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BIODEGRADABILITY OF PHENOL, RESORCINOL AND 5-METHYLRESORCINOL AS SINGLE AND MIXED SUBSTRATES BY ACTIVATED SLUDGE

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The aim of this study was to investigate aerobic biodegradability of phenol, resorcinol and 5-methylresorcinol and their different two-component mixtures using activated sludge sampled from the Kohtla-Järve wastewater treatment plant. The degradation behaviour of phenolic compounds was investigated by respirometry. Non-linear regression analysis was used for determination of the kinetic parameters such as the maximum rate of oxygen uptake ($V_{O_2,max}$), the maximum rate of substrate bio-oxidation (V_{max}) and the half-saturation coefficient (K_S). Various kinetic models were tested to obtain the best curve fit. It was shown that the activated sludge degraded resorcinol and 5-methylresorcinol more slowly than phenol. Among the studied substrates phenol had the highest values of $V_{O_2,max}$, V_{max} as well as the ratio of $V_{O_2,max}$ /K_S. Activated sludge had the highest affinity to phenol with the lowest K_S value. Among all studied bi-substrate systems the highest $V_{O_2,max}$ values were found for phenol (0.1 mM) – 5-methyl-resorcinol. As the kinetic parameters and short-term oxygen demands are functions of the compound undergoing biodegradation and the composition of the microbial community performing the degradation, therefore the results of this study have importance in explanation of effectiveness of wastewater treatment process and of the influence of polluting compounds on it.

Introduction

Phenolic compounds are listed as priority environmental pollutants and they occur in wastewaters from oil refineries, chemical plants, explosives, resins and coke manufacture, coal conversion, pesticide and textile industries [1]. In 2009, the total annual discharge of monohydric and dihydric phenols into the aquatic environment was 2780 kg in Estonia, including 2260 kg in North-Eastern Estonia [2]. One significant source of phenolic pollution in the North-Eastern Estonia is the oil-shale chemical industry where oil is

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produced from oil shale. Wastewater of the oil-shale chemical industry is canalized to the Kohtla-Järve wastewater treatment plant (WWTP). Phenolic compounds contaminate the surrounding surface water as well as underlying aquifers and, therefore, the risk for contamination of groundwater as a source of drinking water in the North-Eastern Estonia is quite high. The presence of phenolic compounds in groundwater is the result of industrial releases or leachate from waste dumps, and the subsequent leaching of phenolic compounds through the soil to the groundwater [3, 4].

Since the phenolic compounds can have serious environmental effects, microbiological and biochemical features of their aerobic degradation are of great interest, and monitoring the oxygen uptake can give valuable data about the processes involved. In spite of their toxic properties, a number of microorganisms can utilize phenolic compounds under aerobic conditions as sole sources of carbon and energy, even at relatively high concentrations [5–7]. However, the biodegradation of phenolic compounds needs a specific microbial population, or degrading bacteria are required to be adapted to the phenolic compounds, in order to induce certain enzymes in the bacteria to be available to take part in the metabolism. Although, the use of pure cultures of microorganisms can be envisaged as an alternative, the activated sludge appears a more attractive solution than a single microbial species because of its various advantages. The main advantage resulting from the microbial consortium formed by acclimated activated sludge is the interaction between all the species present in flocs. Basically, it contains mainly bacteria and protozoa [8–10].

Respirometry is one of the techniques most used to study the aerobic biodegradation of toxic compounds. It has been used to determine the short-term biological oxygen demand [11] and the biokinetic parameters of degradation processes [12–15], to estimate the organic load to the wastewater treatment plant [16], to warn of incoming toxicity [17, 18] and predict effluent quality [19]. The respiration rate of activated sludge is an important variable for the control of activated sludge process, because oxygen consumption is directly associated with both biomass growth and substrate removal [20].

The rate of biodegradation of phenolic compounds depends on the properties of activated sludge (floc composition and size, biomass concentration, microbial community and their metabolic potential), temperature, the concentration and the properties of the substrate. According to the earlier studies concerning the activated sludge taken from the Kohtla-Järve WWTP, this specific sludge was able to oxidize the studied phenolic compounds without adaptation before the performing of the tests [13, 15]. This is possible due to a specific microbial population, which is formed in the treatment plant processing wastewater containing the given compounds. Differences exist in the maximum rate of oxygen uptake ($V_{O_2,max}$) as well as in the amounts of oxygen consumed per mole of substrate [13, 15]. Lepik *et al.* [15] showed that the values of biodegradation parameters with various

activated sludge samples vary considerably and the biodegradability of phenolic compounds is related to their structures. It depends on the properties and the number of substituents of aromatic nucleus. Biodegradation is also affected by the positions of methyl groups of methylphenols. The *p*-substituted phenols are more readily degradable than the *m*- or the *o*-substituted ones. It has been also reported in the literature that resorcinols are potentially more slowly degradable than monohydric phenols [21, 22].

The aim of this study was to investigate the aerobic biodegradability of phenolic compounds characteristic to the oil-shale industry wastewaters – phenol, resorcinol and 5-methylresorcinol as both single and mixed substrates at different concentrations by the activated sludge of the Kohtla-Järve WWTP and to obtain more information how these compounds influence each other's biodegradation. The short-term oxygen uptake measurement was used to assess the biodegradability of phenolic compounds. The biodegradation of the studied compounds was also characterized by the kinetic parameters such as the values of the maximum rate of oxygen uptake ($V_{O_2,max}$), the maximum rate of substrate bio-oxidation (V_{max}) and the half-saturation coefficient (K_S). As the kinetic parameters and short-term oxygen demands are functions of the compound undergoing biodegradation and the composition of the microbial community performing the degradation [11, 23], the respirometric method grants the possibility to assess the potential of activated sludge to oxidize different compounds and predict the rate of their removal in the treatment plant. The knowledge of both the rate and extent of degradation is essential for the understanding of the behaviour of compound and its persistence.

Materials and methods

Chemicals and microorganisms

Phenol, resorcinol and 5-methylresorcinol were used as both single and mixed substrates in the experiments. All the chemicals used for the preparation of substrate solutions were of an analytical grade.

Activated sludge used in the biodegradability tests was sampled from the aeration tank of the Kohtla-Järve WWTP, Estonia, which is also treating phenolic wastewater from the oil-shale chemical industry and, therefore, considered to be acclimated to the phenolic compounds. The activated sludge was stored and pre-aerated at least for 24 h at room temperature to achieve a stable endogenous respiration before it was used for experiments. The concentrations of activated sludge samples were determined gravimetrically and quantified as dry weight of suspended solids (MLSS) in grams per litre. A sample was centrifuged and the deposit was dried for 24 h at 105 °C until a constant weight was reached.

Short-term oxygen uptake measurement

The respirometric method [13, 15] adapted from Čech *et al*. [12] was used for the determination of the short-term oxygen demands (BOD_{st}) and kinetic parameters of phenolic compounds. The BOD_{st} is defined as the sum of oxygen demand for the oxidation of all kinds of readily biodegradable organic compounds in the activated sludge suspension [11].

The open respirometer consisted of a 500-ml working volume reactor, which was continuously stirred with the magnetic stir-bar at a constant rotation speed to ensure the homogeneity of media. The batch system was kept at a temperature of 20 ± 1 °C. The suspension of activated sludge was transferred into the respirometer and aerated. When the dissolved oxygen (DO) concentration reached 8-9 mg/l, the aeration was stopped and the decrease of DO concentration as a function of time was recorded and logged by a computer. The DO concentration was monitored by a submerged electrochemical Clark-type oxygen sensor CellOx 325 and WTW InoLab Oxi Level 2 oxygen meter (WTW, Germany). A low decrease in oxygen concentration at the beginning of the test was due to the endogenous respiration of microorganisms of activated sludge. During the endogenous respiration the heterotrophic microorganisms utilize oxygen at a constant rate over a relatively long period of time. Addition of a certain amount of substrate to the respirometer causes a temporary increase in the respiration rate. As the substrate concentration decreases with time, the respiration rate also decreases, being substrate-dependent at low concentrations. When the substrate has been degraded, the respiration rate returns to the value, which is equal to the original endogenous rate [12]. To avoid oxygen limitations the experiment was stopped when the oxygen level dropped below 1 mg O_2/l. Oxygen uptake rates (OUR) were then calculated from the slope of DO decline with time by using linear regression of all the obtained dissolved oxygen data.

A low ratio of the initial substrate concentration (S_0) to biomass concentration (X_0) was used in short-term measurements. Under these conditions the minimal changes occur in the degrading community and in the biomass concentration over a short period of the experiment and, therefore, the estimated kinetic parameters from those experiments are representative of the existing condition of the biomass in the wastewater treatment plant from which the activated sludge was sampled [24]. The endogenous respiration rate (OUR_{end}) was considered to be constant during the batch respirometric cycle, and the value of OUR_{end} used in all experiments was the original respiration of activated sludge, measured in the sample before the substrate was added.

Modeling

During the process of aerobic biodegradation, the dissolved oxygen (DO) is consumed as one of the substrates by microorganisms. The OUR reflects the

kinetics of aerobic biodegradation of the substrate by heterotrophic organisms. The oxygen profile resulting from the endogenous oxygen uptake and the addition of a substrate is shown in Fig. 1.

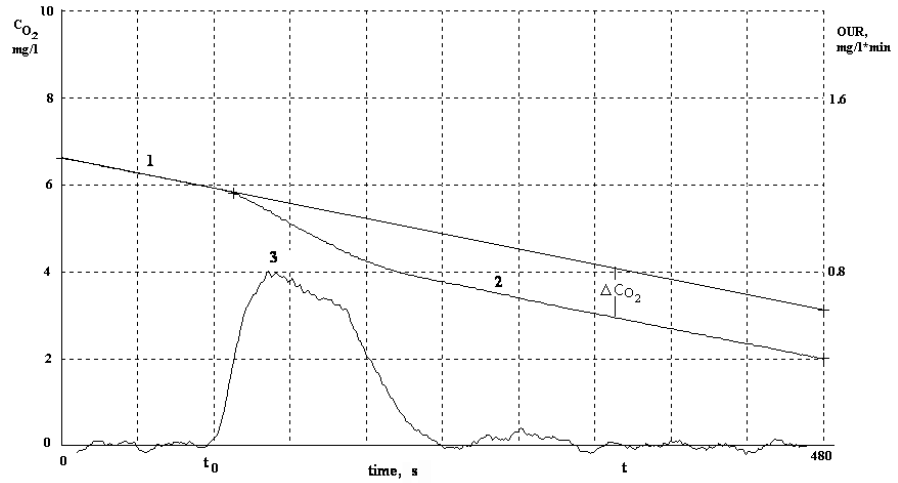

Fig. 1. Oxygen concentration profile due to endogenous respiration of activated sludge (curve 1), total respiration after the addition of substrate (curve 2), and calculated respiration rates (OUR, curve 3) in the case of the addition of phenol at concentration of 0.015 mM.

The OUR can be calculated from the DO data by measuring temporal oxygen concentration changes in the test system. Total decrease in the DO concentration is determined by the microbial oxygen uptake for both the endogenous (OUR_{end}) and exogenous respiration (OUR_{ex}) [13]:

$$\frac{dC_{O_2}}{dt} = -(\mathrm{OUR}_{\mathrm{end}} + \mathrm{OUR}_{\mathrm{ex}}) = -\mathrm{OUR}_{\mathrm{tot}} . \tag{1}$$

The OUR_{end} is calculated by a linear regression from the first part of the respirogram ($t < t_0$), where $OUR_{tot} = OUR_{end}$. The endogenous respiration rate can be regarded practically constant for the time interval of one test and, therefore, could easily be eliminated from the total respiration process.

The rate equation describing the degradation process of an individual substrate by the activated sludge can be based on the kinetics for oxygen and substrate acting as rate-limiting factors at a constant biomass concentration analogous to the Michaelis-Menten kinetics. The equation can be expressed as follows [13]:

$$\frac{dS}{dt} = -V_{\max} \cdot \frac{S}{K_S + S} \cdot \frac{C_{O_2}}{K_{O_2} + C_{O_2}}, \tag{2}$$

where $V_{max} = kX$ and means the maximum rate of substrate degradation; X is the biomass concentration; k is the specific rate of the limiting step in substrate consumption; S is the concentration of substrate; C_{O_2} is the concentration of oxygen; K_S is the half-saturation coefficient of substrate and K_{O_2} the half-saturation coefficient of oxygen.

According to Eq. (2) the rate of substrate degradation is proportional to the biomass concentration and depends hyperbolically on the concentrations of the substrate and oxygen. X can be considered a constant, since in practice, biomass growth due to substrate addition is negligible in the system within the time of the short-term experiment.

Oxygen concentration can be maintained above the concentration of 1 mg/l during the experiment to avoid oxygen becoming a limiting factor. If $C_{O_2} >> K_{O_2}$, Eq. (2) for the substrate degradation can be expressed as follows [13]:

$$\frac{dS}{dt} = -V_{max} \cdot \frac{S}{K_S + S}. \tag{3}$$

Taking into account that the OUR_{ex} is proportional to the rate of substrate degradation, the maximum value of oxygen uptake rate (OUR_{max}) expressing the initial reaction rate for a specific amount of the added substrate, can be determined from the oxygen uptake rate (curve 3) in the range of $t \geq t_0$. OUR_{max} depends on the substrate concentration. If the amount of the added substrate is high enough to cause the plateau in the OUR curve, this indicates that the substrate concentration is in excess and further increase of its concentration does not increase the OUR. When the concentration of the biodegradable substrate is very high, the OUR_{max} value will approximate its maximum value, i.e. the maximum rate of oxygen uptake ($V_{O_2,max}$). OUR_{max} can be related to the initial substrate concentration (S_0) as follows [13]:

$$OUR_{max} = v \cdot V_{max} \cdot \frac{S_0}{K_S + S_0} = V_{O_2,max} \cdot \frac{S_0}{K_S + S_0}, \tag{4}$$

where v is the coefficient that expresses the quantity of oxygen consumed per quantity of substrate in the degradation process and gives an estimate of the short-term oxygen demand (BOD_{st}) caused by the addition of substrate during the test.

In this study the degradation of a single substrate system was modeled by the Michaelis-Menten Eq. (4). The kinetic parameters $V_{O_2,max}$, V_{max} and K_S were determined from the dependence of the values of OUR_{max} on the substrate concentrations by non-linear regression with using the method of the least squares.

Most of the biochemical reactions involve at least two substrates, therefore it is necessary to consider the kinetics of multi-substrate enzyme-catalysed reactions. It is a complex topic, and several mechanisms and interactions are described in the literature [25]. For reasons of simplicity, in

this study the random-order ternary-complex mechanism was used. The general rate equation for two-substrate reaction is presented as follows [25]:

$$V = \frac{V_{\max} \cdot S_1 S_2}{K_d K_{m,2} + K_{m,2} S_1 + K_{m,1} S_2 + S_1 S_2}, \tag{5}$$

where V is the reaction rate; $V_{\max}$ is the maximum possible reaction rate when both substrates are saturated; S_1 and S_2 are the concentrations of both substrates; K_d is the dissociation constant for enzyme-substrate complex; $K_{m,1}$ and $K_{m,2}$ are the half-saturation coefficients of substrate 1 and 2, when other substrate is saturated.

In this study, concerning the bi-substrate systems the Michaelis-Menten Eq. (4), the random-order mechanism (Eq. (5)) and to take into account the possible inhibition effect the Haldane model (Eq. (6)) were applied:

$$\mathrm{OUR}_{\max} = V_{O_2,\max} \cdot \frac{S}{K_S + S + S^2 / K_i}, \tag{6}$$

where K_i is the inhibition coefficient [25].

Biodegradation kinetics for mixtures of homologous substrates is often modeled using no-interaction sum kinetics or purely competitive inhibition kinetics. However, there are many cases in which the interactions between homologous substrates are not purely competitive, even for similar compounds. Potential reasons for deviations from such kinetics include: 1) interactions at the level of substrate transport into the microorganism, 2) interactions between compounds, and 3) the presence of a previously unidentified catabolic pathway or pathway branch [26–28]. For a binary mixture in which the presence of the one substrate does not affect the biodegradation rate of other, the no-interaction sum kinetics model can be described as:

$$\mathrm{OUR}_{\max} = \frac{V_{O_2,\max,1} \cdot S_1}{K_{S,1} + S_1} + \frac{V_{O_2,\max,2} \cdot S_2}{K_{S,2} + S_2}, \tag{7}$$

where the subscripts 1 and 2 refer to each of the two substrates.

However, there are interactions between these substrates which cannot be described by sum kinetics models using only parameters determined in single substrate experiments. For the substrate mixtures, using the values of $V_{\mathrm{O}_2,\max,i}$, $K_{\mathrm{S},i}$, and $\mathrm{K}_{\mathrm{i},i}$ determined from single-substrate experiments, the sum kinetic models on an unspecified type of interaction, proposed by Yoon *et al.* [29] and Abu Hamed *et al*. [30], were used to predict the consumption of the two substrates and to calculate the interaction parameters ($\mathrm{I}_{1,2}$, $\mathrm{I}_{2,1}$) according to the Eq. (8) and (9):

$$\mathrm{OUR}_{\max} = \frac{V_{O_2\max,1} \cdot S_1}{K_{S,1} + S_1 + \mathrm{I}_{2,1} S_2} + \frac{V_{O_2\max,2} \cdot S_2}{K_{S,2} + S_2 + \mathrm{I}_{1,2} S_1}, \tag{8}$$

$$\mathrm{OUR}_{\max} = \frac{V_{O_2\max,1} \cdot S_1}{K_{S,1} + S_1 + \dfrac{S_1^2}{K_{i,1}} + \mathrm{I}_{2,1} S_2} + \frac{V_{O_2\max,2} \cdot S_2}{K_{S,2} + S_2 + \dfrac{S_2^2}{K_{i,2}} + \mathrm{I}_{1,2} S_1}, \tag{9}$$

where the interaction parameter $I_{1,2}$ indicates the degree to which substrate 1 affects the biodegradation of substrate 2. A high value of the parameter indicates a strong inhibition on the substrate uptake by the micro-organism [29].

The best fitting values of kinetic parameters were determined using a non-linear least squares regression method by minimizing the sum of the squares of the differences (residuals) between the measured and the estimated values of OUR_{max} from the regression equation for the entire portion of the response. The suitability of the models was assessed by comparing the theoretical curves obtained with the corresponding experimental data. The standard deviation of the regression ($s_{y/x}$) is the best statistical quantity that measures how well a regression equation fits the measured data. The better the fit, the smaller is $s_{y/x}$. If the data fit the model equation perfectly all the residuals are zero and $s_{y/x} = 0$. For comparison, the coefficient of determination (R^2) (also called the coefficient of regression) was also estimated. If all variance be explained by the model, the value of R^2 is equal to one [31].

Results and discussion

In this study the aerobic biodegradability of phenol, resorcinol and 5-methyl-resorcinol by the activated sludge taken from the Kohtla-Järve WWTP was investigated. The bio-oxidation of the compounds as both single and mixed substrates at different concentrations was studied. To assess the biodegradability of the phenolic compounds, the BOD_{st} and the kinetic parameters estimated from the respirometric data were used. Results from the experiment series run within a period of four years are summarized. This study represents degradation experiments with an undefined adapted mixed population of activated sludge taken from WWTP. From previous studies it is known that in such systems the factors influencing the degradation rate are difficult to quantify and control [15, 32]. The uncertainties of the measurements were calculated as standard deviations of the mean and expressed at the level of confidence of 95% (coverage factor equal to 2). The measurement uncertainty is a non-negative parameter characterizing the dispersion of the quantity values being attributed to a measurand. The parameter may be expressed as a standard deviation called standard measurement uncertainty (or a specified multiple of it), or the half-width of an interval, having a stated coverage probability [33].

Biodegradation of phenolic compounds as single substrates

To estimate the biodegradability of individual phenolic compounds the oxygen uptake measurement was used. The results showed that the BOD_{st} values of these compounds formed 21–28% of the theoretical oxygen demand (thOD) and 26–37% of the biochemical oxygen demand (BOD_7), showing the highest value for phenol. The thOD is the stoichiometric amount of oxygen (expressed as mole of O_2 per mole of the substrate) required to oxidize a particular organic compound into the end products such as carbon dioxide and water. The BOD_7 shows the amount of oxygen used by microorganisms at 20 °C during the incubation period of 7 days in the darkness to metabolize biologically degradable organic compounds [34]. It is worth to mention that in a previous study [15] about 25–43% of thOD was consumed by activated sludge during the short-term measurements, showing the highest value for resorcinol. However, significant differences also existed between samples taken at different times. The discrepancy between the values of the biodegradation parameters obtained with various activated sludge samples could be explained by the variations in the properties of activated sludge (not only MLSS, but also floc composition and size, microbial community, metabolic potential), which in turn are conditioned by the variations in the concentration and the properties of the influents to the WWTP. The mean values of the ratio of BOD_{st}/thOD with the uncertainties of measurement are summarized in Table 1. The BOD_{st}/thOD values achieved for resorcinol, 5-methylresorcinol and phenol in this study correlate well with the results reported by Orupõld *et al*. [13] using the activated sludge taken from the same WWTP.

Table 1. **The mean values of the ratio of the short-term oxygen demand (BOD_{st}) to the theoretical oxygen demand (thOD) and the maximum oxygen uptake rate (OUR_{max}) at 0.015 mM with the uncertainties of measurement (u_c) for the studied phenolic compounds**

Substrate	BOD_{st}/thOD	OUR_{max} mg O_2/(mmol·min·MLSS)
	mean ± u_c	mean ± u_c
phenol	0.28 ± 0.03 (n = 29)	7.27 ± 1.26 (n = 29)
resorcinol	0.22 ± 0.06 (n = 10)	3.85 ± 1.46 (n = 10)
5-methylresorcinol	0.21 ± 0.05 (n = 10)	4.66 ± 0.87 (n = 10)

n – the number of experimental series

More specific respirometric analyses were carried out with phenol, resorcinol and 5-methylresorcinol (hereinafter 5-MR) at concentration of 0.005 mM, 0.01 mM, 0.015 mM, 0.025 mM, 0.04 mM and 0.05 mM. The dependence of OUR_{max} values on substrate concentration was investigated (shown in Fig. 2) and modeled by the Michaelis-Menten kinetics. OUR_{max}

corresponds to the initial rate of degradation reaction for the certain amount of added substrate. The kinetics parameters, such as the maximum rate of oxygen uptake ($V_{O_2,max}$), the maximum rate of substrate oxidation (V_{max}) and the half-saturation coefficient (K_S) for a single substrate were determined. Low values of $s_{y/x}$ and near unity values of coefficient of regression (R^2) for the data sets in Fig. 2(a) ($s_{y/x} = 5.1 \cdot 10^{-3}$; $2.3 \cdot 10^{-3}$; $2.6 \cdot 10^{-3}$ mg O_2/(l·min·MLSS); $R^2 = 0.98$; 0.99; 0.99 for phenol, 5-MR and resorcinol, respectively) and in Fig. 2(b) ($s_{y/x} = 5.2 \cdot 10^{-3}$; $4.2 \cdot 10^{-3}$; $9.0 \cdot 10^{-4}$ mg O_2/(l·min·MLSS); $R^2 = 0.98$; 0.98; 0.99 for phenol, 5-MR and resorcinol, respectively) indicated that the model predictions were in good agreement with the experimental data. The estimated values fitted the measured data reasonably well and the Michaelis-Menten kinetics was able to properly interpret the experimental results in the studied range of substrate concentrations.

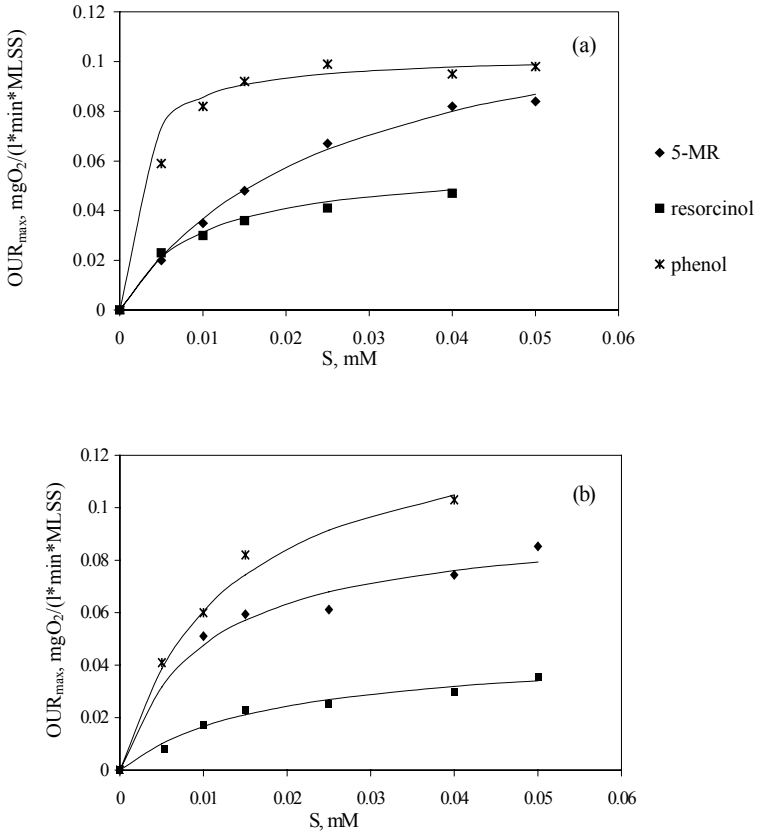

Fig. 2. The relationship between OUR_{max} values and added substrate concentrations for phenol, resorcinol and 5-methylresorcinol (5-MR). The symbols correspond to the measured data, whereas the solid line corresponds to the Michaelis-Menten kinetics. MLSS of specific activated sludge was (a) 6.9 g/l and (b) 6.5 g/l and the samples were taken at the time-interval of 4 months.

The time-dependent variations of the dependence of OUR_{max} values on added substrate concentrations for phenol, resorcinol and 5-MR are also illustrated in Fig. 2(a) and (b). Assuming that the microbial community is the same, the values of $V_{O_2,max}$ and OUR_{end} as indicators of the active biomass concentration suggest that the differences also existed in the capability of microbial culture of the activated sludge samples taken at different times. Variations in the influents of WWTP and in the operating conditions of wastewater treatment can influence the microbial community and the metabolic potential of the activated sludge thereby affecting the kinetic parameters estimated in the tests. The results of estimated kinetic parameters are summarized in Tables 2 and 3.

Table 2. **The mean values of estimated kinetic parameters $V_{O_2,max}$ and V_{max} with the uncertainties of measurement (u_c) for different substrates**

Substrate	$V_{O_2,max}$, mg O_2/(l·min·MLSS)	V_{max}, µmol/(l·min·MLSS)	
	mean ± u_c	mean ± u_c	range
phenol	0.140 ± 0.029	2.4 ± 0.5	1.0–5.7
resorcinol	0.072 ± 0.019	2.0 ± 0.4	0.9–2.5
5-methylresorcinol	0.127 ± 0.036	2.0 ± 0.5	1.6–3.7

Table 3. **The mean values of the half-saturation coefficient K_S and the ratio of $V_{O_2,max}/K_S$ with the uncertainties of measurement (u_c) for different substrates**

Substrate	K_S, µM		$V_{O_2,max}/K_S$, mg O_2/(mmol·min·MLSS)
	mean ± u_c	range	mean ± u_c
phenol	3.7 ± 0.9	0.6–9.0	49.7 ± 14.0
resorcinol	8.0 ± 2.0	3.0–18.0	11.5 ± 5.5
5-methylresorcinol	12.0 ± 5.0	3.0–26.0	12.7 ± 3.6

Among the studied substrates, phenol had the highest values of $V_{O_2,max}$, followed by 5-MR. $V_{O_2,max}$ is the maximum value of OUR_{max} at a particular biomass (enzyme) concentration and is independent of substrate concentration and so cannot be increased by using still higher substrate concentrations [25]. Therefore, $V_{O_2,max}$ is a measure of the metabolic activity of a microbial community, showing the maximum initial rate possible at this enzyme concentration [25]. As oxygen uptake profiles yield the same information as substrate depletion profiles due to the stoichiometric link between these two processes [35], it was also possible to estimate V_{max} by dividing the $V_{O_2,max}$ values by the stoichiometric coefficient ν (Eq. (4)). The mean values of V_{max} were in the range of 2.0–2.4 µmol/(l·min·MLSS),

showing the highest value for phenol. Although the mean values of the degradation rates of 5-MR and resorcinol were somewhat lower compared to those of phenol, the obtained results showed that the studied biomass was able to oxidize also the resorcinolic compounds. In this study, the dry weight of suspended solids (MLSS) was used for the estimation of the concentration of biomass. Consequently, the obtained results may underestimate the values of the specific oxygen uptake rate and the maximum rate of substrate oxidation because of the overestimation of the phenol-consuming bacteria that are present in the tested activated sludge. While $V_{O_2,max}$ varies with the total concentration of enzyme present in microorganisms, K_S is independent of enzyme concentration and is characteristic of the system being investigated. K_S is the value of S_0, which gives an initial rate equal to $0.5\times V_{O_2,max}$. K_S gives an indication of the affinity of the biomass for the substrate: a low K_S value indicates a high affinity for substrate, whereas a high K_S value shows a low affinity [25]. Considering that K_S is related to the affinity of the microbial community for specific substrate, the activated sludge showed the highest affinity to phenol and the lowest affinity to 5-MR. The mean values of K_S for these compounds were 3.7 μM and 12 μM, respectively. The ratio of $V_{O_2,max}/K_S$, indicating the slope of the linear part of the curve OUR_{max} *vs* substrate concentration, was the highest for phenol with the average value of 49.7 mg O_2/(mmol·min·MLSS). The ratio of $V_{O_2,max}/K_S$ refers to specific affinity and was proposed by Healey [36] as a more suitable parameter that should reflect both the affinity and catalytic activity.

With regard to the half-saturation coefficients of phenolic compounds, there is a broad range of reported values in the literature. The K_S values for phenol from this study have been proven by a correspondence with the findings of earlier studies using mixed microbial cultures [12, 32]. However, comparison of the achieved K_S values for phenol with the data reported by the other authors show that some studies have revealed K_S values to be higher [37] and some lower [24, 38] than those obtained in this study. The K_S values ranging from 8.3 μM to 16.9 μM for phenol corresponding to an adapted activated sludge on phenolic compounds [13] and ranging from 6.2 μM to 13.3 μM for phenol uptake rate by *Pseudomonas putida* [39] were similar to the values obtained in this study. The K_S values for 5-MR and resorcinol reported in this study were similar or slightly lower than those reported in the other studies [13, 40]. The differences between the coefficients obtained using respirometry and batch growth techniques reported in the literature could be due to the different features of the bio-oxidation process that are actually tested. It may be assumed that respirometry measures enzyme activities related to the first oxidation steps of the tested compound, especially when oxygenases are involved in the aerobic oxidation pathway [41].

Biodegradation of phenolic compounds as mixed substrates

The biodegradability of three different bi-substrate systems of phenolic compounds was studied: phenol – resorcinol, phenol – 5-MR and resorcinol – 5-MR containing both components at equal concentrations from 0.005 mM to 0.05 mM (hereinafter referred also as (1:1)). Moreover, the biodegradability of the bi-substrate systems of phenol – resorcinol and phenol – 5-MR containing one substrate at a concentration of 0.1 mM and the other varied in the range of 0.005–0.05 mM, was also studied. The Michaelis-Menten Eq. (4), the random-order mechanism (Eq. (5)) and the Haldane model (Eq. (6)) were used to model the measured data. To determine the interaction parameters the sum kinetic models of the unspecified type of interaction (Eq. (8) and (9)) were applied.

The results showing the estimated kinetic parameter $V_{O_2,max}$ for different simulation models are summarized in Table 4. According to Eq. (7), it was also assumed that, if no interactions between substrates occur, the OUR_{max} and $V_{O_2,max}$ for the bi-substrate system is an additive sum of the corresponding parameters of the individual substrates. This assumption was proven to be correct only for the mixtures of phenol – 5-MR (94% from the additive sum) and resorcinol – 5-MR (95% from the additive sum) containing both substrates at equal concentrations, when the Haldane model was used. It could be explained by the fact that these substrates were degraded by different enzymes via different metabolic pathways. On the other hand, the Haldane model could be taking into account a possible substrate inhibition

Table 4. **The mean values of kinetic parameter $V_{O_2,max}$ with the uncertainties of measurement (u_c) and % of additive sum of the parameters of the individual substrates for the studied bi-substrate systems containing both components at equal concentrations and calculated from different kinetic models**

Bi-substrate system (1:1)	Random-order mechanism		Haldane kinetics		Michaelis-Menten kinetics	
	$V_{O_2,max}$, $mgO_2/(l \cdot min \cdot MLSS)$	% of additive sum	$V_{O_2,max}$, $mgO_2/(l \cdot min \cdot MLSS)$	% of additive sum	$V_{O_2,max}$, $mgO_2/(l \cdot min \cdot MLSS)$	% of additive sum
phenol – resorcinol	0.196 ± 0.051 (n = 10)	75	0.239 ± 0.079 (n = 10)	89	0.197 ± 0.051 (n = 10)	76
phenol – 5-MR	0.234 ± 0.065 (n = 10)	83	0.294 ± 0.113 (n = 10)	94	0.247 ± 0.073 (n = 10)	88
resorcinol – 5-MR	0.134 ± 0.001 (n = 4)	58	0.217 ± 0.014 (n = 4)	95	0.152 ± 0.004 (n = 4)	67

n – the number of experimental series

effect at a higher concentration even though the dependence $OUR_{max} = f(S)$ was described by the hyperbolic curve. In the cases when the Michaelis-Menten kinetics or the random-order mechanism were used the mean values of $V_{O_2,max}$ for all bi-substrate (1:1) systems were in the range of 58–88% of the assumed additive values. The results are shown in Table 4. In order to ascertain the role played by an interaction between two substrates, the interaction parameters were determined using the sum kinetic models mentioned above. The values of interaction parameters $I_{1,2}$ and $I_{2,1}$ for the studied bi-substrate systems containing both components at equal concentrations are given in Table 5.

In the case of the bi-substrate system containing resorcinol – 5-MR, the mean value of the actual $V_{O_2,max}$ formed 58–67% of the additive sum of the $V_{O_2,max}$ values of the individual components when the Michaelis-Menten and the random-order mechanism were applied. However, it should be noted that at a mixture concentration of 0.005 mM for both components the actual OUR_{max} value was up to 99% of the additive sum. If the Haldane model was used, the actual $V_{O_2,max}$ value formed 95% of the assumed additive sum. This finding is up to expectation because it has been shown that the degradation of resorcinol and 5-MR in bacteria follows different metabolic pathways [40, 42–44]. However, the interaction parameters determined indicated that the 5-MR had a greater effect of inhibition on the resorcinol biodegradation than resorcinol had on the biodegradation of 5-MR (shown in Table 5). Orcinol hydroxylase is not very substrate-specific and can also catalyze the hydroxylation of resorcinol [42]. The typical dependence of the OUR_{max} values on added substrate concentrations in the case of the bi-substrate system containing resorcinol and 5-MR at equal concentrations is shown in Fig. 3. Taking into account the values of $s_{y/x}$ (varied between $7.0 \cdot 10^{-4}$–$8.4 \cdot 10^{-3}$ mg O_2/(l·min·MLSS) and the high values of the coefficient of regression ($R^2 = 0.97$–0.99) for the data sets indicated that all the applied models were fit well and agreed with the experimental data.

Concerning all of the simulation models, the highest $V_{O_2,max}$ values were obtained for the system of phenol – 5-MR among the studied bi-substrate (1:1) systems. The mean value of the actual $V_{O_2,max}$ formed 83–88% of the additive sum of $V_{O_2,max}$ values of the individual components when the

Table 5. **The values of interaction parameters $I_{1,2}$ and $I_{2,1}$ for the studied bi-substrate systems containing both components at equal concentrations**

Bi-substrate system (1:1)	$I_{1,2}$ [a)]	$I_{2,1}$
	range	range
phenol – 5-MR	–0.49–2.16	–0.24–5.17
phenol – resorcinol	–0.59–0.14	0.40–3.76
resorcinol – 5-MR	0.04–0.44	0.56–0.88

a) Subscript "1" denotes phenol and subscript "2" denotes resorcinol or 5-MR. In the case of the system of resorcinol – 5-MR subscripts "1" and "2" mean resorcinol and 5-MR, respectively.

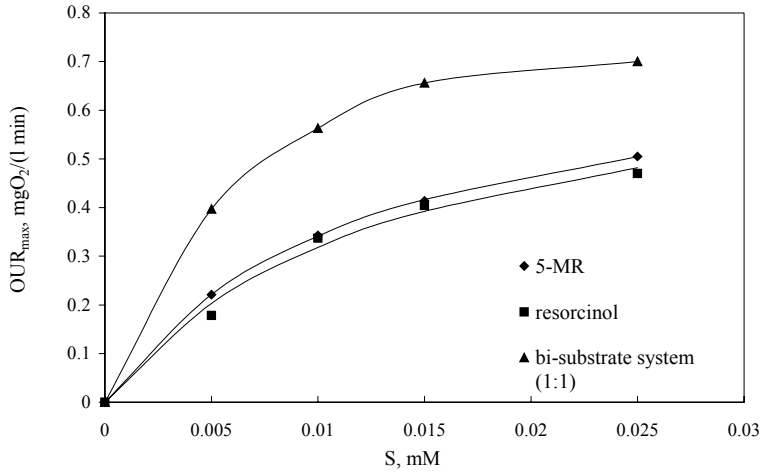

Fig. 3. The dependence of the OUR_{max} values on added substrate concentrations for the bi-substrate system containing resorcinol and 5-methylresorcinol (5-MR) at equal concentrations. The symbols correspond to the measured data, whereas the solid line corresponds to the Michaelis-Menten kinetics. MLSS of specific activated sludge was 5.9 g/l.

Michaelis-Menten and the random-order mechanism were applied. If the Haldane model was used, the actual $V_{O_2,max}$ value was 94% of the assumed additive sum. However, the interaction parameter determined indicated that 5-MR had a greater effect of inhibition on the biodegradation of phenol than phenol had on 5-MR, but in some experiments these compounds enhanced each other's biodegradation. The typical dependence of the OUR_{max} values on an added substrate concentration in the case of bi-substrate system containing phenol and 5-MR at equal concentrations is shown in Fig. 4. According to the values of $s_{y/x}$ (varied between $2.6 \cdot 10^{-3}$–$3.7 \cdot 10^{-2}$ mg O_2/(l·min·MLSS) and the values of the coefficient of regression ($R^2 = 0.89$–0.99) it was concluded that the regression curves were consistent with the measured data. The results illustrated that the models proposed adequately described the dynamic behaviours of biodegradation.

In the case of the bi-substrate (1:1) system containing phenol – resorcinol, the mean value of the actual $V_{O_2,max}$ formed 75–89% of the additive sum of the $V_{O_2,max}$ values of the individual components, showing the highest value for the Haldane model. The values of $V_{O_2,max}$ lower than the assumed values may be explained by the possibility that there could be a competition for binding to the active site of an initial enzyme such as phenol monooxygenase (hydroxylase), which is also active on resorcinol. Furthermore, other enzymes, including resorcinol monooxygenase, could also be involved in the degradation of resorcinol [13, 42, 43]. The obtained interaction parameters indicated that phenol had a stimulatory effect on the biodegradation of resorcinol, shown by the negative values, and that resorcinol

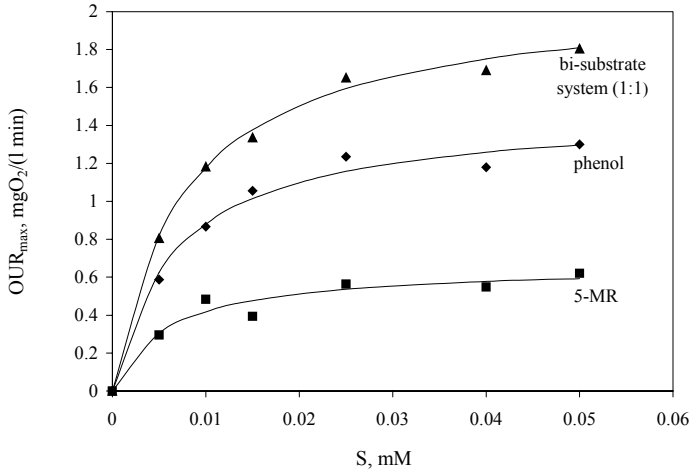

Fig. 4. The dependence of OUR_{max} values on added substrate concentrations for phenol, 5-methylresorcinol (5-MR) and their bi-substrate system containing both components at equal concentrations. The symbols correspond to the measured data, whereas the solid line describes the Michaelis-Menten kinetics and in the case of the bi-substrate system the Michaelis-Menten ($s_{y/x}$ = 0.05, R^2 = 0.99) as well as the random-order mechanism ($s_{y/x}$ = 0.06, R^2 = 0.99) curves. MLSS of specific activated sludge was 5.9 g/l.

inhibited the biodegradation of phenol. The values of $s_{y/x}$ (varied between $3 \cdot 10^{-3}$–$4.3 \cdot 10^{-2}$ mg O_2/(l·min·MLSS) and the values of coefficient of regression (R^2 = 0.92–0.99) for the data sets indicated that the predicted values obtained from all applied models were in acceptable agreement with the measured data.

Among the bi-substrate systems containing one substrate at 0.1 mM, the highest $V_{O_2,max}$ values were found for phenol (0.1 mM) – 5-MR, followed by the systems of phenol (0.1 mM) – resorcinol, phenol – 5-MR (0.1 mM) and phenol – resorcinol (0.1 mM). The results are presented in Table 6. The Michaelis-Menten kinetics and the Haldane model did not describe adequately the biodegradation process due to poor agreement between the predicted and the experimental data.

The mean value of the $V_{O_2,max}$ obtained by the random-order mechanism was higher for the system of phenol (0.1 mM) – resorcinol than that for the system of phenol – resorcinol (0.1 mM). The obtained interaction parameters (shown in Table 6) for the system of phenol (0.1 mM) – resorcinol showed that resorcinol had a stronger effect on the biodegradation of phenol than *vice versa*, although the concentration of resorcinol was lower in the mixture. A similar trend was also observed for the system containing phenol and resorcinol (0.1 mM), where phenol had a stronger inhibition effect on the biodegradation of resorcinol than resorcinol had on the degradation of phenol (even though the concentration of phenol was lower). Taking into

Table 6. **The values of kinetic parameter $V_{O_2,max}$ and the interaction parameters $I_{1,2}$ and $I_{2,1}$ for the studied bi-substrate systems containing one substrate at a concentration of 0.1 mM and the other varied in the range of 0.005 mM–0.05 mM**

Bi-substrate system	Random-order mechanism	Sum kinetics	
	$V_{O_2,max}$, $mgO_2/(l \cdot min \cdot MLSS)$	$I_{1,2}$ [b)] range	$I_{2,1}$ range
phenol (0.1 mM) – resorcinol	0.343 ± 0.130 (n = 4)	0.13–0.20	0.28–0.82
phenol – resorcinol (0.1 mM)	0.302 ± 0.107 (n = 4)	–0.20–7.10	0.06–0.07
phenol (0.1 mM) – 5-MR	0.359 ± 0.037 (n = 4)	0–0.04	–1.10–9.89
phenol – 5-MR (0.1 mM)	0.315 ± 0.071 (n = 4)	–0.66–1.06	0.01–3.44

[b)] Subscript "1" denotes phenol and subscript "2" denotes resorcinol or 5-MR;
n – the number of experimental series

account the statistical parameters used for estimation of the model's suitability and adequacy, the random order mechanism indicated good agreement with the experimental data in the case of the system of phenol (0.1 mM) – resorcinol ($s_{y/x} = 1.9 \cdot 10^{-2}$–$5.2 \cdot 10^{-2}$ mg $O_2/(l \cdot min \cdot MLSS)$; R^2 = 0.94–0.96) and the system of phenol – resorcinol (0.1 mM) ($s_{y/x} = 6.9 \cdot 10^{-3}$–$8.1 \cdot 10^{-3}$ mg $O_2/(l \cdot min \cdot MLSS)$; R^2 = 0.98–0.99). The dependence of the OUR_{max} values on substrate concentrations for different bi-substrate systems containing phenol – resorcinol is given in Fig. 5. The values of OUR_{max} at the concentration ranges of 0.005–0.025 mM were higher for the system of

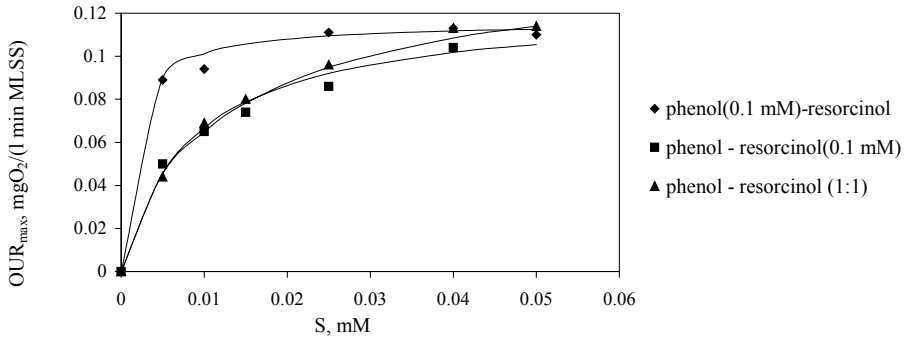

Fig. 5. The dependence of OUR_{max} values on added substrate concentrations for different bi-substrate systems containing phenol and resorcinol. The symbols correspond to the measured data, whereas the solid line corresponds to the random-order mechanism. MLSS of specific activated sludge was 6.5 g/l.

phenol (0.1 mM) – resorcinol than those values for other mixtures shown in Fig. 5. In lower concentrations of the mixture, the concentration of phenol was considerably higher than the concentration of resorcinol, and therefore the biodegradation of phenol was preferred. The results of the biodegradation of single substrates indicated the higher values of OUR_{max} and $V_{O_2,max}$ for phenol than those for resorcinol. The values of OUR_{max} for the bi-substrate systems of phenol – resorcinol (0.1 mM) and phenol – resorcinol (1:1) were almost equal in the concentration range of 0.005–0.025 mM.

The mean value of $V_{O_2,max}$ was higher for the system of phenol (0.1 mM) – 5-MR than that of system of phenol – 5-MR (0.1 mM), showing the highest value of $V_{O_2,max}$ among all of the studied bi-substrate systems. The obtained interaction parameters for the system of phenol (0.1 mM) – 5-MR showed that 5-MR had an inhibition effect and in some experiments also an enhancing effect on the biodegradation of phenol. Phenol had almost no effect ($I_{1,2}$ values were in the range of 0–0.04) on the biodegradation of the 5-MR despite the fact that the concentration of phenol was higher in the mixture. In the case of the system containing phenol – 5-MR (0.1 mM), 5-MR had also a stronger effect on the biodegradation of phenol than *vice versa*. Phenol had the mild inhibition and in some tests even enhancing effect on the biodegradation of 5-MR. The values of $s_{y/x}$ (varied between $1.3 \cdot 10^{-3}$–$4.3 \cdot 10^{-2}$ mg O_2/(l·min·MLSS) and the values of coefficient of regression ($R^2 = 0.91$–0.98) for the data sets were received by the application of the random order mechanism.

Conclusions

Respirometry has been widely used to assess the ability of a bacterial population to remove substances from wastewater, to determine the effect of the substances on the bacteria and also to quantify the substrate depletion [45]. The results of this study offer the opportunity to compare the biodegradability of phenol, resorcinol and 5-methylresorcinol as both single and mixed substrates by the activated sludge taken from the Kohtla-Järve wastewater treatment plant (WWTP). The short-term oxygen demand (BOD_{st}) was determined and the biodegradability was assessed by using the kinetics parameters characteristic of the biodegradation process, such as the maximum rate of oxygen uptake ($V_{O_2,max}$), the maximum rate of substrate bio-oxidation (V_{max}) and the half-saturation coefficient (K_S).

The obtained results indicated that the microbial community of activated sludge was able to oxidize phenol, 5-methylresorcinol and resorcinol. However, it was shown that the activated sludge degraded resorcinol and 5-methylresorcinol more slowly than phenol. Among the studied substrates $V_{O_2,max}$, BOD_{st}/thOD and V_{max} showed the highest values for phenol. The activated sludge had better affinity to phenol with the lowest value of K_S and the highest value of $V_{O_2,max}/K_S$. Kinetic parameters are constants specific to

the particular system under study. Kinetic studies allow the prediction the reaction rate in response to different conditions specific to the particular system and also render it possible to estimate how efficiently the microbial community is carrying out its function in biodegradation processes [13].

The biodegradability of different bi-substrate systems: phenol – resorcinol, phenol – 5-methylresorcinol and resorcinol – 5-methylresorcinol containing both components at equal concentrations in the range of 0.005–0.05 mM, and alternatively containing one substrate at a concentration of 0.1 mM and the other varied in the above-mentioned range, was also studied. Concerning all of the simulation models, the highest $V_{O_2,max}$ values were obtained for the system of phenol – 5-methylresorcinol among the studied bi-substrate (1:1) mixtures. In the case of the systems containing one substrate at 0.1 mM, the highest $V_{O_2,max}$ values were found for phenol (0.1 mM) – 5-methylresorcinol, having the highest value of $V_{O_2,max}$ among all studied bi-substrate systems, followed by the systems of phenol (0.1 mM) – resorcinol, phenol – 5-methylresorcinol (0.1 mM), phenol – resorcinol (0.1 mM). Specific oxygen uptake rates depended on the relative concentrations of both components. Concerning these systems the results showed that the Michaelis-Menten kinetics and the Haldane model did not fit the measured data. The obtained interaction parameters indicated that the interaction between substrates depended on the samples of activated sludge and the concentrations of both substrates in the mixture. The interactions between the substrates depend on whether these compounds compete with each other for the binding to their degrading enzymes or the degradation of these compounds will be initiated by the different substrate-specific enzymes. However, in higher concentrations of the mixture, the active site of enzymes may become saturated with the molecules of substrates and an inactive complex could be formed.

The results of this study could be used for the optimization of the operation of a wastewater treatment plant, because the kinetic parameters and BOD_{st}, as functions of the compound undergoing biodegradation and the composition of the microbial community performing the degradation, yield information about activated sludge process and the biodegradability of substrate in the WWTP. When the reliable estimates of short-term oxygen demands are available, the necessary oxygen consumption reserve can be estimated for the operation of the plant. It is known that oil-shale processing wastewater is unique due to its content of resorcinolic compounds [13]; therefore, in order to avoid too high concentrations in the effluents, these results are important with respect to practical application in the treatment plant. Biodegradation in mixed substrate systems, such as in wastewater treatment plant, is likely more extensive than the estimated parameters would indicate. As known, activated sludge systems involve extremely complex structures having many inputs which may influence the degradation of compounds and many different compounds may be present in the influents that could also affect the biodegradation processes. In the case of

the biodegradation of mixed substrates several factors would play important roles: the interaction between substrates; the microbial community of activated sludge; and whether the compounds are degraded simultaneously or sequentially; and through which metabolic pathways the degradation occurs.

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