

Evaluation of biodegradation kinetic constants for aromatic compounds by means of aerobic batch experiments

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Abstract

Kinetics of aerobic biodegradation have been investigated for twenty aromatic species using sludges collected from the aeration basin of municipal sewage treatment plants. The reproducibility of the results is tested with respect to the sludges period of collection and the wastewater treatment plant where they are taken.

The comparison of kinetic constants, estimated for the investigated chemicals, allows to evaluate the reactivity effect of single groups (i.e., –OH, –CH₃, –Cl, –NO₂) into the aromatic structures. The search for easy structure-reactivity relationships is also attempted by means of contributing group methods.

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1. Introduction

The knowledge of aerobic biodegradation kinetic constants of chemicals is important both in the prediction of their persistence in the environment as in the design and in the verification of wastewater treatment plants (WWTPs). During recent years several attempts were done to develop predictive models for aerobic biodegradation of chemical substances, by relating the rate of biotransformation to the structure of the compounds. These models are based on the use of proper chemical descriptors as Hammett substituent constants, *n*-octanol/water partition coefficient, acid dissociation con-

stants and many others (Desai et al., 1990; Tabak and Govind, 1993; Okey and Stensel, 1996; Loonen et al., 1999; Tunkel et al., 2000). Each of the proposed models has its own advantages and disadvantages, none of them being so far recognized as the most reliable (Raymond et al., 2001). However it is noteworthy to stress that these models were in many cases tested by using sets of data obtained through the adoption of quite different experimental procedures. In fact, it is possible to find in the literature many papers (Nyholm et al., 1996; Pagga, 1997) dealing with the assessment of reaction kinetics of aerobic biodegradation, in which different approaches were adopted to follow the decay of the substrate, for the initial substrate/sludge ratio and for the acclimation. Moreover it is necessary to consider that to carry out these experiments the need of using sludges coming from municipal WWTPs often sampled in different periods

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Table 1
Chemicals and working conditions

	Compound name	Molecular formula	HPLC analysis conditions		k_{bio} [h ⁻¹]	$k_{\text{bio}}/\text{VSS}$ [l g ⁻¹ h ⁻¹]	Y_1	Y_2
			Eluents	λ [nm]				
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	
1	1,3-Dinitrobenzene	(O ₂ N) ₂ C ₆ H ₄	65% A, 35% B	254	0.04	0.013	0.06	3.39
2	2,4-Dinitrophenol	(O ₂ N) ₂ C ₆ H ₃ OH	65% A, 35% B	220	0.03	0.01	0.17	3.41
3	3,5-Dinitrobenzoic acid	(O ₂ N) ₂ C ₆ H ₃ COOH	75% A, 25% B	220	0.01	0.003	0.21	3.33
4	4-Nitroaniline	NH ₂ C ₆ H ₄ NO ₂	80% A, 20% B	220	0.36	0.12	0.14	3.43
5	2-Chloro-4-nitrophenol	ClC ₆ H ₃ (NO ₂)OH	65% A, 35% B	220	0.07	0.02	0.29	3.36
6	2,6-Dichlorophenol	Cl ₂ C ₆ H ₃ OH	63% A, 37% B	220	0.25	0.08	0.42	3.32
7	4-Nitrophenol	O ₂ NC ₆ H ₄ OH	70% A, 30% B	300	0.33	0.11	0.49	3.58
8	Nitrobenzene	(O ₂ N)C ₆ H ₅	65% C, 35% B	280	0.24	0.08	0.51	3.57
9	3-Nitrobenzoic acid	(O ₂ N)C ₆ H ₄ COOH	73% A, 27% B	220	0.61	0.20	0.54	3.51
10	4-Chlorophenol	ClC ₆ H ₄ OH	65% A, 35% B	220	0.36	0.13	0.62	3.54
11	<i>o</i> -Cresol	CH ₃ C ₆ H ₄ OH	60% C, 40% B	220	1.68	0.56	0.87	3.66
12	2,3-Dimethylphenol	(CH ₃) ₂ C ₆ H ₃ OH	60% C, 40% B	220	2.43	0.81	0.91	3.57
13	Phenol	C ₆ H ₅ OH	85% A, 15% B	220	7.32	2.44	0.95	3.75
14	4-Hydroxybenzoic acid	OHC ₆ H ₄ COOH	90% A, 10% B	254	7.25	2.42	0.97	3.90
15	Benzoic acid	C ₆ H ₅ COOH	75% A, 25% B	220	11.4	3.80	0.99	3.68
16	3,4-Dihydroxybenzoic acid	(OH) ₂ C ₆ H ₃ COOH	95% A, 5% B	254	4.45	1.48	1.08	3.71
17	Benzene	C ₆ H ₆	60% C, 40% B	200	2.01	0.67	1.03	3.39
18	Gallic acid	(OH) ₃ C ₆ H ₂ COOH	100% A	220	1.31	0.44	1.19	3.08
19	Orthophthalic acid	C ₆ H ₄ 1,2-(COOH) ₂	90% A, 10% B	220	1.16	0.39	1.02	3.62
20	Terephthalic acid	C ₆ H ₄ 1,4-(COOH) ₂	87% A, 13% B	254	0.47	0.16	1.02	3.62

may have a deleterious influence on the reproducibility of the results.

In the present work the aerobic biodegradation of twenty aromatic species (Table 1) is investigated with the aim of assessing their kinetic constants. In particular, the reproducibility of the results is checked with respect to the origin of the sludges and the period of sampling. An attempt to correlate the values found for single kinetic constant to the structure of the molecules is also done.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Aldrich and used as received. Aromatic species, used in the experiments, are listed in Table 1 (column 1) along with their molecular formula (column 2). The following components (purchased from Carlo Erba) were used in the mobile phase for high performance liquid chromatography (HPLC): (A) phosphate buffer solution (pH 2.5, 0.4% phosphoric acid, 5% methanol); (B) acetonitrile; (C) water.

2.2. Experimental tests

The experimental tests were carried out in triplicate for each aromatic compound in batch conditions, using

as inoculum activated sludge taken from the biological oxidation basins of two different WWTPs (Napoli Ovest and Minturno, in Italy).

Each sample solution was prepared by adding a mixture of bi-distilled water and activated sludge to the inorganic test medium reaching a total reaction volume of 250 ml: the ratio between the bi-distilled water and the activated sludge was set in order to achieve 3 g dry matter for liter in the reactor. The samples were stirred and aerated (by bubbling air) at fixed temperature (25 °C). The pH of each solution was regulated at a value close to 7.0 by adding HCl or NaOH.

The concentration decay of the tested substrate was followed until its complete degradation. After having stopped the stirring the sludge was allowed to settle and an appropriate volume of the supernatant was drawn off. The reactor was refilled to the initial volume with an aqueous solution containing the mineral medium and the substrate at the right concentrations to restore the original conditions (10 g m⁻³). The system was newly stirred under air bubbling.

This procedure was repeated several times (7–9 reloads) in order to acclimate the biomass, until no lag-phase was observed.

The composition and dose of the inorganic test medium (Urano and Kato, 1986) were: 5 ml of a mixture of K₂HPO₄ (25.75 × 10⁻³ g m⁻³), KH₂PO₄ (8.5 × 10⁻³ g m⁻³), Na₂HPO₄·12H₂O (44.6 × 10⁻³ g m⁻³), NH₄Cl (1.7 × 10⁻³ g m⁻³); 0.5 ml of an aqueous solution

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ($22.5 \times 10^{-3} \text{ g}^{-3}$); 0.5 ml of CaCl_2 ($27.5 \times 10^{-3} \text{ g m}^{-3}$) and 1 ml of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ($0.25 \times 10^{-3} \text{ g m}^{-3}$).

Sample solutions were taken from flasks at fixed intervals of time, filtered with 0.45 μm pore size Cellulose Syringe Filters (Tracer) to remove cellular material, analyzed by means of a Knauer HPLC pump 64 equipped with a UV Detector K-2001 Knauer using a Synergi 4 μ MAX-RP 8A (Phenomenex) column. The injection volume was 50 μl and the flow rate 1.0 ml min^{-1} . The eluent composition and the detector wavelength λ adopted for each single compound are reported in the columns 3 and 4 of Table 1.

3. Results and discussion

Biodegradation kinetic constants, k_{bio} , were derived from the analysis of the curve showing the concentration decay against time. For each biodegradation test of a single substrate, more reloads were performed until no further changes of the biodegradation rate were observed.

The biodegradation results for each aromatic compound, reported in a semi-log plane, have been fitted with a first order curve in order to determine the k_{bio} values (Fig. 1 related to the 3,4-dihydroxybenzoic acid). In fact, the value of k_{bio} was obtained from the slope of the relationship between $\ln(C/C_0)$ versus time because the biodegradation process can be considered as a pseudo-first order kinetics according to a simplified Michaelis-Menten equation (Bailey and Ollis, 1986). Following this procedure, average kinetic constants for all investigated substances were derived, whose values are reported in columns 5 and 6 of Table 1.

Moreover the experiments were repeated for four chemicals (Fig. 2) from two to four times with the same activated sludge (stored at 4 °C during the tests time) in

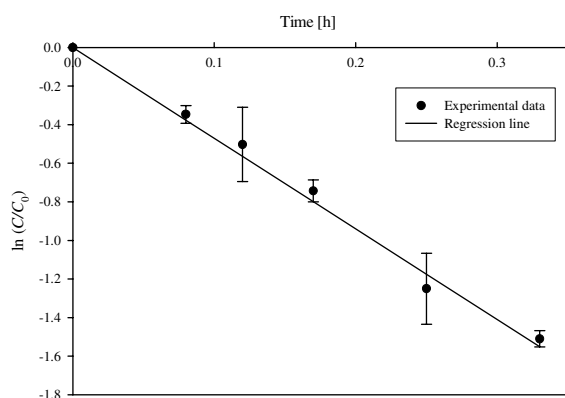


Fig. 1. Normalized concentration values of the 3,4-dihydroxybenzoic acid against time in logarithmic scale.

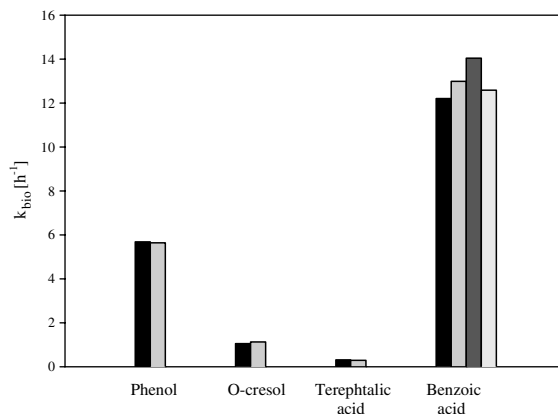


Fig. 2. k_{bio} values for phenol, *o*-cresol, terephthalic and benzoic acids, with the same sludge.

order to check the reproducibility of the results. The tests were also repeated using the activated sludge taken at different time from Napoli Ovest WWTP (Fig. 3) in order to check the influence of collection time on the biodegradation kinetics.

The results shown in the Fig. 2 indicate that a good reproducibility is achieved using the present experimental procedure on the same sludge. However in the case of sludges coming from the same treatment plant but taken in different period a standard deviation upto 35% was found.

Furthermore, biodegradation experiments on benzoic acid and *o*-cresol have been carried out using the sludge taken from the Minturno WWTP in order to check the influence of the sludge origin on the biodegradation kinetic constants. Preliminary results have shown a good reproducibility for *o*-cresol (1.53 h^{-1} for Minturno WWTP, 1.68 h^{-1} for Napoli Ovest WWTP). For benzoic acid, the kinetic constant values are not

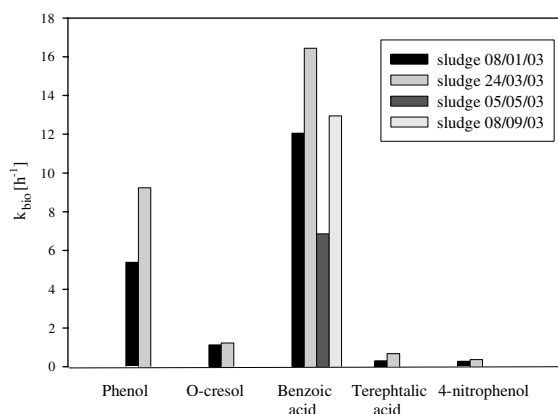


Fig. 3. k_{bio} values of five chemicals with Napoli Ovest WWTP sludge taken in different days.

comparable (5.21 h^{-1} for Minturno WWTP, 11.4 h^{-1} for Napoli Ovest WWTP). Similar results in biodegradation tests of organic substrates with sludges from different municipal STP plants have been previously reported in the literature (Van Ginkel et al., 1995).

As a general remark on the above reported results it can be observed that the introduction in an aromatic ring of a nitro group or a chlorine atom results into a reduction of the biodegradation rate. This behaviour well agrees with that expected on the basis of the electron-withdrawing character of these substituents and of the electrophilic nature of the oxygen transfer to the reacting molecules.

On the other hand, the introduction of a hydroxyl group requires a particular discussion. In fact, it is expected that its introduction in an aromatic ring would result into an increase of the rate of biodegradation. This was really observed in the case of phenol since its kinetic constant (7.32 h^{-1}) is almost four times that found for benzene (2.01 h^{-1}). However the introduction of hydroxyl groups in an aromatic ring already containing an activating substituent shows just the opposite trend (see the results found for benzoic acid and its hydroxyderivatives). This apparent discrepancy can be removed if one considers that the biodegradative process generally proceeds through three different steps: (i) migration of the substrate from the bulk solution towards the microorganism; (ii) migration of the substrate through the membrane of the microorganism; (iii) enzymatic oxidation of the substrate inside the microorganism. The overall rate of the process is thus determined by the slowest among these steps. In the case of hydroxybenzoic acid series, the introduction of hydroxyl group in the structure of benzoic acid may result into two effects: (1) the increase of the enzymatic reaction rate (electron-donor effect of the hydroxyl group); (2) a decrease of the migration rate of the species, due to an increase of molecular weight (or the volume of the molecule).

Therefore the introduction of hydroxyl groups in the structure of benzoic acid could result into a change in the rate controlling step which could be represented for hydroxybenzoic acids by the transport (migration) from the bulk solution to the microorganism or through the membrane. This result seemed to be confirmed by different tests carried out on 3,4-dihydroxybenzoic acid changing the temperature. In fact, two additional tests have been carried out at the temperature of 32 and 37 °C with the same sludge, obtaining values of 4.53 h^{-1} and 4.57 h^{-1} respectively. This result shows that no change occurred in biodegradation kinetics, at changing the temperature, thus confirming that the rate controlling step is not represented by the enzymatic oxidation.

Similar considerations can be reported for each species characterised by the insertion of an activating sub-

stituent in an already too reactive structure (for example, in the case of methylphenols, or dicarboxylic aromatic acids). Therefore, it is important to stress that kinetic constants previously estimated in the case of hydroxybenzoic derivatives or methylphenols could represent overall volumetric mass transfer coefficients.

An attempt to find a reliable correlation between the kinetic constant for biodegradation and the structure of investigated compounds was done using the probability of ready biodegradation (Y_1) as introduced by Horward et al. (1991), by means of the following expression:

$$Y_1 = a_0 + a_1f_1 + a_2f_2 + a_3f_3 + \dots + a_n f_n + a_m \text{MW} + e_j \quad (1)$$

where Y_1 = probability that chemical is readily biodegradable; f_n = the number of n th substructure in the j th chemical; a_0 = the intercept; a_n = the regression coefficient for the n th substructure; MW = the molecular weight; a_m = the regression coefficient for MW; e_j = the error term.

For the chemicals tested, Y_1 have been easily estimated by means of the calculus code named Biowin (column 7 of Table 1), developed by the Syracuse Research Corporation according with USEPA (USEPA, web site). The Biowin code also allows to calculate the primary biodegradation values (Y_2), obtained modifying expression (1) and recently also used by Khan and Ongerth (2004) to model chemicals residues in Australian sewage. The values calculated for Y_2 related to chemicals used in this work are summarised in column 8 of Table 1.

In Fig. 4 natural logarithms of k_{bio} 's are plotted against Y_1 , showing that for many investigated chemicals a proportionality exists. However, on the basis of the above reported considerations it could be expected that not all the estimated constants may correlate with Y_1 which substantially reflects the electronic effects of the substituents present in the structure.

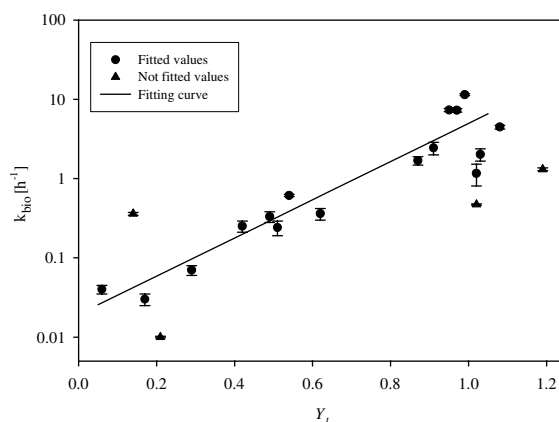


Fig. 4. k_{bio} versus Y_1 for investigated substrates.

However a better linear correlation between $\ln(k_{\text{bio}})$ and Y_1 can be easily obtained by ruling out from the original pool hydroxybenzoic acids and methylphenols and dicarboxylic aromatic acids (full circles, Fig. 4).

Worse results were found by employing the *primary biodegradation value*, Y_2 , following the procedure suggested by Khan and Ongerth (2004). This behaviour could be due to the fact that the contribution of the groups to the Y_2 function was derived by the authors by using biodegradation data collected according to MITI-I test protocol (Tunkel et al., 2000). In fact, this protocol states that a single run has to be performed with a substance concentration of 100 g m^{-3} , an inoculum of 30 g m^{-3} solid sludge and without any acclimation, by scoring the tested compound as readily or not readily biodegradable depending on the capability of the species to reduce its ThOD (theoretical oxygen demand) for more or less than 60% within 28 days (in case of primary biodegradation the values of Y_2 , ranging from 0 to 5, are attributed according to the characteristic reaction time). That is the condition and the evaluation of the data recommended in the MITI-I test protocol are quite different from those adopted in the present investigation.

Considering the results that are described above, another approach to find a “structure-reactivity” relationship for aerobic biodegradation has been adopted, for which the contribution of each group to the kinetic constants was accounted for by using a linear relationship of the following type:

$$\ln(k_{\text{bio}})_i = \ln(k_{\text{bio}})_0 + \sum f_j n_j \quad (2)$$

where n_j = number of substituents of one type (i.e. the number of nitro group, or chlorine substituents, etc.); f_j = effect of the substituent on the reactivity of studied compound.

In Fig. 5 an example of application of the above reported relationship (2) to the data collected on three

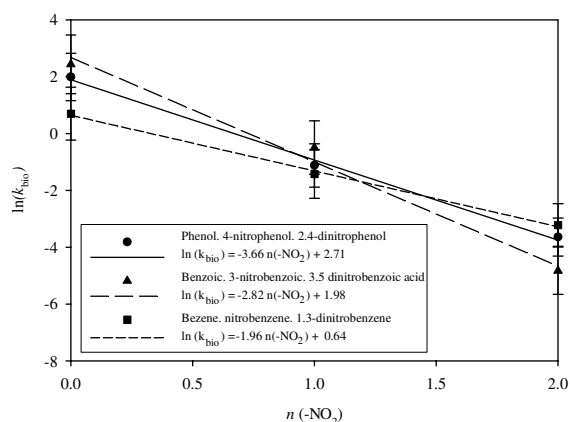


Fig. 5. $\ln(k_{\text{bio}})$ versus nitro groups number.

groups of nitroderivates is given. It is clear from the figure that although for each nitroderivates series the linear relationship holds, the three straight lines are characterized by quite different slopes which do not allow to estimate a single value for the contribution of the nitro group to the kinetic constant of aerobic biodegradation. The difference found among the slopes for the three sets of data is more large that expected on the basis of statistical uncertainties associated to the determination of a single constant (for example, in the case in which sludges coming from different plants are used). This observation supports the hypothesis that a single substituent, as a nitro group, influences the value of the kinetic constant in different ways depending upon the presence in the structure of other groups. That is, the nitro group exerts different effects when introduced in an unsubstituted aromatic ring (as benzene) or in the phenol structure. This idea which has been put forward in the past also by other authors (Tabak and Govind, 1993), indicates a different way to find a reliable structure-reactivity relationship, that needs more confirmation, investigating the effects of other groups (chlorinated molecules, aminoderivatives etc.).

4. Conclusion

The biodegradation of aromatic compounds is, nowadays, a research issue, both for the development of reliable biodegradation tests and the evaluation of the biodegradation kinetics.

In the present work aerobic kinetic biodegradation constants of twenty aromatic compounds have been estimated, by means of batch biodegradation tests using activated sludge, taken from the aeration basin of two WWTPs at different times.

The effects, on the kinetic constants, due the presence of a single group (e.g. $-\text{OH}$, $-\text{CH}_3$, $-\text{Cl}$, $-\text{NO}_2$, etc.) into the molecule structure, have been investigated. In the case of nitro groups or chlorine atoms the results have shown that the kinetic biodegradation constants decrease increasing the number of groups in the molecule structure, as expected. Contrary the activating actions of hydroxyl and methyl groups have been only partially observed; in fact their introduction in an already activated structures (phenol, benzoic acid, etc.) have shown an inactive trend probably because the whole process became transport controlled.

The interpretation of all experimental results have shown that the effects of each group also depends on the presence of other groups in the molecule structure. This result has been not clearly pointed out by other authors. Thus the mutual influence of each group should be more intensely investigated, validating the kinetics constants results, also by means of continuous flow reactors.

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