fischeri*): a comparison of three commercial systems. Water Res 35:3448–3456

Kahru A, Tomson K, Pall T, Kulm I (1996) Study of toxicity of pesticides using luminescent bacteria *Photobacterium phosphoreum*. Water Sci Technol 33:147–154

Kaiser KLE, Palabrica VS (1991) Photobacterium phosphoreum toxicity data index. Water Poll Res J Can 26:361–431

Kargi F, Eker S (2006) Effect of sludge age on performance of an activated sludge unit treating 2,4 dichlorophenol-containing synthetic wastewater. Enzyme Microb Technol 38:60–64

Lapertot M, Ebrahimi S, Oller I, Maldonado MI, Gernjak W, Malato S, Pulgarín C (2008) Evaluating Microtox© as a tool for biodegradability assessment of partially treated solutions of pesticides using Fe^3 + and TiO_2 solar photo-assisted processes. Ecotoxicol Environ Saf 69:546–555

Liu D, Thomson K, Kaiser KLE (1982) Quantitative structure-toxicity relationship of halogenated phenols on bacteria. Bull Environ Contam Toxicol 29:130–136

Maldonado MI, Malato S, Pérez-Estrada LA, Gernjak W, Oller I, Doménech X, Peral J (2006) Partial degradation of five pesticides and an industrial pollutant by ozonation in a pilot-plant scale reactor. J Hazard Mater 138:363–369

McNicholl BP, McGrath JW, Quinn JP (2007) Development and application of a resazurin-based biomass activity test for activated sludge plant management. Water Res 41:127–133

Meric S, Eremektar G, Ciner F, Tunay O (2003) An OUR-based approach to determine the toxic effects of 2,4-dichlorophenoxyacetic acid in activated sludge. J Hazard Mater 101:147–155

Monsalvo VM, Mohedano AF, Casas JA, Rodríguez JJ (2009) Cometabolic biodegradation of 4-chlorophenol by

sequencing batch reactors at different temperatures. Bioresour Technol 100:4572–4578

OECD (1993a) 209 Activated sludge, respiration inhibition test, OECD guidelines for testing of chemicals. Organisation for Economic Co-operation and development, Paris

OECD (1993b) 301 ready biodegradability, OECD guidelines for testing of chemicals. Organisation for Economic Cooperation and development, Paris

OECD (1993c) 302 B Zahn-Wellens/EMPA test, OECD guidelines for testing of chemicals. Organisation for Economic Co-operation and development, Paris

OECD (1993d) 303 a simulation test—aerobic sewage treatment, OECD guidelines for testing of chemicals. Organisation for Economic Co-operation and development, Paris

Oller I, Malato S, Sánchez-Pérez JA, Maldonado MI, Gassó R (2007) Detoxification of wastewater containing five common pesticides by solar AOPs—biological coupled system. Catal Today 129:69–78

Pagga U (1997) Testing biodegradability with standardized methods. Chemosphere 35:2953–2972

Ren S (2004) Assessing wastewater toxicity to activated sludge: recent research and developments. Environ Int 30:1151–1164

Reuschenbach P, Pagga U, Strotmann U (2003) A critical comparison of respirometric biodegradation tests based on OECD 301 and related test methods. Water Res 37:1571–1582

Ricco G, Tomei MC, Ramadori R, Laera G (2004) Toxicity assessment of common xenobiotic compounds on municipal activated sludge: comparison between respirometry and Microtox (R). Water Res 38:2103–2110

Santos-Juanes L, Amat AM, Arques A, Bernabeu A, Silvestre M, Vicente R, Añó E (2008) Activated sludge respirometry to assess solar detoxification of a metal finishing effluent. J Hazard Mater 153:905–910

Smejkal CW, Seymour FA, Burton SK, Lappin-Scott HM (2003) Characterisation of bacterial cultures enriched on the chlorophenoxyalkanoic acid herbicides 4-(2,4-dichlorophenoxy) butyric acid and 4-(4-chloro-2-methylphenoxy) butyric acid. J Ind Microbiol Biotechnol 30:561–567

Strotmann UJ, Keinath A, Hüttenhain SH (1995) Biological test systems for monitoring the operation of wastewater treatment plants. Chemosphere 30:327–338

Svenson A, Zhang LL (1995) Acute aquatic toxicity of protolyzing substances studied as the Microtox effect. Ecotoxicol Environ Saf 30:283–288

Tamer E, Hamid Z, Aly AM, Ossama ET, Bo M, Benoit G (2006) Sequential UV-biological degradation of chlorophenols. Chemosphere 63:277–284

Tomei MC, Annesini MC, Bussoletti S (2004) 4-nitrophenol biodegradation in a sequencing batch reactor: kinetic study and effect of filling time. Water Res 38:375–384

Vasseur P, Ferard JF, Vial J, Larbaigt G (1984) Comparaison des tests microtox et daphnie pour l'evaluation de la toxicité aigue d'effluents industriels. Environ Poll A Ecol Biol 34:225–235

Vismara C, Garavaglia A (1997) 4-chloro-2-methylphenoxyacetic acid containing compounds. Genotoxicity evaluation by Mutatox(R) assay and comparison with acute (Microtox(R)) and embryo (FETAX) toxicities. Bull Environ Contam Toxicol 58:582–588

Yoshioka Y, Nagase H, Ose Y, Sato T (1986) Evaluation of the test method activated-sludge, respiration inhibition test proposed by the OECD. Ecotoxicol Environ Saf 12: 206–212

Zona R, Solar S (2003) Oxidation of 2,4-dichlorophenoxyacetic acid by ionizing radiation: degradation, detoxification and mineralization. Radiat Phys chem 66:137–143