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Biodegradability of phenolic compounds as single and mixed substrates by activated sludge

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I. **Lepik, R**., Orupõld, K., Viggor, S., Tenno, T. 2003. Study of Biodegradability of Methyl- and Hydroxyphenols by Activated Sludge. *Oil Shale* 20 (2), 99–112.

II. **Lepik, R**. and Tenno, T. 2011. Biodegradability of Phenol, Resorcinol and 5-Methylresorcinol as Single and Mixed Substrates by Activated Sludge. *Oil Shale* 28 (3). In Press.

III. **Lepik, R**. and Tenno, T. 2011. Determination of Biodegradability of Phenolic Compounds Characteristic to Wastewater of Oil-Shale Industry on Activated Sludge by Oxygen Uptake Measurement. *Environmental Technology*, doi:10.1080/09593330.2011.572923. In Press.

Author's contribution

The author of the current PhD thesis was a principal investigator in all papers I–III, being fully responsible for the planning the research and conducting the experiments, interpretation of data and writing the manuscript.

ABBREVIATIONS AND SYMBOLS

ATP	adenosine triphosphate
BOD	biochemical oxygen demand
BOD_{st}	short-term oxygen demand
C12O	catechol 1,2-dioxygenase
C23O	catechol 2,3-dioxygenase
CFU	colony forming unit
COD	chemical oxygen demand
DMP	dimethylphenol
DO	dissolved oxygen
FAD	flavin adenine dinucleotide
G12O	gentisate 1,2-dioxygenase
$I_{i,j}$	interaction parameter
K_i	inhibition coefficient
K_S	half-saturation coefficient
MLSS	mixed liquor suspended solids, dry weight of biomass
MR	methylresorcinol
NADPH	nicotinamide adenine dinucleotide phosphate
NADH	nicotinamide adenine dinucleotide
OUR	oxygen uptake rate
OUR_{end}	endogenous respiration rate
OUR_{ex}	exogenous respiration rate
OUR_{max}	maximum oxygen uptake rate at a given concentration of substrate
PC34O	protocatechuate 3,4-dioxygenase
PC45O	protocatechuate 4,5-dioxygenase
PCMH	*p*-cresol methylhydroxylase
POB	*p*-hydroxybenzoate
POBH	*p*-hydroxybenzoate hydroxylase
q	specific rate of substrate removal
R^2	coefficient of determination
$s_{y/x}$	standard deviation of the regression
thOD	theoretical oxygen demand
u_c	uncertainty of measurement
V_{max}	maximum rate of substrate bio-oxidation
$V_{O_2,max}$	maximum rate of oxygen uptake
WWTP	wastewater treatment plant
$Y_{X/S}$	growth yield, yield coefficient
μ	specific growth rate
μ_{max}	maximum specific growth rate

1. INTRODUCTION

Contamination of the environment with hazardous chemicals is one of the major concerns facing the industrialized societies worldwide today. Phenolic compounds are listed as priority environmental pollutants (U.S. EPA, 1996). They are found in many industrial wastewaters, e.g. from pharmaceutical, plastic, pulp and paper industry. In Estonia, the greatest source of phenolic pollution is the oil-shale chemical industry where oil is produced from oil shale. Oil shale is among the most important natural resources in Estonia, being widely used in the chemical industry as well as in the production of energy. Great quantities, over 95% of hazardous waste are generated in the production of oil shale energy and shale oil (Estonian Environment Information Centre, 2001 and 2010). 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. In 2002, the corresponding annual discharge of these phenols was 7413 kg and 7264 kg, respectively. In order to decrease the pollution, the discharges of monohydric phenols shall be reduced to one ton per year by 2014 in accordance with the „Program concerning reduction of discharges of phenols into the water bodies until 2014" (Minister of Environment of Estonia, 2010).

Phenol, methylphenols, resorcinols and dimethylphenols are considered major pollutants in the wastewater of the oil-shale chemical industry which is canalized to the Kohtla-Järve wastewater treatment plant (WWTP). Phenolic compounds contaminate the surrounding surface water as well as underlying aquifers and, therefore, are a potential threat to the groundwater that is one of the sources of drinking water in the North-Eastern Estonia. 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 (Estonian Environment Information Centre, 2001 and 2010). Because of the adverse health effects of phenols, the total content of monohydric phenols in groundwater, which could be used as a source of drinking water, should be below 1 μg/l (WHO, 2008). Phenols are toxic to several biochemical functions of human beings. Methylphenols are both carcinogenic and mutagenic, whereas phenol appears to be potentially genotoxic, although this may be more a result of the action of its metabolites (hydroquinone, catechol) than the parent compound. The presence of phenolic compounds in the environment poses a significant risk to biota; disturbing and destroying the life of species and their habitats. In general, the *para* substituted compounds are more toxic than *ortho* and *meta* compounds (Moore and Ramamoorthy, 1984; U.S. EPA, 2002; ATSDR, 2008).

Biodegradability and toxicity of organic compounds are two basic criteria determining their behaviour in natural environment and during the biological treatment of wastewater. Since phenolic compounds can have serious environmental effects, microbiological and biochemical features of their aerobic degradation are of great interest, and monitoring 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 (e.g. Paller *et al.*, 1995; Aleksieva *et al.*, 2002). 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 invoke enzyme induction mechanism in the bacteria. Respirometry is a simple and convenient method most used to study the aerobic biodegradation of toxic compounds. 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 (Marsili-Libelli and Tabani, 2002). It provides information about the biodegradation kinetics and the biochemical oxygen demand of the substrate, the microbial activity and it can also indicate toxic effects on the activated sludge (Spanjers *et al.*, 1993 and 1994; Brouwer *et al*., 1994). Therefore, the respirometric measurements can be used for the purpose of controlling and monitoring the wastewater treatment process (Grady *et al.*, 1989; Brown *et al.*, 1990, Vanrolleghem *et al.*, 1994; Ince *et al.*, 2008). As organic matter in wastewater is highly heterogenous, multiple enzymes are normally required to achieve the desired biochemical conversion of such complex substrate and a microorganism generally contains different enzymes with their cofactors and its cell envelop protects and minimizes deactivation and poisoning of the organism (Qian and Tan, 1998).

Due to the practical importance of elimination of phenolic compounds by activated sludge processes from the oil-shale industry wastewaters, containing phenol and different methyl-, dimethyl- and hydroxyphenols, the aerobic biodegradability of these compounds both as single and mixed substrates at different concentrations was studied in this thesis. The results of this study could provide some useful information for the application in wastewater treatment. The 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 (Orupõld *et al.*, 2001) and permit the detection of possible interference between various components in multi-substrate media (Limbert and Betts, 1995).

2. LITERATURE OVERVIEW

2.1. Biodegradation of organic compounds

Biodegradation can in principle be complete or partial. Biodegradation of a compound in an environment may occur as a simple biotransformation by an enzyme, rendering the parent structure into a new one by removing or altering substituents, with or without making relevant changes to the carbon backbone. Because the biodegradation reactions may break intramolecular bonds of compounds, their toxicity and transport properties are commonly altered. Biodegradation may happen by the action of an extracellular enzyme in an aqueous solution or by the intracellular enzyme system of an organism. In the latter case the parent compound has to enter the cell through a complex cell wall and membrane system. This will be by passive diffusion of predominantly non-polar substances (i.e. hydrocarbon). More polar and, especially, dissociated compounds like aromatic acids need an active transport system for uptake. These transport proteins are highly specific for single compounds and often exclude structural isomers or analogues (Wittich, 1996).

Biodegradation can result in the transformation of chemicals into innocuous compounds which are readily assimilated into the environment; but on the other hand, an innocuous parent chemical could be transformed into a toxic intermediate (dead-end metabolites) which prevent further degradation (Klopman, 1996; Wittich, 1996). Biodegradation may lead to the complete mineralization of compound, transforming the organic compound to carbon dioxide and water, with a concomitant formation of microbial biomass (Painter, 1995; Pitter and Sýkora, 1996). However, the complete mineralization of xenobiotic compounds in treatment systems is rare and the term biotransformation more accurately describes the potential changes to the composition and molecular structure of such compounds (Byrns, 2001). In order to remove pollutants from natural environments by the biodegradation, the ability of pure and mixed cultures of microorganisms to metabolize xenobiotic compounds under both aerobic and anaerobic conditions has received much attention due to the environmental persistence and toxicity of these chemicals (Singleton, 1994).

The biodegradability of organic substances is the degree of the changes in the physical and chemical characteristics and in the molecular structure of organic substances under degradation by microorganisms. Figure 1 illustrates the biodegradation processes of organic compounds under aerobic conditions (Jiang *et al*., 2002).

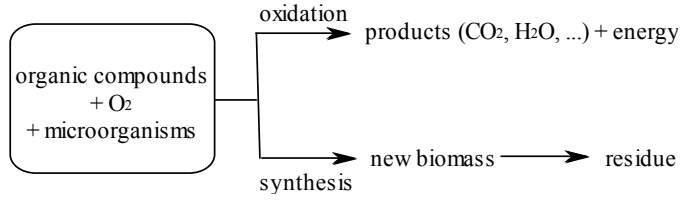


Figure 1. Aerobic biodegradation of organic compounds (based on Jiang *et al*., 2002).

The biodegradability of a compound is influenced by its physical characteristics, such as solubility in water and vapour pressure; and by its chemical properties, including molar mass, molecular structure and the presence of various kinds of functional groups (Neilson *et al.*, 1985). Aromatic compounds can be either totally or partly degraded by microorganisms depending on the type of substituent(s) and on the number of benzene rings. Alterations in chemical structure modify the susceptibility of organic compounds to biodegradation. The important point in degradation of aromatic compounds is whether substituents remain intact or are either transformed or eliminated before the ring cleavage, – and it depends on the bacterial species. Elimination of side chains can occur before or after ring-cleavage reactions, whereas substituent(s) often decrease biodegradability of the compounds (Harwood and Parales, 1996). The electronegative groups (e.g halogen atoms, nitro groups) as substituents of the aromatic nucleus lower the reactivity of the resonance structure towards oxygen and prevent either activation or ring cleavage, the two initial steps in the degradation of arenes (Kieslich, 1984). The nature of the substituent as well as the position of the substituent in the benzene ring, in relation to phenolic -OH group exerts a greater influence in deciding the degradative ability of phenolic compounds. 4-nitrophenol showed less growth compared to chlorophenols due to resonance effect of $-NO_2$, which decreases induction of phenol hydroxylase. In the case of chlorophenols, the order of degradability was found to be 4-chlorophenol > 2-chlorophenol > 3-chlorophenol. The presence of more than one substituent in the benzene ring exerts steric effect, thereby influencing the degradation of phenolic compounds (Chitra and Chandrakasan, 1996).

Thus, the biodegradability and the uptake rate of a compound by microbial community depend on its structure and physical form, aqueous solubility and total substrate concentration and the time that has been available for acclimation. Although the structure of the substrate has a major importance in determining its biodegradability, the potential of the compound to associate with both organic and inorganic material in the environment, as well as physico-chemical factors such as the temperature, pH, nutrient (nitrogen and phosphorus) availability, salinity and oxygen concentration (Neilson *et al.*, 1985; Leahy and Colwell, 1990), the strain of microbe and the amount of inoculum used (Singleton, 1994) are essential parameters for successful biodegradation. Hill and Robinson (1975) stated that not only the inhibitory effect of the substrate but also the amount of the inoculum used might affect the duration of the lag phase. The biodegradation of organic compounds by microbes may result in production of toxic intermediates. It could be minimized by using mixed cultures or microbial consortia which have a wider spectrum of metabolic properties (Singleton, 1994). Annadurai *et al.* (2002) showed that mixed liquors had a better ability for phenol degradation than activated sludge and *Pseudomonas (P.) putida* alone.

2.2. Environmental factors affecting microbial metabolism

The environmental conditions, including the presence of microbes capable of degrading a specific compound, microbial population density, nutrient supply, compound bioavailability, oxygen availability, temperature, pH, and type of media, can be as important as structure in determining the actual degradation of compound (Klopman, 1996). Environmental factors can influence degradation in a variety of ways; by preventing the growth of organisms, by the availability of xenobiotics; and more subtly, by affecting gene expression (Singleton, 1994).

pH. The effect of pH on the overall oxidation process is associated with specific enzymatic processes affecting the activity of microbial enzymes. A relatively narrow effective range of pH 5 to 9, with the optimum pH around 7 exists for most bacteria (Roš, 1993; Bitton, 1994; Singleton, 1994). Lallai *et al.* (1988) obtained with phenol-acclimatized microbial mixed population that the specific growth rate of microorganisms, the specific substrate consumption rate and the yield factor are influenced by pH according to bell-shaped curves (parabolic profile) with maximum values at pH 6–7. The bacterial activity decreases as the pH deviates from neutral conditions (Kim and Armstrong, 1981) and the optimum pH varies according to the particular enzyme considered (Lallai *et al.*, 1988). pH affects the ionization of chemicals and thus plays a role in the transport of nutrients and toxic chemicals into a bacterial cell (Bitton, 1994). The solubility of compounds at different pH values is also involved in determining the rate of degradation (Singleton, 1994). The degradation of organic matter affects pH due to: (a) the uptake of the carbon source through the cell wall of the bacteria, (b) the release of CO_2 resulting from respiration processes in the liquid phase, and (c) the uptake of ammonium for growth (San and Stephanopoulos, 1984; Iversen *et al*., 1994; Siano, 1995). The biodegradation of phenol produces CO_2, which contributes to a decrease of the solution pH due to the formation of carbonic acid (El-Naas, 2009), and consequently, phenol degradation deteriorates as the medium pH deviates from a neutral condition (Annadurai *et al.*, 2002).

pH also affects the surface charge of the cells of the activated sludge biomass. The surface charge of biomass is predominantly negative over the pH range of 3–10 (Aksu and Gönen, 2004). Cells with large negative surface charge appear more hydrophilic (Palmgren *et al*., 1998). Below the pH of 3, the overall surface charge on cells becomes positive due to the isoelectric point of activated sludge, so the electrostatic attraction between phenol and the activated sludge biomass will be insignificant. Above the pH of 9, phenol could be expected to become negatively charged in a phenoxide ion (Aksu and Gönen, 2004).

Temperature. Temperature influences the rate of all chemical and biological reactions. Most microbes isolated and studied degrade optimally at mesophilic temperatures. Their ability to degrade xenobiotics at a temperature range of

25°C to 37°C is often overlooked (Singleton, 1994). Regarding the temperature effect, authors (Mordocco *et al*., 1999; Kira *et al.*, 2000; Monteiro *et al.*, 2000; Sá and Boaventura, 2001; Annadurai *et al*., 2002) are almost unanimous and find the higher phenol removal efficiency to be near 30°C. However, the rate and the extent of degradation are relatively sensitive to deviations outside the optimal temperature. A variation of 5°C may cause a decrease in the phenol degradation rate of at least 50% at the lower end and almost 100% at the higher end (Sá and Boaventura, 2001). The decreased specific growth rate at high temperatures is due to the thermal denaturation of proteins, particularly enzymes, as well as changes in membrane structure, which leads to alterations in cell permeability (Bitton, 1994). On the other hand, exposure to lower temperatures is expected to slow down the bacterial activity. In addition, the inhibitory effect of phenol on the bacteria is known to be enhanced at low temperatures (Kira *et al*., 2000).

Substrate bioavailability and toxicity. One of the main factors affecting the bioavailability of xenobiotics is their sorption to particulate matter (Singleton, 1994). Leedjärv *et al.* (2006) pointed out the influence of the sample matrix (particles present in natural samples) on the bioavailability of phenolic compounds. The bioavailable amount of phenolic compounds mainly depends on the characteristics of the sample in general, and not only on its phenolic composition. Additional compounds may interfere with phenolic compounds; the compounds present in the sample may be toxic to the bacteria; and phenolic compounds may absorb to the matrix components, hence affecting the bioavailability of phenolic compounds (Leedjärv *et al*., 2006). In many natural waters and particularly in sediments, substrate may be bound to a variety of macromolecules such as polysaccharides, proteins, nucleic acids, lipids, and humic material. This results in an apparent reduction in the available concentration (Neilson *et al.*, 1985). Another factor determining the bioavailability of a particular pollutant is its water solubility. Low water solubility will limit the transport of a pollutant into the microbial cell, thereby hindering its degradation (Singleton, 1994). Small molecules with a high affinity to water are preferred for degradation. The number in the degradation sequence tended to increase by decreasing the dissociation constant (pK_a) and by increasing the octanol-water partitioning constant (K_{ow}) of compound (Nielsen and Christensen, 1994).

The toxicity of aromatic compounds is frequently attributed to the modification of membrane structure by hydrophobic interactions with the lipid bilayer structure, caused by the lipophilic nature of such a compound (Sikkema *et al.*, 1994). The partitioning of lipophilic compounds into the lipidic bilayer of membranes might be expected to provoke significant changes in the structure, the integrity and the function of the membranes, thereby modifying the activities of enzymes directly associated with these membranes (Sikkema *et al*., 1995). Heipieper *et al.* (1992) have shown that phenol induced an increase in the degree of saturation of the membrane lipids of *P. putida* and also provoked

the isomerization of the unsaturated fatty acids from *cis* to *trans* isomers. Such studies indicate that the cell membrane is a probable site of growth inhibition. In the light of the results presented by Léonard and Lindley (1999), it would appear that the inhibitory effect of phenol is most likely caused by a biochemical phenomenon modifying the *in vivo* enzyme activity and notably decreasing the phenol hydroxylase activity, indicating that inhibition was probably associated with modified membrane fluidity by a change in the fatty acid composition of cellular lipids. The results of Páca and Martius (1996) revealed that in the presence of nutrients, the phenol degradation rate is higher and in excess of oxygen the cells are less sensitive to the inhibitory effect of phenol. Humic matter is also shown to be effective in suppressing an inhibitory effect of phenol on the biomass and the application of humic matter can have great potential in wastewater treatment, as it can serve as a source of additives, stimulating biological activity and removing toxic inhibitors of biological processes (Lipczynska-Kochany and Kochany, 2008). Liu *et al.* (2009) showed a serious toxic effect of phenol on sludge flocs, as microbial activity decreased linearly with the increase in phenol concentration, and suggested that the application of aerobic granules in wastewater treatment could provide an improved ability to tolerate toxic chemical shock, particularly at longer exposure times. A granular structure provided microbial cells with a beneficial protection from phenol due to the concentration gradient, developed within granules because of the diffusional resistance, by reducing the concentration of chemicals below some threshold value to avoid inhibition and larger sized granules showed a higher tolerance (Liu *et al.*, 2009).

Oxygen availability. Aerobic biodegradation by microorganisms require the presence of molecular oxygen to initiate enzymatic attack on the aromatic rings (Semple and Cain, 1996), which suggests that the dissolved oxygen may be a limiting factor of the treatment process (Contreras *et al*., 2008). Oxygen may become a limiting factor due to its low water solubility and thus the use of chemicals (hydrogen peroxide) which release oxygen and therefore improve aeration has been suggested (Singleton, 1994). Martius *et al.* (1996) showed that at the dissolved oxygen concentration of 0.35 mg/l the clear oxygen limitation can be recorded and the rate of aerobic metabolism is reduced to 70%. Since molecular oxygen exists in the triplet state with two unpaired electron spins, it reacts only slowly with organic compounds, which generally exist in a singlet state. Only after enzymatic activation to the singlet state can oxygen act in biological oxidation (Kieslich, 1984).

The dissolved oxygen concentration has a key factor in the inhibition effect of phenol on the aerobic microbial degradation. Páca and Martius (1996) proved that an excessive amount of oxygen suppresses the effect of phenol inhibition on the cells and that the rate of phenol degradation was higher in comparison with the conditions of oxygen limitation. Despite a slight decrease of phenol affinity to the cell (cf. the increase of the half-saturation coefficient K_S), an excess of oxygen decreases cell sensitivity to substrate inhibition by phenol (cf.

the increase of the inhibition coefficient K_i) (Páca and Martius, 1996). The toxic effect of the biodegradable phenolic compounds causes a maintenance metabolism which leads to higher oxygen demand and to increased sensitivity with respect to the oxygen. The higher sensitivity to oxygen can be explained by the role of molecular oxygen as a co-substrate for both the hydroxylation and the subsequent cleavage of the aromatic nucleus. It is assumed that a higher energy demand for maintenance and endogenous metabolism could be caused by the higher activation energy by the toxic substrate inactivated cells and the changes of the fatty acid composition of the cell lipids in the presence of phenol. The increase of maintenance metabolism needs an adequate oxygen availability for the cells which allows them to overcome the toxic effect of substrates (Keweloh *et al*., 1991; Martius *et al.*, 1996). However, periodic determination of the activity of catechol 2,3-dioxygenase showed that in highly oxygenated (100 ml O_2/min) reactions, dioxygenase activity was rapidly lost. The loss of catechol 2,3-dioxygenase activity was attributed to oxidative inactivation and the inactivation rate increased with the increasing O_2 concentration (Ali *et al.*, 1998).

2.3. Degradation of mixed substrates

2.3.1. General aspects

In microbial communities many processes may occur which enhance microbial metabolism. Many species are able to use more than one carbon source simultaneously. This phenomenon is known as co-utilization. Attention has been drawn to the possibility that, under conditions of low substrate concentration, the regulation of catabolic enzymes may be altered so that a simultaneous utilization of more than one substrate occurs (Neilson *et al.*, 1985). Microorganisms can attain a considerable growth rate by using multiple carbon sources simultaneously, even when each of them is present in a very low concentration (Egli, 1995). As it is the amount of biomass that determines nutrient requirements, co-utilization influences the biodegradation rates of the involved substrates. Co-utilization can thus enhance biodegradation simply by increasing the biomass of the degraders.

Concurrent metabolism occurs when two substances are simultaneously degraded or transformed. The substrate may not necessarily be transformed stoichiometrically into a single metabolite, and the additional substrate (co-substrate) may not increase either the rate or the yield of the transformation. In natural situations, concurrent metabolism may play a role which is at least as significant as that of co-metabolism (Neilson *et al.*, 1985).

The presence of easily degradable carbon sources can enhance the biodegradation of more persistent chemicals. This is best illustrated by the process of co-metabolism, that is; the transformation of a compound, which cannot serve as a primary energy source for the microorganisms, by cells growing on other substrate (Committee on *In Situ* Bioremediation, 1993; Committee on Intrinsic Remediation, 2000). Co-metabolism in particular has been identified as

a beneficial process for the metabolism of xenobiotic (Singleton, 1994). For instance, co-metabolic transformations can produce compounds which are readily degradable by other microorganisms in the environment (Singleton, 1994). During co-metabolism one organism which is growing on a particular substrate also oxidizes a secondary substrate that it is unable to assimilate or be used as a carbon and energy source (Singleton, 1994). The concentration of a secondary substrate may have toxic effects causing inhibition or deactivation of enzymes (Fiedler and Lau, 1998; Alexander, 1999). Degradation of a secondary substrate may provide nutritional benefit to microorganisms, but the cells are unable to utilize it in the absence of a primary substrate (Brandt *et al*., 2003). However, degradation of the secondary substrate can continue after depletion of the primary substrate. In some situations, oxidation of dead biomass provides the necessary energy for background degradation; in other situations intracellular reserves provide it (Brandt *et al*., 2003). Microbial co-metabolism involves the transformation of a secondary substrate (non-growth substrate, the compound that does not support growth) in the presence of the primary substrate (growth substrate) and can occur if the catabolic enzymes are not induced by the secondary substrate. Often, the non-growth substrate (e.g. chlorophenol) itself exerts an inhibitory effect on its own transformation in the presence of the growth substrate (e.g. phenol) (Hao *et al*., 2002). In cometabolic degradations, the effect of a toxic substrate on bacterial activity is usually associated with enzyme inhibition and inactivation (Ely *et al.*, 1995a and 1995b).

Organic chemical mixtures are prevalent in wastewaters from industrial and municipal sources as well as in contaminated groundwater. The occurrence of contaminants in mixtures is an important problem because the removal or degradation of one component can be inhibited by other compounds in the mixture or the other carbon sources will be available for the microorganisms, and because different conditions may be required to treat different compounds within the mixture. Researchers have noted that microbial degradation of a compound in a mixture can be strongly impacted by other components of the mixture (Meyer *et al.*, 1984; Klečka and Maier, 1988; Saéz and Rittmann, 1993). This has been observed not only for mixtures of toxic chemicals (bioremediation) but also for mixtures of pollutants and readily degraded compounds (wastewater treatment). To understand mixture effects, the metabolic role that each compound plays for the microorganisms should be considered. The terms “homologous” and “heterologous” have been proposed by Harder and Dijkhuizen (1982) for compounds that serve the same or different roles, respectively. The effects of other compounds in a mixture of homologous (mixture of substrates serving the same purpose) carbon and energy substrates on the biodegradation of a chemical can be positive, as in the case of increased growth at low substrate concentrations (McCarty *et al.*, 1984; Schmidt and Alexander, 1985) or induction of required degradative enzymes (Alvarez and Vogel, 1991). More commonly, negative interactions are reported. Reasons for decreased biodegradation rates include competitive inhibition (Chang *et al.*,

1993; Oh *et al.*, 1994; Bielefeldt and Stensel, 1999), toxicity (Haigler *et al.*, 1992), and the formation of toxic intermediates by non-specific enzymes (Klečka and Gibson, 1981; Bartels *et al.*, 1984).

Understanding the degradation of complex mixtures of substrates and to improve degradation qualitatively and quantitatively will necessitate analysis of substrate flux into the different productive and unproductive pathways and, identification of the critical metabolic steps (Pieper *et al*., 1995). As the result of substrate interactions that occur in the biodegradation of multiple substrates, it is expected that patterns of biodegradation of single compound systems generally differ from the biodegradation of mixtures occurring *in situ* (Neilson *et al*., 1985). The utilization pattern can change with different mixture compositions, depending on the chemical nature and concentration of the substrate, oxygen concentration and microbial growth rates (Okpokwasili and Nweke, 2005). Many substrate interactions have been observed during hydrocarbon biodegradation (Neilson *et al*., 1985).

2.3.2. Biodegradation kinetics

A variety of biodegradation kinetics and microbial growth models have been proposed and used by many researchers. Such models allow the prediction of chemicals that remain at a certain time, calculation of the time required to reduce chemical to a certain concentration and on the other hand, it can be used to predict the amount of biomass production achievable at a given time (Okpokwasili and Nweke, 2005). The basic hypothesis of biodegradation kinetics is that substrates are consumed via catalyzed reactions carried out by the microorganisms with the requisite enzymes. Therefore, rates of substrate degradation are generally proportional to the concentration of microorganisms able to degrade the substrate and dependent on the substrate concentration characteristic of saturation kinetics (e.g. Michaelis-Menten and Monod kinetics) (Okpokwasili and Nweke, 2005). Saturation kinetics suggest that at low substrate concentrations (relative to the half-saturation coefficient), rates are approximately proportional to substrate concentration (first order in substrate concentration), while at high substrate concentrations, rates are independent of substrate concentration (zero order in substrate concentration). The kinetic equations, which describe the activity of an enzyme or a microorganism on a particular substrate, are crucial in understanding many phenomena in biotechnological processes (Okpokwasili and Nweke, 2005).

The most commonly used biodegradation kinetic model for a non-inhibitory substrate is the Monod kinetics, which relates the rates of bacterial growth and substrate removal to the concentration of the substrate being removed (Monod, 1949; Magbanua *et al*., 1994):

$$q = \frac{\mu}{Y_{X/S}} = \frac{\mu_{\max}}{Y_{X/S}} \cdot \frac{S}{K_S + S}, \tag{1a}$$

$$q = -\frac{1}{X} \cdot \frac{dS}{dt} = q_{\max} \cdot \frac{S}{K_S + S}, \tag{1b}$$

where q is the specific rate of substrate removal, μ is the specific growth rate, $\mu_{\max}$ is the maximum specific growth rate, $q_{\max}$ is the specific substrate removal rate, S is the substrate concentration, K_S is the half-saturation coefficient of substrate, X is the biomass concentration, t is time, and $Y_{X/S}$ is the growth yield (an increase in biomass concentration synthesized per amount of substrate removed).

A characteristic of enzyme-catalyzed reactions is, that for a given enzyme concentration, the initial reaction rate increases with increasing initial substrate concentration to a limiting value. At still higher substrate concentrations, the initial rate is sometimes found to be less than the maximum value. For example, phenol at sufficiently high concentrations has been observed to exhibit an inhibitory effect on pure microbial cultures (e.g. Hill and Robinson, 1975; Yang and Humphrey, 1975) and activated sludge systems (e.g. Pawlowsky and Howell, 1973; D'Adamo *et al.*, 1984). Several mathematical models have been developed to quantify the inhibitory effects of toxic substrates on microbial growth kinetics (e.g. Edwards, 1970; Meriç *et al.*, 2002). Most of these equations have been adopted from the models of the substrate inhibition of enzymatic reactions, and involve a common substrate inhibition coefficient. The Haldane equation is one of the most widely used inhibition models describing the growth-linked biodegradation kinetics of a substrate that is inhibitory to its own biodegradation (Andrews, 1968):

$$\mu = \mu_{\max} \cdot \frac{S}{K_S + S + \frac{S^2}{K_i}}, \tag{2a}$$

$$q = q_{\max} \cdot \frac{S}{K_S + S + \frac{S^2}{K_i}}, \tag{2b}$$

where K_i is the inhibition coefficient.

The specific growth rate (μ) is dependent on the substrate concentration, temperature, pH and inoculum (Hill and Robinson, 1975; D'Adamo *et al.*, 1984). $\mu_{\max}$ and Y values depend on the microorganism type and the oxidation state of the organic matter in wastewater (Ince *et al.*, 2008). The inhibition coefficient (K_i) characterizes a loss of the cell ability to oxidize a substrate due to its inhibitory effect. At low concentrations, inhibition is negligible ($S^2/K_i << K_S$) and the Haldane equation simplifies to the Monod equation. The half-saturation coefficient (K_S) is defined as the substrate concentration at which

μ is equal to the half of μ_{max}. The smaller the K_S, the lower is the substrate concentration at which μ approaches μ_{max}. Also, the K_S value shows the affinity of microorganisms to the substrate. However, if the substrate is inhibitory it is not possible to observe an actual μ_{max}, thus K_S takes on a hypothetical meaning. Thereby the degree of inhibition should be determined by the K_S/K_i ratio, and not just by K_i alone. The larger is the K_S/K_i, the greater the degree of inhibition (Brown *et al.*, 1990; Grady *et al.*, 1999). Though originally developed for pure cultures and a single substrate, the Monod and the Haldane models have often been used for mixed cultures or activated sludge and multi-component substrates or wastewaters. In these cases, however, μ_{max} and K_S are not constant but variable, depending on a composition of mixed cultures (Čech *et al.*, 1984).

The Haldane equation has been used to describe the specific degradation rate of phenol in cultures of *P. putida* (Hill and Robinson, 1975; Kumar *et al.*, 2005), *Ralstonia eutropha* (Léonard *et al.*, 1999) and activated sludge (Kumaran and Paruchuri, 1997; Nuhoglu and Yalcin, 2005). Nevertheless, some authors have reported that the production and accumulation of several metabolic intermediates of the phenol degradation, such as the 2-hydroxymuconic acid semialdehyde (2-HMAS), makes inadequate the use of this model, because the Haldane model does not take into account the effect of metabolic intermediates on phenol degradation (Mörsen and Rehm, 1990; Léonard *et al.*, 1999; Wang and Loh, 1999). Moreover, analytical determination of these intermediates may be difficult. Since several intermediates are involved in the degradation of phenol, it is highly difficult to quantify the effect of each. The concentration of the major intermediate, 2-HMAS, in the *meta*-cleavage of phenol has been found to be proportional to the amount of phenol removed in phenol degradation (Mörsen and Rehm, 1990). Thus, the concentration of the degraded amount of phenol could be introduced into the model equations (Nuhoglu and Yalcin, 2005).

Environment rarely encounters a single pollutant, as the source of pollution is usually multi-substrate in nature. Most of the biochemical reactions involve at least two substrates; therefore, it is necessary to consider the kinetics of multi-substrate enzyme-catalyzed reactions. It is a complex topic, and several mechanisms and interactions are described in the literature (e.g. Palmer, 1995). For reasons of simplicity, only some specific examples of two-substrate reactions are mentioned as follows. Two-substrate enzyme-catalyzed reactions may proceed by a variety of mechanisms, including the ping-pong, compulsory-order ternary-complex and random-order ternary-complex mechanisms. The reaction mechanism may be sequential, where both substrates (A, B) bind to the enzyme (E) to form a ternary complex before the first product (P, Q) is formed, or it may be non-sequential (ping-pong mechanism). For reasons of simplicity, in this study the random-order ternary-complex mechanism was considered. A random-order mechanism is a sequential mechanism where the order of binding to and leaving the enzyme is not specified. Any substrate can bind first to the enzyme and any product can leave first. The general rate equation for two-substrate reaction is presented as follows (Palmer, 1995):

$$V = \frac{V_{\max} \cdot S_A S_B}{K_d K_{m,B} + K_{m,B} S_A + K_{m,A} S_B + S_A S_B}, \tag{3}$$

where V is the reaction rate; $V_{\max}$ is the maximum possible reaction rate when both substrates are saturating; S_A, S_B are the concentrations of both substrates; K_d is the dissociation constant for enzyme-substrate complexes; $K_{m,A}$, $K_{m,B}$ are the half-saturation coefficients of substrate A and B when other substrate is saturating.

Biodegradation in mixed substrate environments, such as in treatment systems, is likely to be more extensive than the parameters would indicate. Inhibitors affect substrate removal by microorganisms in a manner similar to the way they affect enzyme activity (Palmer, 1995). Biodegradation kinetics for mixtures of homologous growth substrates are often modelled using no-interaction sum kinetics or purely competitive inhibition kinetics. The applicability of these models seems reasonable, particularly for similar compounds (e.g. benzene and toluene) that can be catabolized by the same pathways. If the same enzymes are used in the transformation of each substrate, competitive inhibition kinetics would be expected. However, there are many cases in which the interactions between homologous substrates are not purely competitive, even for similar compounds. For example, in experiments with *P. putida* O1 and a mixed culture Oh *et al.* (1994) found that benzene consumption was inhibited by the presence of toluene to a much greater extent than predicted by the ratio of K_S values, and toluene consumption was far less inhibited by the presence of benzene than would be the case in purely competitive inhibition. In many other reports, unusual substrate interactions in mixtures have been reported but not modelled (Arvin *et al.*, 1989; Smith *et al*., 1991; Millette *et al*., 1995). Potential reasons for deviations from such kinetics include: 1) interactions at the level of substrate transport into the microorganism, 2) interactions/competition between compounds and/or biodegradable intermediates, and 3) the presence of a previously unidentified catabolic pathway or pathway branch (Arvin *et al.*, 1989; Smith *et al*., 1991; Millette *et al*., 1995).

The most common model type for growth on homologous mixtures is one in which the specific growth rate is the additive sum of the specific growth rates of each substrate i (μ_i), yielding a model in which the substrates do not interfere with each other in binding process and the presence of one substrate does not affect the biodegradation rate of the other (Brandt *et al.*, 2003). The rate of consumption for substrate i is described as (Reardon *et al.*, 2000):

$$\frac{dS}{dt} = -\frac{\mu X}{Y_{X/S}}, \tag{4}$$

where S is the substrate concentration, t is time, μ is the specific growth rate, X is the biomass concentration and $Y_{X/S}$ is the biomass yield.

Supposing that the Monod model is suitable for biodegradation of each of the substrate individually, the no-interaction sum kinetics model for a binary mixture can be described as follows (Reardon *et al.*, 2000):

$$\mu = \frac{\mu_{\max,1} \cdot S_1}{K_{S,1} + S_1} + \frac{\mu_{\max,2} \cdot S_2}{K_{S,2} + S_2}, \tag{5}$$

where the subscripts 1 and 2 denotes parameters for each of the two substrates.

If the same metabolic pathway is used in the catabolism of structural analogues compounds, it is possible that these substrates and/or their metabolites compete for the active site(s) on the enzyme(s) involved in the pathway. A sum kinetics model incorporating purely competitive substrate kinetics is (Yoon *et al.*, 1977):

$$\mu = \frac{\mu_{\max,1} \cdot S_1}{K_{S,1} + S_1 + \left(\frac{K_{S,1}}{K_{S,2}}\right) \cdot S_2} + \frac{\mu_{\max,2} \cdot S_2}{K_{S,2} + S_2 + \left(\frac{K_{S,2}}{K_{S,1}}\right) \cdot S_1}. \tag{6}$$

Eqution (6) indicates that each substrate exhibits a competitive inhibition effect on the utilization of the other substrate due to the competition of structurally analogous substrates for the same binding site (Segel, 1993). The competitive substrate kinetics can be used to describe simultaneous and sequential substrate consumption for mixtures of substrate (Okpokwasili and Nweke, 2005).

Another form of dual-substrate interaction with an enzyme is non-competitive inhibition, characterized by the formation of a non-reactive complex when both substrates are simultaneously bound to the enzyme. The cell growth model based on a non-competitive type of interaction is expressed as (Segel, 1975):

$$\mu = \frac{\mu_{\max,1} \cdot S_1}{\left(K_{S,1} + S_1\right)\left(1 + \frac{S_2}{K_{S,2}}\right)} + \frac{\mu_{\max,2} \cdot S_2}{\left(K_{S,2} + S_2\right)\left(1 + \frac{S_1}{K_{S,1}}\right)}. \tag{7}$$

Uncompetitive enzyme inhibition model has also been used to describe dual substrate interaction. It differs from non-competitive inhibition in that one of the compounds (the inhibitor) can bind only to the enzyme-substrate complex and not to the free enzyme. A cell growth model based on an uncompetitive type of interaction is (Segel, 1975):

$$\mu = \frac{\mu_{\max,1} \cdot S_1}{K_{S,1} + S_1\left(1 + \frac{S_2}{K_{S,2}}\right)} + \frac{\mu_{\max,2} \cdot S_2}{K_{S,2} + S_2\left(1 + \frac{S_1}{K_{S,1}}\right)}. \tag{8}$$

In the sum kinetic models, kinetic parameters determined in the single substrate experiments are used for curve fitting. However, Reardon *et al.* (2000) concluded that there are interactions between these substrates which cannot be described by sum kinetics models using only parameters determined in single substrate experiments; and that the interaction may not be a competitive, non-competitive or uncompetitive enzyme inhibition. To account for these mixture effects, an alternative model was formulated by incorporating an unspecified type of interaction into the sum kinetics framework (Reardon *et al.*, 2000):

$$\mu = \frac{\mu_{\max,1} \cdot S_1}{K_{S,1} + S_1 + I_{2,1} S_2} + \frac{\mu_{\max,2} \cdot S_2}{K_{S,2} + S_2 + I_{1,2} S_1}, \tag{9}$$

where $I_{1,2}$ and $I_{2,1}$ are interaction parameters.

In general, the interaction parameter $I_{i,j}$ indicates the degree to which substrate *i* affects the biodegradation of substrate *j*; a larger value of the parameter indicates stronger inhibition on the substrate uptake by the microorganism (Yoon *et al*., 1977; Reardon *et al*., 2000). Since the sum kinetics with an interaction parameters model reduces to a purely competitive inhibition model if $I_{i,j} = K_{S,j}/K_{S,i}$, comparison of these quantities indicates whether the kinetics are statistically different from competitive inhibition (Reardon *et al.*, 2000). In dual-substrate system, sequential substrate utilization is represented by a large value of $I_{1,2}$ and a small value of $I_{2,1}$ (Okpokwasili and Nweke, 2005).

Abu Hamed *et al.* (2004) slightly modified the sum kinetics model, first proposed by Yoon *et al*. (1977), to fit the experimental data on specific degradation rates of the substrates and taking also into account an inhibition effect, as represented in equation (10):

$$q = \frac{q_{\max,1} \cdot S_1}{K_{S,1} + S_1 + \frac{S_1^2}{K_{i,1}} + I_{2,1} S_2} + \frac{q_{\max,2} \cdot S_2}{K_{S,2} + S_2 + \frac{S_2^2}{K_{i,2}} + I_{1,2} S_1}, \tag{10}$$

where q_{max}, K_S, K_i are the maximum substrate degradation rate, the half-saturation and inhibition coefficients, respectively, for any single substrate system. The subscripts 1 and 2 refer to each of the two substrates. Equation (10) was utilized to evaluate and estimate the relative interaction effects on the individual degradation rates (Abu Hamed *et al.*, 2004).

As the kinetic parameters are constants specific for the particular system under study, the biodegradative performance of different systems could be compared on the basis of kinetic features (Yang and Humphrey, 1975).

2.3.3. Interaction between phenolic compounds

Many pseudomonads have the ability to utilize a wide variety of organic substrates, including aromatics and phenolic compounds for growth. The degradation of a number of different organic substrates by *P. putida* can be expected to proceed simultaneously (Hutchinson and Robinson, 1988). The convergence of catabolic pathways allows for the efficient utilization of a wide range of growth substrates, while the non-specificity of the induced enzymes allows for the simultaneous utilization of several similar substrates without an excess of redundant genetic coding for enzyme induction. In addition, the substrate utilization rates cannot be assumed to be independent of one another, especially if there were a rate controlling enzymatic reaction at the convergence of the catabolic pathways (Hutchinson and Robinson, 1988). Clarke and Ornston (1975) have shown that in the case of phenol and methylphenols the substrates share common steps in their respective metabolic pathways. Under these circumstances, the specific uptake rates depend on the relative concentrations of the two components. Hutchinson and Robinson (1988) showed that phenol and 4-methylphenol (hereafter also *p*-cresol) were simultaneously metabolized by *P. putida*. *P. putida* did not appear to metabolize either of the two substrates preferentially. *P. putida* was found to degrade a particular substrate at a rate proportional to its fraction of the total substrate mixture. The pathways involved in the degradation of phenol and *p*-cresol by *P. putida* are almost identical and the same enzymes are utilized in metabolizing both substrates (Sala-Trepat *et al.*, 1972). The results of Heinaru *et al.* (2001) showed that the degradation of phenol and *p*-cresol from their mixtures leads to the reversible formation of *p*-hydroxybenzoate (POB), which repressed the consumption of phenol, and therefore determines the sequential decomposition of phenolic compounds. Strains PC18 and PC24 of *P. fluorescens* were able to use the protocatechuate *ortho* branch for the degradation of methylated phenols (Heinaru *et al.*, 2001). Phenol also induced a synthesis of *p*-cresol methylhydroxylase (PCMH) in strain PC18 of *P. fluorescens* (Heinaru *et al.*, 2000). Phenol and *p*-cresol were utilized simultaneously during the growth of *P. mendocina* PC1 and *P. fluorescens* PC24 on the mixture of phenol and *p*-cresol. Simultaneous utilization of these compounds can be explained by the absence of metabolic conflict: strain PC1 degraded the above-mentioned compounds via the *meta* pathway by catechol 2,3-dioxygenase (C23O), and strain PC24 via the *ortho* pathway (phenol by catechol 1,2-dioxygenase (C12O) and *p*-cresol by protocatechuate 3,4-dioxygenase (PC34O)) (Heinaru *et al*., 2001).

Viggor *et al.* (2002) reported that the degradation of phenolic compounds from mixtures indicated a flux of substrates into different catabolic pathways. In the case of methylsubstituted phenols, multiple distinct pathways will often be involved in the catabolism of structural isomers. Catechol 2,3-dioxygenase activity was induced by dimethylphenols in *P. mendocina* PC1, where *meta* cleavage pathway was functional during the degradation of *p*-cresol. In the case of strains PC18 and PC24 of *P. fluorescens*, the degradation of *p*-cresol occurred via the protocatechuate *ortho* pathway; and the key enzyme for this pathway, PCMH,

was also induced by dimethylphenols. 2,4- and 3,4-dimethylphenols were converted into the dead-end products 4-hydroxy-3-methylbenzoic acid and 4-hydroxy-2-methylbenzoic acid. In the degradation of 3,4-dimethylphenol, the transient accumulation of 4-hydroxy-2-methylbenzaldehyde repressed the consumption of phenol from substrate mixtures. A mixed culture of strains with different catabolic types made it possible to overcome the incompatibilities with the degradation of the studied substrate mixtures (Viggor *et al.*, 2002).

The inhibition of biodegradation of the phenolic compounds has also been reported in literature. Kar *et al*. (1997) observed that phenol and *p*-cresol mutually inhibited their biodegradation by *Arthrobacter*; the inhibition of *p*-cresol to phenol degradation was found stronger than that of *o*-cresol (2-methylphenol), but *o*-cresol marginally enhanced the phenol degradation. However, Paraskevi and Euripides (2005) reported that the addition of *o*-cresol strongly inhibited phenol degradation by the indigenous soil bacterium *Pseudomonad*. Jiang *et al*. (2006) demonstrated that the inhibition of phenol biodegradation by *Candida tropicalis* was much stronger due to *m*-cresol (3-methylphenol) than *vice versa*. 2,4-dimethylphenol was found to be converted by *Chlorella* (even at a concentration of 1000 mg/1) to an isomer of dimethylbenzenediol that was in some cases accumulated in the medium (Klekner and Kosaric, 1992a). However, phenol in mixture with more toxic derivatives did not stimulate the degradation of 2,4-dimethylphenol and 2-chlorophenol by cells of *Chlorella* but improved the removal of 2,4-dichlorophenol. However, these derivatives clearly inhibited biodegradation of phenol itself (Klekner and Kosaric, 1992b).

Hollender *et al.* (1994) demonstrated that both substrates in the mixtures consisting of 4-chlorophenol and 2-methylphenol (2-MP) or 4-chlorophenol and 3-methylphenol (3-MP) were mineralized simultaneously by *Comamonas testosteroni* JH5. But 2-methylphenol and 3-methylphenol persisted in the presence of 3-chlorophenol (inactivation of the *meta*-cleaving enzyme was observed) and were degraded only to a small extent (2-MP 37%; 3-MP 10%) in combination with 2-chlorophenol. The mineralization of 4-chlorophenol and 4-methylphenol (4-MP) occurred successively and was accompanied by diauxic growth. The degradation of 4-chlorophenol was repressed until 4-MP or 4-hydroxybenzoic acid (as intermediate) was completely degraded. The degradation of 4-MP proceeds via 4-hydroxybenzoic acid and a protocatechuate, which was *meta* cleaved by a 4,5-dioxygenase. The results obtained suggest that the active methylhydroxylase exclusively oxidizes aromatic methyl substituents in the *para* position (Hollender *et al.*, 1994).

2.4. Main metabolic pathways of phenolic compounds

Studies of major pathways for aerobic catabolism of aromatic compounds in bacteria have revealed that, while different enzymes carry out initial conversion steps, compounds are transformed into a limited number of central intermediates. These common metabolites are further degraded through a few central pathways to finally provide intermediates of the citrate cycle and are mineralized to carbon dioxide and water (Harayama and Renik, 1989; van der Meer *et al*., 1992; Harwood and Parales, 1996). The efficiency of a certain catabolic pathway often depends on the properties of the involved key enzyme(s). The crucial step in aromatic metabolism is the cleavage of the resonance structure of the benzene nucleus. All aromatic compounds must be transformed into *ortho*- or *para*-dihydroxybenzenes before ring cleavage can occur. The microbial degradation of aromatic compounds by ring cleavage is predominantly an aerobic process and is invariably achieved by monooxygenase- or dioxygenase-catalyzed reactions (Kieslich, 1984). These enzymes often exhibit a broad substrate spectrum, which in nature allows the funnelling of a variety of structurally similar hydrocarbons into a few central metabolic pathways (Schmid *et al*., 1998). Within the peripheral pathway, the aromatic compound is modified in a number of steps, including the action of monooxygenase or dioxygenase resulting in the formation of a dihydroxylated benzene ring. The main resulting metabolites are catechol and protocatechuate with hydroxyl groups at positions 1,2 and gentisate with hydroxyl groups at positions 1,4. The ring cleavage of both catechol and protocatechuate catalyzed by dioxygenases occurs either between the hydroxyl groups (intradiol or *ortho*-cleavage) or adjacent to one of the hydroxyl groups (extradiol or *meta*-cleavage) (Figure 2). The *ortho*-cleavage pathway of catechol and protocatechuate (see also Figure 3), catalyzed by catechol 1,2-dioxygenase (C12O) and protocatechuate 3,4-dioxygenase (PC34O), respectively, converges at 3-oxoadipate, which is in two further reactions converted to acetyl-coenzyme A and succinyl-coenzyme A. Thus it follows that the *ortho*-cleavage pathway is also called the 3-oxoadipate (*β*-ketoadipate) pathway (Ornston and Stanier, 1966; Kieslich, 1984; Harwood and Parales, 1996; van der Meer, 1997). The *meta* cleavage of catechol and protocatechuate are catalyzed by catechol 2,3-dioxygenase (C23O) and protocatechuate 4,5-dioxygenase (PC45O), respectively. The *meta*-cleavage pathways of protocatechuic acid are shown in Figure 4. Protocatechuic acid is formed by different reactions from 1,2-, 1,3-, and 1,4-substituted aromatic compounds such as, e.g., *m*- and *p*-cresol (Kieslich, 1984; Heinaru *et al*., 1997).

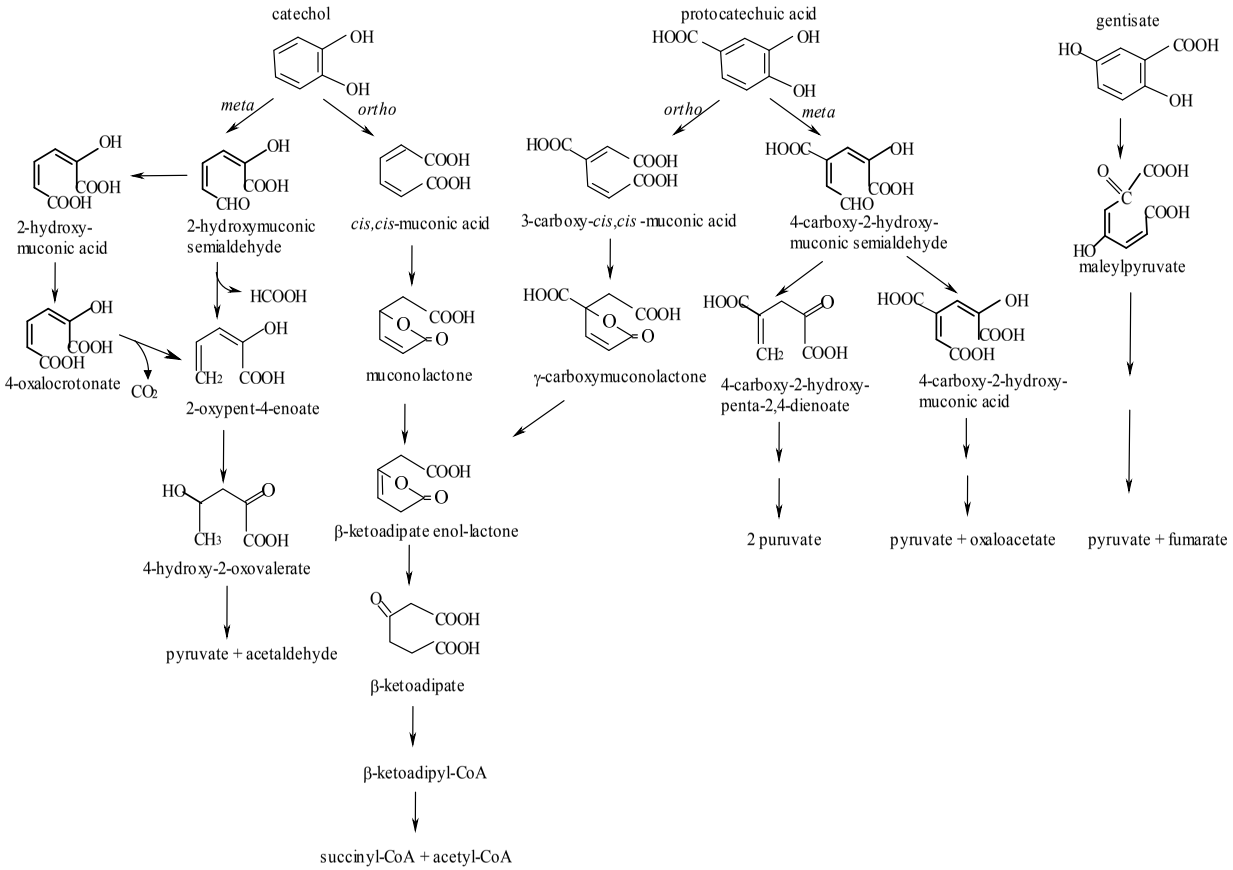


Figure 2. Central pathways of the catabolism of aromatic compounds (based on Chapman, 1972; Kieslich, 1984; Harwood and Parales, 1996; Martínková *et al.*, 2009).

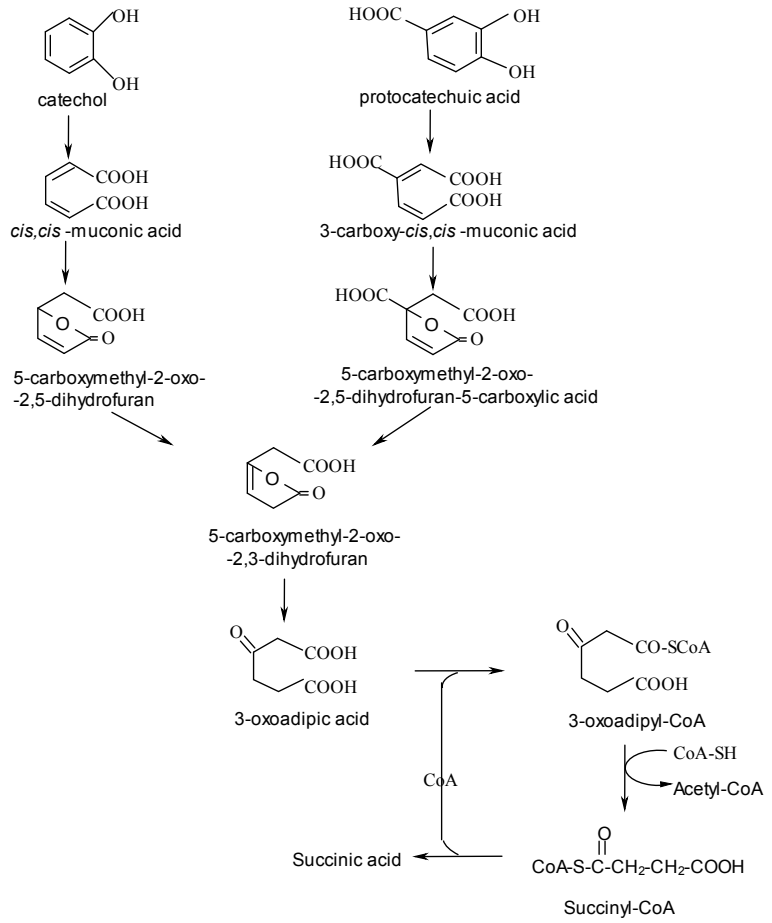


Figure 3. The *ortho*-cleavage pathways of catechol and protocatechuic acid in bacteria (Kieslich, 1984).

The degradation of gentisate (an intermediate of *m*-cresol, naphthalene, salicylate and 3-hydroxybenzoate degradation) is initiated by gentisate 1,2-dioxygenase (G12O), which cleaves the aromatic ring between the carboxyl and the adjacent hydroxyl group to form maleylpyruvate (Dagley, 1971; Chapman, 1972) as shown also in *Rhodococcus erythropolis* (Suemori *et al.*, 1995). Two further reactions catalyzed by maleylpyruvate isomerase and fumarylpyruvate hydrolase finally convert the intermediates to pyruvate and fumarate (Figure 2). G12O shows a broad substrate range towards alkylsubstituted gentisic acids (Crawford *et al*., 1975).

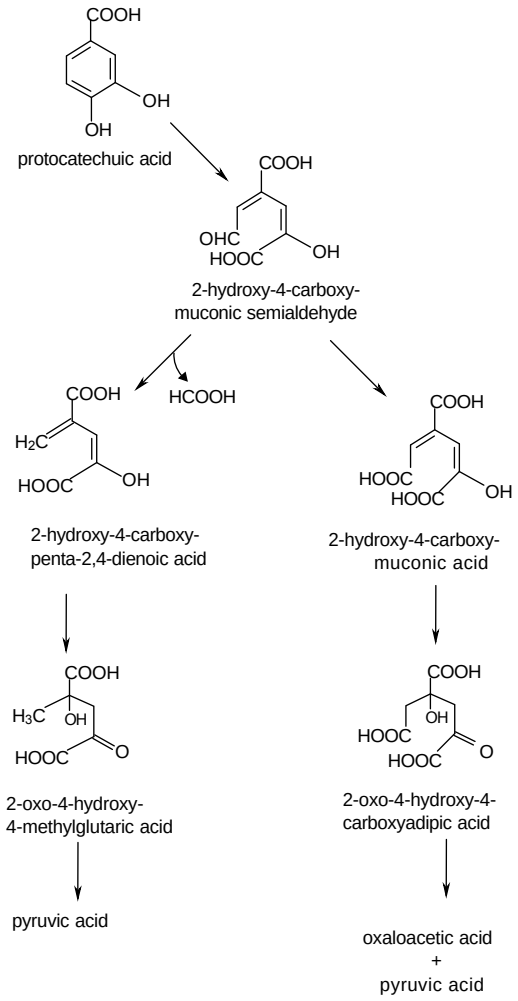


Figure 4. The *meta*-cleavage pathways of protocatechuic acid metabolism (from Chapman, 1972).

Phenolic compounds are usually biotransformed by oxidation to the corresponding catechols before *ortho*- or *meta*-cleavage of the aromatic ring (Chapman, 1972; Dagley, 1987). However, important factors in the rate and extent of oxidation of aromatics are the number, position and chemical nature of the substituents on the aromatic ring. Knackmuss (1981) postulated that one of the reasons for the major effects of substituents on the biodegradation of the aromatic ring was primarily due to enzyme specificity. The substituted compound must not only be a structural analogue of the normal enzyme substrate if it is to be converted, but must also be of comparable reactivity, and must have functional groups of similar polarity and size to those of the parent compounds. The enzyme must also catalyze the same reaction on the substituted analogue as

on its parent compound, as the reaction product is specific to a particular enzyme reaction (Knackmuss, 1981). In some cases the presence of substituents on the aromatic ring may prevent oxidation of the compound. The electronegative groups (e.g. halogen atoms, nitro groups) as substituents lower the reactivity of the resonance structure towards oxygen and prevent either activation or ring cleavage (Kieslich, 1984).

In the typical metabolic pathway phenol is converted by the phenol hydroxylase to catechol, which is further degraded through *ortho*- or *meta*-cleavage. This FAD-dependent phenol hydroxylase was found to be induced by phenol and other aromatic compounds. The broad substrate specificity enables this enzyme to convert also *p*-chlorophenol, *p*-nitrophenol, resorcinol and *p*-cresol (Fialová *et al*., 2003). Toluene dioxygenase has also been identified as the enzyme responsible for the initial step of the metabolism of phenol by *P. putida* F1 (Spain and Gibson, 1988; Spain *et al.*, 1989). The production and accumulation of metabolic intermediates during phenol degradation has been commonly observed. Among these, 2-hydroxymuconic acid semialdehyde is the first product of the catechol ring cleavage in the *meta*-pathway degradation of phenol (Mörsen and Rehm, 1990; Allsop *et al.*, 1993). The degradation of phenol via *meta*-cleavage utilizes a sequence of (a) hydroxylation to catechol, (b) ring cleavage by catechol 2,3-dioxygenase to 2-hydroxymuconic semialdehyde, and (c) either oxidation to 4-oxalocrotonate or hydrolysis to 2-oxopent-4-enoate (Ornston and Stanier, 1966). The first reaction, phenol + NADPH + O_2 → catechol + NADP + H_2O, seems to be the rate-limiting step in the biodegradation of phenolic compounds (Neujahr and Kjellen, 1978).

Concerning the metabolism of methylsubstituted phenols the action of phenol hydroxylase will lead to formation of the corresponding catechols, which can be subject to both *ortho* and *meta* cleavage (Hopper and Chapman, 1971). In the case of *ortho* cleavage of methylcatechols, however, methylmuconolactones are usually formed as dead-end products (Knackmuss *et al.*, 1976). Methylsubstituted aromatic substrates are generally degraded via the *meta* pathway, catalyzed by catechol 2,3-dioxygenases, whereas the degradation of non-substituted and chloro-substituted aromatic substrates tends to occur through *ortho*-cleavage catalyzed by *ortho*-cleaving 1,2-dioxygenases (Knackmuss, 1981; Klečka and Gibson, 1981; Schmidt *et al*., 1985). Since 2,3-dioxygenase, the key enzyme of the *meta*-cleavage pathway, is irreversibly inactivated by acyl halides (a dead-end metabolite) generated from 3-chlorocatechol, bacteria which utilize haloaromatics use generally *ortho* cleavage dissimilatory pathways (Klečka and Gibson, 1981; Knackmuss, 1981; Bartels *et al.*, 1984). However, the complete degradation of 4-chlorophenol via *meta* cleavage of 4-chlorocatechol is postulated by Hollender *et al*. (1994). Also the degradation of phenol via the *meta* pathway has been described by many authors, for example in the case of *P. putida* (Yang and Humphrey, 1975). In pseudomonads, degradation of *p*-cresol also occurs via the catechol *meta* pathway and phenol can be degraded by both catechol *ortho* and *meta* pathways (Ampe and Lindley, 1996). But Kolomytseva *et al*. (2007) showed that in

Rhodococcus opacus *p*-cresol (4-methylphenol) is degraded via 4-methyl-catechol and through the *ortho* pathway. This is in contradiction with the general assumption that phenol, as a non-substituted aromatic compound, should be degraded via the *ortho* pathway in phenol-degrading bacteria, whereas *p*-cresol, as an alkyl-substituted aromatic compound, should be catabolized via the *meta* pathway in *p*-cresol-degraders (Müller *et al.*, 1996a). This can easily be explained on the basis that catabolism of phenol and *p*-cresol must use two different pathways for the aromatic ring cleavage of catechol. It is well known that the *ortho* and *meta* pathways of catechol are metabolic alternatives and the use of one or the other depends on the nature of the growth substrates as well as on the microbial species (Shingler *et al*., 1992). As reported in the literature, the one subfamily of C12O enzymes initiating the cleavage of the aromatic ring shows high enzymatic activity for chlorocatechols (Eulberg *et al.*, 1997 and 1998; Suvorova *et al.*, 2006) and another subfamily of C12O exhibits a high specificity for methylcatechols (Murakami *et al*., 1997; Kolomytseva *et al*., 2007).

An alternative catabolic route to ring hydroxylation has been described for methylated phenols in which metabolism is initiated by the oxidation of a methyl group to a corresponding carboxyl group, followed by conversion of the ring cleavage product of protocatechuate to *β*-ketoadipate (Dagley and Patel, 1957). The first enzyme for the degradation of *p*-cresol and dimethylphenols via the protocatechuate branch of the *β*-ketoadipate pathway is *p*-cresol methyl-hydroxylase (PCMH) (Hopper and Taylor, 1977; Keat and Hopper, 1978). Reactions catalyzed by PCMH are shown in Figure 5 (Cunane *et al*., 2000). PCMH has a limited substrate range and requires an alkyl-substituted phenolic ring with a hydroxyl group in the *para* position (Bossert *et al*., 1989). Protocatechuate, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate (POB) have been identified as intermediates in *p*-cresol degradation by *P. putida* (O'Reilly and Crawford, 1989). Catabolism of the intermediate *p*-hydroxybenzoate (POB) to protocatechuate is catalyzed by *p*-hydroxybenzoate hydroxylase (POBH) (van der Meer, 1997).

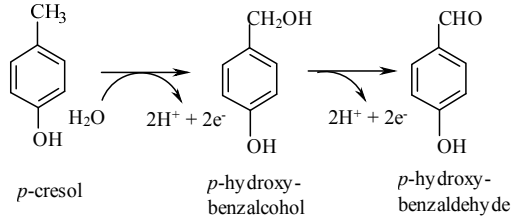


Figure 5. Reactions catalyzed by *p*-cresol methylhydroxylase (Cunane *et al.*, 2000).

However, this was not detected in *Alcaligenes eutrophus* JMP 134 (Pieper *et al.*, 1995). In the case of *ortho*-methylsubstituted phenols, hydroxylation is directed

at the 6-position; both 2- and 6-hydroxylation seems to be possible in *meta*- and *para*-substituted phenolic compounds like 3,4-dimethylphenol. Nevertheless, C-2 hydroxylation of 3,4-dimethylphenol by *Alcaligenes eutrophus* JMP 134 phenol-hydroxylating enzymes is preferred over C-6 hydroxylation (Pieper *et al.*, 1995). Mineralization of methylphenols via ring hydroxylation and subsequent *meta* cleavage has been reported for monomethylphenols and 2,3- and 3,4-dimethylphenol (Bayly *et al*., 1966; Ribbons, 1970).

As described by Pieper *et al.* (1995) the different dimethylmuconolactones resulted from the metabolism of 2,4-, 2,5-, 3,4- and 3,5-dimethylphenol via dimethylcatechol, followed by *ortho* cleavage. The extent of dimethylmuconolactone accumulation in the presence of both *ortho*- and *meta*-cleavage activities can be regarded as an indication of the effectiveness of catechol 2,3-dioxygenase. Thus, in the presence of both *ortho*- and *meta*-cleavage activities, 3,4-dimethylcatechol is quantitatively channelled into the *meta*-cleavage pathway, whereas 3,5-dimethylcatechol is quantitatively subjected to *ortho* cleavage even when only low levels of catechol 1,2-dioxygenase are induced (Pieper *et al.*, 1995). It is proposed that enzymes of the 3,5-dimethylphenol pathway and those for conversion of *p*-cresol to *p*-hydroxybenzoate are plasmid encoded, that the methyl-oxidizing enzymes are expressed constitutively, and that the later enzymes are inducible (Hopper and Kemp, 1980).

Chapman and Hopper (1968) proved that the catabolism of 2,4-dimethylphenol (2,4-DMP) by pseudomonads was initiated by the oxidation of a methyl group situated *para* to the hydroxyl group and accompanied by the accumulation of 4-hydroxy-3-methylbenzoic acid (4H3MBA) and the noticeable increase of PC34O activity (see Figure 6).

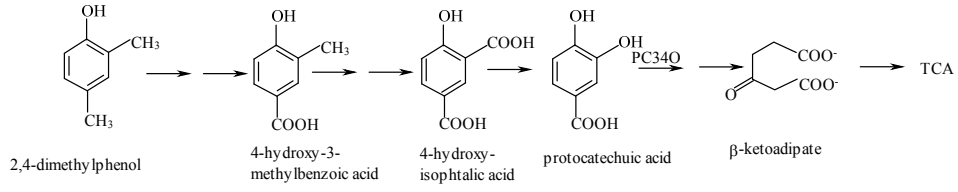


Figure 6. Proposed pathway for 2,4-dimethylphenol (Chapman and Hopper, 1968).

In *Comamonas testosteroni* JH5, 2,4- and 3,4-DMP were transformed into the dead-end metabolites 4H3MBA and 4-hydroxy-2-methylbenzoic acid (4H2MBA) (Hollender *et al.*, 1994). However, through catechol *meta* cleavage, this strain also mineralized 2,3-DMP completely, but 2,5- and 3,5-DMP only partially. The complete degradation of 2,5-DMP and 3,5-DMP via the gentisate pathway has been demonstrated in pseudomonads. A methyl group situated *meta* position to hydroxyl group was oxidized first, and a second hydroxyl group then was introduced into *para* position to the first group; gentisic acid (2,5-dihydroxybenzoic acid) or an alkyl-substituted gentisate was formed, and

this compound served as a substrate for the ring-cleavage dioxygenase. The aromatic ring of methylgentisate was cleaved by G12O (Hopper and Chapman, 1971; Hopper *et al.*, 1971; Hopper and Kemp, 1980). *o*-Cresol and 2,6-DMP were not attacked, possibly because they do not possess a methyl group situated *meta* to the hydroxyl group and therefore cannot furnish the carboxyl group of a gentisic acid (Hopper and Chapman, 1971). The induction of the *meta* cleavage pathway by 2,3-DMP and 3,4-DMP was also revealed in *Alcaligenes eutrophus* JMP 134 (Pieper *et al*., 1995). The oxidation of DMPs into dimethylcatechols depends on the position of the methyl groups present in the aromatic ring (Semple and Cain, 1997). Therefore, not all dimethylcatechols formed from DMPs were cleaved by C23O (Arenghi *et al.*, 2001). As one possible oxidation product of 3,4-DMP, 4,5-dimethylcatechol does not have a methyl group adjacent to any of the hydroxyl groups in the aromatic ring; this resulted in the complete degradation of 3,4-DMP. In contrast to that, the oxidation products of 2,3-, 2,4- and 3,5-DMP have one methyl group in *ortho* position with respect to the hydroxyl group that could cause their slow degradation. As an oxidation product of 2,3- and 3,4-DMP, 3,4-dimethylcatechol has been reported to be also degraded via a catechol *meta* pathway (Shingler *et al.*, 1989; Pieper *et al*., 1995). Viggor *et al.* (2002) showed that in the case of strains PC18 and PC24 of *P. fluorescens*, 2,4- and 3,4-DMPs were converted into the dead-end products as 4-hydroxy-3-methylbenzoic acid and 4-hydroxy-2-methylbenzoic acid.

Chapman (1972) was able to isolate the degraders of all the DMP isomers except 2,6-DMP, a result which he attributed to the methyl groups filling positions adjacent to the hydroxyl group which would normally be hydroxylated, thus preventing the formation of the appropriate catechol. However, it was found that 2,6-DMP was degraded by a much more complex route in which it was converted to 2,6-dimethylhydroquinone and then 2,6-dimethyl-3-hydroxyhydroquinone which cleaved to citraconate and propionate (Ewers *et al.*, 1989). Evidently, this was because of the unavailability of sites for hydroxylation due to the presence of the two methyl groups on either side of the aromatic ring's hydroxyl group.

1,3-dihydroxybenzene (resorcinol) and 3,5-dihydroxytoluene (5-methylresorcinol aka orcinol) are produced in the processing of oil-shale. Many microorganisms like *Azotobacter vinelandii*, *Trichoporon cutaneum*, pseudomonads are capable of using resorcinols as sole source of carbon (Chapman and Ribbons, 1976a and 1976b; Groseclose and Ribbons, 1981). A necessary condition for the degradation of 1,3-dihydroxybenzene and its derivatives is the formation of 1,2,4-trihydroxy derivatives which are then substrates for ring cleavage enzymes (Chapman and Ribbons, 1976a). These studies indicated that resorcinol was degraded via three different pathways in bacteria. In *Azotobacter vinelandii* resorcinol catabolism (Figure 7) occurs via pyrogallol (1,2,3-trihydroxybenzene) which is then cleaved by pyrogallol 1,2-dioxygenase to give isomers of 2-hydroxymuconate (oxalocrotonate). Further degradation to pyruvate and acetaldehyde is analogous to the *meta*-cleavage pathway of catechol (Groseclose and Ribbons, 1981).

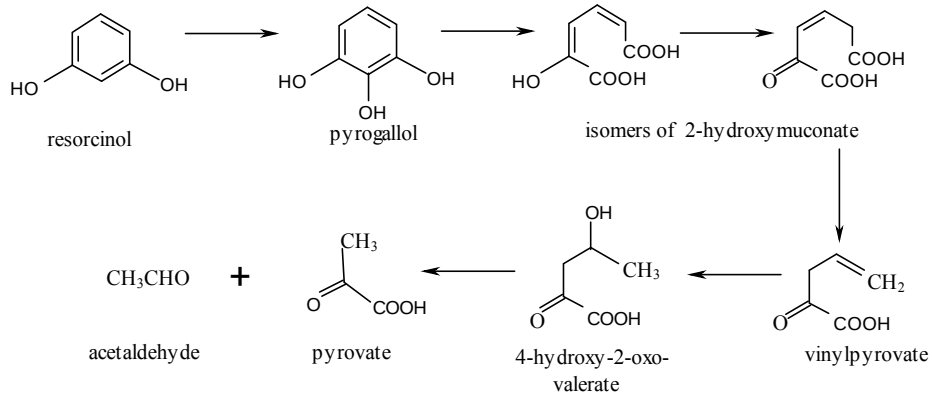


Figure 7. Pathway for resorcinol catabolism by *Azotobacter vinelandii* (Groseclose and Ribbons, 1981).

P. putida apparently adopted two different pathways: resorcinol was converted into 1,2,4-trihydroxybenzene (hydroxyquinol), and hydroxyquinol was subsequently degraded by (a) 2,3,5-trihyrdoxytoluene 1,2-dioxygenase (*meta* cleavage) (Chapman and Ribbons, 1976a) and (b) hydroxyquinol 1,2-dioxygenase (*ortho* cleavage) (Chapman and Ribbons, 1976b). Chapman and Ribbons (1976a) showed that orcinol is metabolized in *P. putida* O1 and *P. putida* ORC by hydroxylation to give 2,3,5-trihydroxytoluene, which undergoes a *meta* cleavage between carbon atoms 1 and 2 substituted by methyl and hydroxyl groups, with consequent formation of acetate and pyruvate by the two successive hydrolytic reactions of the ring cleavage product. Chapman and Ribbons (1976a) observed that orcinol-grown cells of the strains of *P. putida* O1 and ORC were also able to oxidize resorcinol and reciprocally, resorcinol-grown cells oxidized orcinol, but a difference existed both in the relative rates of oxidation and the amount of oxygen consumed per mole of substrate. It was shown that resorcinol and orcinol were substrates for the hydroxylase reaction. The expected hydroxylated products (2,3,5-trihydroxytoluene and 1,2,4-trihydroxybenzene) were shown to be substrates for a ring cleavage enzyme induced during growth on orcinol. The ring cleavage dioxygenases which are known to act on substituted 1,2,4-trihydroxybenzene rings are of the *meta* or extradiol type, whereas that which cleaves hydroxyquinol is of the intradiol or *ortho* category (Chapman and Ribbons, 1976a). However, in certain strains of *P. putida* (O1) both *ortho* and *meta* cleavage of hydroxyquinol can occur (Chapman and Ribbons, 1976b). It is possible that resorcinol may be completely catabolized by the enzymes of the orcinol pathway in *P. putida* O1. It is concluded that hydroxyquinol 1,2-dioxygenase does not oxidize 2,3,5-trihydroxytoluene showing a substrate specificity in common with certain other *ortho* cleavage dioxygenases (Stanier and Orston, 1973). But on the contrary,

2,3,5-trihydroxytoluene 1,2-dioxygenase cleaves the ring of some substrate analogues (for example hydroxyquinol and 3-methylcatechol, as observed for the other *meta* cleavage oxygenase) (Dagley, 1971). The proposed metabolic sequence for 5-methylresorcinol and resorcinol catabolism is shown in Figure 8.

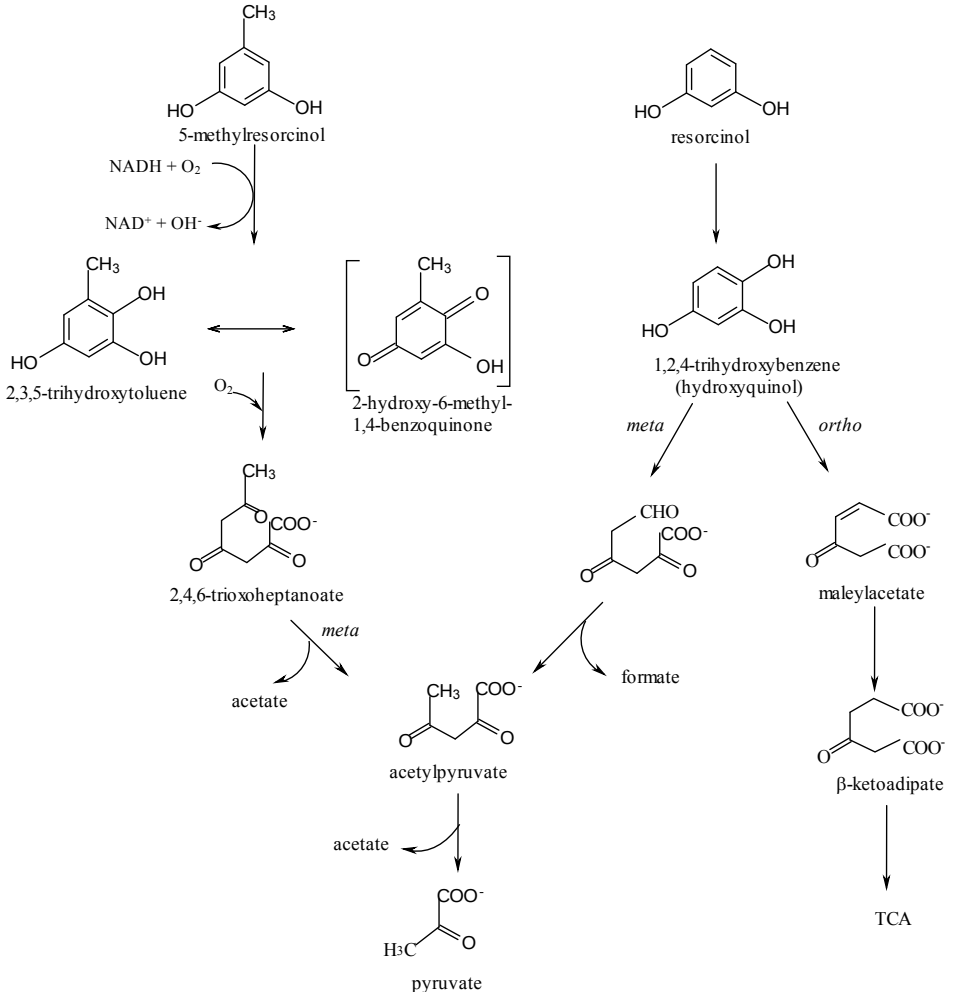


Figure 8. Metabolic sequence for 5-methylresorcinol and resorcinol catabolism (based on Ohta *et al*., 1975; Chapman and Ribbons, 1976a and 1976b; Ohta and Ribbons, 1976).

2.5. Respirometry

The measurement of dissolved oxygen (DO) concentration is widely applied in environmental applications. Respirometry is the measurement and interpretation of the biological oxygen consumption rate of microorganisms under well-defined experimental conditions (Vanrolleghem, 2002).

In biochemical terms, respiration is the adenosine triphosphate (ATP) generating metabolic process in which either organic or inorganic compounds (H_2, Fe^{2+}, NH_4^+ or S^0) serve as the electron donor and inorganic compounds such as O_2, NO_2^-, NO_3^-, SO_4^{2-}, CO_2 etc. serve as the ultimate electron acceptor (Bitton, 1994; Vanrolleghem, 2002). Respiration can be aerobic (an oxygen is the ultimate electron acceptor) or anaerobic; anaerobic respiration releases less energy than aerobic respiration (Bitton, 1994). ATP is generated as electrons removed from the substrate are transferred along the electron transport chain from one metabolic carrier to the next and, ultimately, to oxygen. The biomass converts the energy of intramolecular bonds in the substrate to the high-energy phosphate bonds of ATP. Cells use energy (ATP) to make building blocks, synthesize macromolecules (proteins, lipids, polysaccharides, etc.), and repair damage to cells (maintenance energy) and maintain movement and active transport across the cell membrane (Bitton, 1994; Vanrolleghem, 2002). Only a portion (1-*Y*) of the consumed organic substrate is oxidized to provide energy. The remainder, typically about half of the substrate (on a weight/weight basis) is converted into a new biomass (Vanrolleghem, 2002).

Respirometry is a simple, convenient and widely used technique for the characterization of wastewater and activated sludge (Henze, 1992; Brouwer *et al*., 1994; Orupõld *et al.*, 1999), because oxygen consumption is directly associated with both biomass growth and substrate removal. The oxygen uptake rate (OUR) is a function of both viable cell concentration and specific respiratory activity (Guwy *et al.*, 1998). OUR reflects the state of microbial activity in breaking down organic matter and the specific OUR for a given sludge type has been shown to be a good indicator of active biomass and to vary with the substrate concentration (Marsili-Libelli and Tabani, 2002). Measurements of the oxygen consumption of activated sludge have been used to determine the short-term biological oxygen demand (Spanjers *et al*., 1994; Papers I-III) and the biokinetic parameters of degradation processes (Čech *et al*., 1984; Orupõld *et al.*, 2001; Vanrolleghem, 2002; Papers I-III), to estimate the organic load to the wastewater treatment plant (Brouwer *et al*., 1994), to warn of incoming toxicity (Kim *et al.*, 1994; Vanrolleghem *et al.*, 1994) and predict effluent quality (Guwy *et al.*, 1998). An on-line application of respirometry is a useful tool for monitoring, modelling and control of the activated sludge process (Spanjers and Vanrolleghem, 1995; Watts, 2000; Vanrolleghem, 2002). The activity of aerobic biomass is affected by (Watts, 2000):

a) the types of organisms present;
b) the biomass viability, i.e. the actual concentration of living cells available to respire;

c) readily and/or less readily biodegradable materials;
d) toxic or inhibitory components;
e) temperature;
f) the presence of oxygen.

The majority of the respirometric techniques based on the measurement of oxygen in the liquid phase using electrochemical dissolved oxygen (DO) sensor. A large number of different factors cause uncertainty in DO measurement using amperometric sensors (Helm *et al*., 2010). Jalukse and Leito (2007) have carried out in-depth analysis and modelling of amperometric (DO) sensors. They identified 16 separate uncertainty sources and found that the relative expanded uncertainties at the level of confidence of 95% (coverage factor k equal to 2) obtained by experts under laboratory conditions varied between 1% and 9%. The magnitudes of uncertainty sources are strongly dependent on the analyte, the matrix, sensor design and measurement condition (Jalukse and Leito, 2007). At DO concentrations lower than below 4 mg/l (depending on other conditions) the background current of the sensor becomes the dominating uncertainty source (Jalukse and Leito, 2007). It is obvious that operation and design of the wastewater treatment plant (WWTP), including the composition of incoming wastewater, and sludge age of the activated sample source, play an important role in producing the experimental results obtained by respirometry (Dircks *et al*., 1999). There are several system parameters which may cause inaccuracy of the oxygen uptake rate (OUR). They are grouped in two sets: *design parameters*, including the sizing of the device (flow rate and volume) and its operation (sampling time, duty cycle, numerical algorithm for DO data processing); and *environmental parameters*, concerning the physico-chemical conditions of the experiment, i.e. uncontrolled oxygen transfer rate (accidental air-leak in the case of closed respirometer), pH, sludge condition on start-up behaviour and size of sample injection. The consistency of these parameters is important to ensure that all measurements are taken under exactly the same operating conditions (Marsili-Libelli and Tabani, 2002). Wastewater and sludge samples are to be representative of the treatment system under study; temperature and pH must be kept constant in the measuring system (Marsili-Libelli and Tabani, 2002). Reliable results can be obtained from full respirograms, where the measurement of respiration occurs before substrate addition, in an endogenous state, and is continued until all substrate added is oxidized and endogenous respiration is achieved again (Spanjers *et al*., 1999). The endogenous respiration rate of activated sludge can be defined as the oxygen uptake rate in the absence of substrate from external sources and is indicative of the concentration of active biomass (Vanrolleghem, 2002). Endogenous respiration can change due to the changes in biomass activity and due to the dilution effect (volume variations because of sample addition). Being exactly known, this dilution effect is not strictly a random error, nevertheless it may constitute a systematic inaccuracy if neglected (Marsili-Libelli and Tabani, 2002). The endogenous respiration rate, which is generally low and practically doesn't change during the

course of a respirogram, is derived from the respiration rate prior to the addition of the substrate and after the substrate has been completely oxidized. The area under the respirogram between dosage and the time instant of the second endogenous respiration rate represents the short-term BOD of the added substrate. The initial concentration of the substrate should be at least twice the substrate K_S value in order to obtain a fully reliable estimate of maximum oxygen uptake rate and the half-saturation coefficient, because low initial concentration of the substrate leads to larger errors in these estimated parameters (Spanjers *et al*., 1999).

Respirometry is shown to be a suitable and quick technique for investigating the kinetics of primary substrate metabolism for a wide number of compounds and the main advantages of the respirometric method are as follows (Čech *et al*., 1984; Limbert and Betts, 1995; Ellis *et al*., 1996):

1) Practical realization is simple and inexpensive;
2) It could be also used for those substrates that cannot be easily determined analytically;
3) It determines kinetic parameters of mixed culture at given conditions without changing its qualitative and quantitative composition.

The kinetic parameters are functions of the compound undergoing biodegradation and the composition of the microbial community performing the degradation (Brown *et al*., 1990). During batch experiments the ratio of the initial substrate concentration (S_0) to the initial biomass concentration (X_0) is one of the major factors affecting the values of the kinetic parameters obtained for that substrate, because of its effect on the composition of the microbial community and on the physiological state of culture, which is the sum of a cell's macromolecular composition and determines how rapidly it can synthesize enzymes, as well as how rapidly those enzymes react (Grady *et al*., 1996). In batch kinetic tests using low S_0/X_0 ratios, the shifts in biomass such as cell multiplication and growth are prevented, maintaining the physiological state existing in the environment from which the biomass was taken and the kinetic parameters' values obtained are "extant" (Grady *et al*., 1996). The higher S_0/X_0 ratios allow biomass growth to take place with a concomitant adjustment in macromolecular composition and cell physiology. The larger the value of S_0/X_0, the greater the change in the community structure and the physiological state of the cells, and the more the measured kinetics will reflect the maximum capability of the members of the microbial community rather than the characteristics of the original culture. Kinetic parameters determined under this condition are "intrinsic" and represent the inherent maximal activity of the biomass at a given temperature (Grady *et al*., 1996). The values of the intrinsic kinetic parameters are independent of the biomass concentration, the substrate concentration and of the reactor system employed (Brown *et al*., 1990). Typically, an S_0/X_0 ratio greater than 20 on a chemical oxygen demand basis will provide intrinsic kinetic parameters (Grady *et al*., 1996) and the S_0/X_0 value less than 0.025 gives extant kinetics (Grady *et al*., 1996; Ellis *et al.*, 1996). The

extant values of kinetic parameters are therefore expected to be lower than intrinsic values (Ellis *et al*., 1996).

Ready biodegradability tests are often limited because of a lack of definition in inoculum source and the variations in the inoculum quality and quantity which can be critical for reproducibility and efficiency. Therefore, quantitative measurement of the S_0/X_0 ratio, or alternatively its setting at a certain value, could enhance the reliability of test results (Vazquez-Rodriguez *et al.*, 1999 and 2003). The levels of metabolites are also higher as the S_0/X_0 ratio increases (Chudoba *et al.*, 1985). In order to improve the comparison between different analytical procedures, a low S_0/X_0 ratio is deemed advantageous (Vazquez-Rodriguez *et al.*, 1999 and 2003).

3. AIMS OF THE STUDY

The general objective of this thesis was to assess the aerobic biodegradability of phenol and different methyl-, dimethyl- and hydroxyphenols characteristic to the oil-shale industry wastewaters both as single substrates and bi-substrate mixtures at different concentrations by the activated sludge adapted for the processing of phenolic wastewater from the oil-shale chemical industry. Various kinetic models were applied to describe the behaviour of the biodegradation process of substrates.

The specific aims were:

1. To investigate the potential of the activated sludge from the Kohtla-Järve wastewater treatment plant to degrade various phenolic compounds.
2. To evaluate the efficiency of phenol, *o*-cresol, *p*-cresol, resorcinol, 5-methylresorcinol, 2,4-, 2,6-, 3,4- and 3,5-dimethylphenol's removal in the treatment plant.
3. To obtain information about interactions among phenol, *o*-cresol, *p*-cresol, resorcinol and 5-methylresorcinol during the biodegradation of these compounds as mixed substrates and how these compounds may influence each other's biodegradation at low initial sustrate concentrations.

4. EXPERIMENTAL

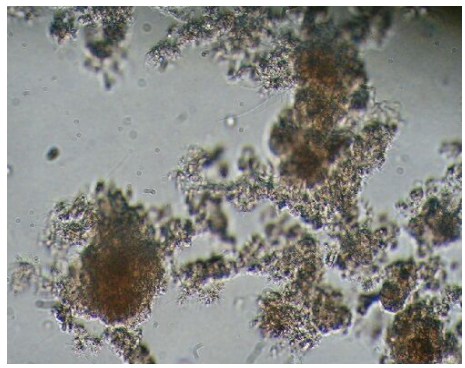
4.1. Description of apparatus and measurement conditions

4.1.1. Chemicals and media

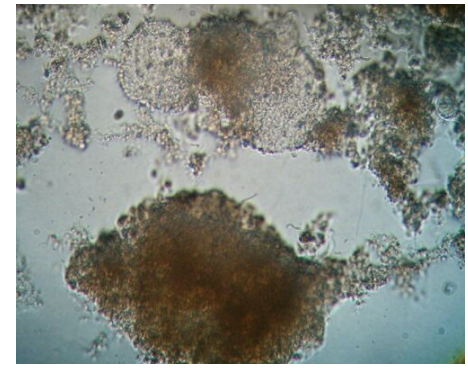
Phenol, *p*-cresol, *o*-cresol, resorcinol, 5-methylresorcinol, 2,4-, 2,6-, 3,4- and 3,5-dimethylphenols were used both as single and mixed substrates in the experiments. All the chemicals used for the preparation of substrate solutions were of an analytical grade.

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 activate or induce certain enzymes in the bacteria to be available to take part in the metabolism reaction. Activated sludge appears as 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 (Painter and King, 1985; Joshi *et al.*, 1999; Marrot *et al.*, 2006). The dominant heterotrophs in the microbial composition of activated sludge are found to be species of *Achromobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Comamonas*, *Flavobacterium*, *Micrococcus*, *Nocardia* and *Pseudomonas* (Painter and King, 1985).

The inoculum used in this thesis was the activated sludge taken from the aeration tank of the Kohtla-Järve wastewater treatment plant (WWTP), Estonia, which also treats phenolic wastewater from the oil-shale chemical industry and, therefore, is considered to be adapted to the phenolic compounds. The activated sludge was stored and pre-aerated for at least 24 h at room temperature to remove residual organic substances and to achieve a stable endogenous respiration before it was used for the experiments (Papers I-III). The concentration of activated sludge samples was determined gravimetrically and quantified as mixed liquor suspended solids (MLSS) in grams per litre according to the standard method (APHA, 1989). A sample of 10 ml of sludge was centrifuged for 10 min at 4000 rpm, and the deposit was dried for 24 h at 105°C until a constant weight was reached. The MLSS of the activated sludge was in the range of 3.5 g/l to 10.8 g/l. In earlier study, Kahru *et al*. (1998) have shown that the activated sludge microbial consortium of the Kohtla-Järve WWTP contained $1 \cdot 10^6$ CFU/ml (CFU – colony forming unit) of phenol-degrading bacteria and $3 \cdot 10^8$ CFU/ml of heterotrophic bacteria; and *Rhodococcus sp*., *Pseudomonas sp*. and *Kurthia sp*. were isolated from this sludge. The size of the flocs of activated sludge, taken from the Kohtla-Järve WWTP, was between 21 µm–130 µm, about 64% of the flocs were falling in the range of 41 µm–70 µm (Kullapere, 2004). In Figure 9, a micrograph of an activated sludge floc is shown.



(a)



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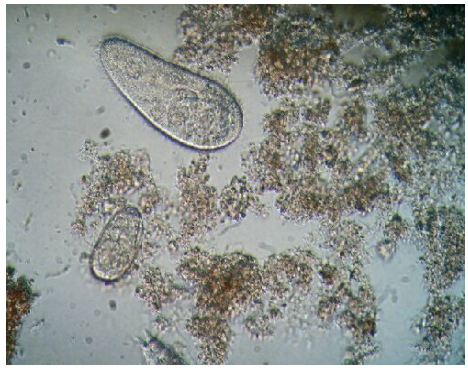

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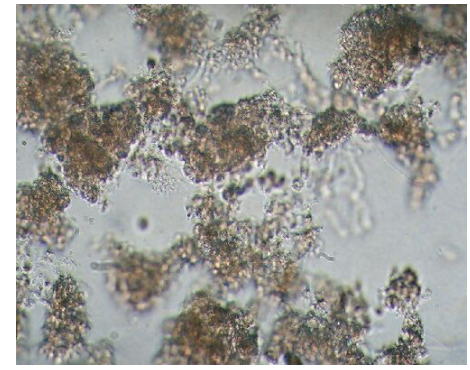

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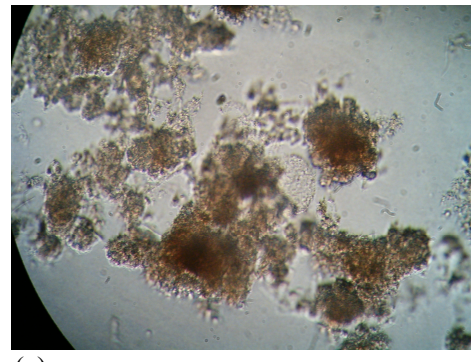

(e)

Figure 9 (a–e). Flocs of activated sludge, taken from the Kohtla-Järve WWTP, observed via microscopy. Image is zoomed 984 times. Courtesy of Marko Kullapere.

4.1.2. Measuring instrument

An amperometric measuring instrument used for monitoring the dissolved oxygen (DO) concentration and measuring the uptake of oxygen by the activated sludge consists of the WTW Inolab Oxi Level 2 oxygen meter with

sensor CellOx 325 (Wissenschaftliche-Technische Werkstätten GmbH, Germany). The DO sensor consists of the electrode system (cathode and anode), the electrolyte solution and the polymeric membrane. The DO sensor is based upon the electrochemical reduction of oxygen on the cathode of the sensor. The following reactions take place on the cathode: $O_2 + 4e^- + H_2O \rightarrow 4OH^-$ and on the anode: $2Cd + 4OH^- \rightarrow 2Cd(OH)_2 + 4e^-$. The output current of the sensor is proportional to the diffusion flux of oxygen through the membrane (which is impermeable to ionic salts) and electrolyte solution to the cathode. Under normal measurement conditions, the amperometric sensor operates in the diffusion-limited mode. Thus, at a constant temperature, the diffusion flux is proportional to the concentration of DO in the measured solution (Jalukse *et al.*, 2004; Jalukse and Leito, 2007). The DO sensor is connected to the DO meter to visualize the DO concentration value (mg/l). The DO sensor has a built-in temperature sensor for measuring the solution temperature, an important parameter for converting the output current of the sensor into oxygen concentration. Instrument has four-digit display (two digits after the decimal point) and measuring range 0–50 mg/l. In operating manual, the stated accuracy in the full temperature range is 0.5% of the measured value at an ambient temperature of 5...30°C. The range for temperature measurement and automatic temperature compensation is –1...40°C. The accuracy of temperature compensation in the whole temperature range is ±2% of the measured DO concentration value. The stated accuracy of temperature measurement is ±0.1 K.

4.1.3. Procedures

Two methods based on the oxygen uptake measurement – the biochemical oxygen demand and the short-term oxygen uptake – were used. The biochemical oxygen demand (BOD) is one of the most important and widely used parameters in the measurement of organic pollution. The conventional standard method for the determination of BOD measures the microorganisms' oxygen consumption over a period of 5 to 7 days, denoted as BOD_5 or BOD_7, respectively (ISO 5815-1:2003). While BOD_7 is a good indicator of the concentration of biodegradable organic pollutants in water, its determination is extremely slow and hence not suitable for the process control and operation in wastewater treatment plants or expeditious determination of the concentration of biodegradable organic pollutants in polluted water (Liu and Mattiasson, 2002; Thevenot *et al.*, 2001). The measurement of the short-term oxygen demand (BOD_{st}) has some advantages over the conventional BOD method due to the simplicity of procedure and the relative quickness with which the results are obtained, and BOD_{st} is determined under conditions imitating the processes in the aeration tank, from which the biomass obtained, in consideration of the ratio of the initial substrate concentration (S_0) to the biomass concentration (X_0).

4.1.3.1. Biochemical Oxygen Demand Measurement

The biochemical oxygen demand (BOD) measurements of individual phenolic compounds by the conventional method (ISO 5815-1:2003; Paper I) were carried out. The biochemical oxygen demand (BOD_5, BOD_7) is defined as the amount of oxygen (mg/l) used by microorganisms at 20°C during the incubation period of 5 to 7 days in the dark to metabolize biologically degradable organic compounds. The unit of BOD_5 and BOD_7 used in this thesis was a mole of O_2 per mole of the substrate.

The test solutions containing an individual phenolic compound at 0.015 mM were prepared with air-saturated distilled water containing a phosphate buffer (pH = 7.2) and mineral nutrients (N, Ca, Mg, Fe, S) for microbial growth. 1 ml of activated sludge suspension was added per 1 l of the medium. A nitrification inhibitor (allylthiourea) at the final concentration of 0.5 mg/l was also added to impede the ammonia oxidation. The 300-ml BOD bottles were incubated at 20°C in the dark to prevent any photochemical reaction. The dissolved oxygen concentration was measured by an oxygen meter at the beginning and after that every day during the incubation period of 7 days. Two aerobic processes are involved in the oxygen decrease in the test bottles – the endogenous oxygen consumption of the microorganisms and the biodegradation of the phenolic compound by the activated sludge. Suitable blank controls were run with each test to measure the endogenous respiration of the microorganisms. Biodegradation was followed by the depletion of oxygen concentration in the test bottles, corrected for the blank value and divided by the expected theoretical oxygen demand (thOD) to calculate the percentage of biodegradation. The thOD is the stoichiometric amount of oxygen (expressed as a mole of O_2 per mole of the substrate) required to oxidize a given organic compound into end products such as carbon dioxide and water (Baker *et al.*, 1999). Oxygen uptake rates (OUR expressed in $mgO_2/l \cdot h$) were also calculated for each substrate.

4.1.3.2. Short-Term Oxygen Uptake Measurement

The respirometric method (Hellat *et al*., 1997; Orupõld *et al*., 2001) adapted from Čech *et al*. (1984) was used for the determination of the short-term oxygen demand (BOD_{st}) and the kinetic parameters characteristic to the biodegradation process. The BOD_{st} is defined as the sum of oxygen demand for oxidation of readily biodegradable organic compounds in the activated sludge suspension (Spanjers *et al.*, 1994; Lukasse *et al.*, 1997).

The 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 the media. The batch system was kept at a temperature of 20°C ± 1°C. The suspension of activated sludge was transferred into the respirometric cell and aerated. When the dissolved oxygen (DO) concentration was 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 oxygen sensor CellOx 325 and WTW InoLab Oxi Level 2 oxygen meter (WTW, Germany). At the beginning of the test a low decrease in oxygen concentration due to heterotrophic endogenous respiration was recorded, after that substrate was added. Addition of a certain amount (0.1–1.0 ml) 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, or slightly different from, the original endogenous rate (Čech *et al*., 1984). To avoid oxygen limitations the experiment was stopped when the oxygen concentration dropped below 1 mg/l. The oxygen uptake rates (OUR) were calculated from the slope of DO decline with time by using linear regression of all the obtained dissolved oxygen data and were corrected by the endogenous oxygen consumption (OUR_{end}) of microorganisms measured in the absence of substrate.

The endogenous respiration is normally assumed to be caused by maintenance of the biomass, including the decay and concomitant growth of bacteria and also oxygen consumption by protozoa. Dircks *et al.* (1999) found that the oxygen uptake rate response from activated sludge due to the addition of a single organic substrate can be divided into two phases. The first phase reflects the primary metabolism of the added substrate, but the OUR curve can have a secondary phase, i.e., the tail phase before the original level of endogenous respiration is reached. In the case of phenolic compounds, this effect might reflect the oxidation of metabolic intermediates such as catechol, 2-hydroxymuconic semialdehyde or *cis,cis*-muconate (Nuhoglu and Yalcin, 2005) or caused by an adsorption or accumulation of the substrate in the flocs (Dircks *et al*., 1999).

Since the applied method uses a low ratio of the initial substrate concentration (S_0) to the biomass concentration (X_0), the minimal changes occur in the degrading community and in the biomass concentration over a short period of the experiment. 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 (Ellis *et al.*, 1996). In kinetic experiments, where the substrate concentration is low relative to the concentration of the degrading organisms, the biomass growth is negligible during parameter estimation and biomass concentration may be assumed to remain constant during the short duration of the experiment (Nakhla and Al-Harazin, 1993; McAvoy *et al.*, 1998). Also in this thesis, the endogenous respiration rate (OUR_{end}) and the concentration of biomass were considered to be constant during the batch respirometric cycle. 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.

4.2. Modelling

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 Figure 10 (Paper II). In the test, the substrate concentration changes continuously, while the reaction rate decreases with a decrease in the substrate concentration and, hence, the OUR also decreases in time.

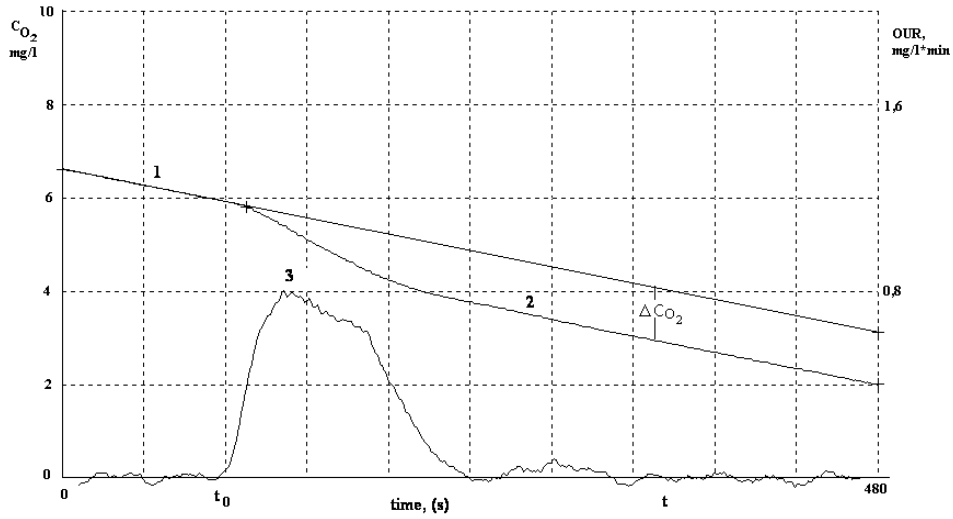

Figure 10. Oxygen concentration profile due to endogenous respiration of activated sludge (curve 1), total respiration after the addition of substrate (curve 2), and calculated respiration rates (OUR, curve 3) in the case of the addition of phenol at concentration of 0.015 mM (Paper II).

The OUR can be calculated from the DO data by measuring temporal oxygen concentration changes in the test system:

$$\mathrm{OUR} = -\frac{dC_{O_2}}{dt} = \frac{C_{1(O_2)} - C_{2(O_2)}}{t_2 - t_1} \ . \tag{11}$$

Total decrease in the DO concentration is determined by the microbial oxygen uptake for both the endogenous ($\mathrm{OUR_{end}}$) and exogenous respiration ($\mathrm{OUR_{ex}}$) (Orupõld *et al.*, 2001):

$$\frac{dC_{O_2}}{dt} = -(\mathrm{OUR_{end}} + \mathrm{OUR_{ex}}) = -\mathrm{OUR_{tot}}. \tag{12}$$

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 total respiration process.

The kinetics of oxygen uptake in substrate degradation processes by activated sludge at a constant temperature depends on three important factors: the concentration of DO, biomass concentration and substrate concentration in the test media. For the description of the degradation of substrate by activated sludge it is rather convenient to use a formulation based on analogy with saturation kinetics in monomolecular adsorption. Most biochemical reactions according to the Monod or the Michaelis-Menten kinetics have been actually modelled by using the above-mentioned saturation kinetics. According to these models, at high substrate concentration the reaction rate is of zero order and proceeds at maximum velocity, while at low substrate concentration the rate becomes first order and concentration-dependent (Palmer, 1995; Hellat *et al.*, 1997; Okpokwasili and Nweke, 2005).

The rate equation, analogous to the Michaelis-Menten kinetics, describing the degradation process of an individual substrate by activated sludge based on the kinetics for oxygen and substrate acting as rate-limiting factors at a constant biomass concentration, can be expressed as follows (Orupõld *et al.*, 2001):

$$\frac{dS}{dt} = -V_{\max} \cdot \frac{S}{K_S + S} \cdot \frac{C_{O_2}}{K_{O_2} + C_{O_2}} \ , \tag{13}$$

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 and K_S is the half-saturation coefficient of substrate and K_{O_2} is the half-saturation coefficient of oxygen. According to the equation (13), the rate of substrate degradation is proportional to the biomass concentration and depends hyperbolically on the concentrations of the substrate and dissolved oxygen. X can be considered as a constant, since in practice, biomass growth due to the substrate degradation is negligible in the system within the time of the short-term experiment.

Oxygen concentration should be maintained above the concentration of 1 mg/l during the experiment to avoid oxygen becoming a limiting factor. If $C_{O_2} >> K_{O_2}$, the equation (13) for the substrate degradation can be expressed as follows (Orupõld *et al.*, 2001):

$$\frac{dS}{dt} = -V_{\max} \cdot \frac{S}{K_S + S} \ . \tag{14}$$

The measured OUR_{ex} is proportional to the rate of substrate degradation:

$$OUR_{ex} = -v \cdot \frac{dS}{dt} \quad , \tag{15}$$

where v is the coefficient that expresses the quantity of oxygen consumed per quantity of substrate in the degradation process. The coefficient v gives an estimate of the short-term oxygen demand (BOD_{st}) caused by the addition of substrate during the test (Orupõld *et al.*, 2001):

$$v = \frac{C_{O_2}(t_0) - C_{O_2}(t) - OUR_{end} \times (t - t_0)}{S_0} = \frac{\Delta C_{O_2}}{S_0} = BOD_{st} \, , \tag{16}$$

where t_0 is the time of the substrate addition, t is the time at $t > t_0$, when $OUR_{tot} = OUR_{end}$ and $C_{O_2}(t)$ is the concentration of dissolved oxygen at time t. The BOD_{st} is the amount of oxygen utilized for the bio-oxidation of the added substrate.

At a constant biomass concentration and under the conditions where oxygen would not be a limiting factor, and 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 $t \geq t_0$ (see Figure 10). OUR_{max} depends on the substrate concentration and the dependence of the values of OUR_{max} on the substrate concentration is investigated. 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 ($V_{O_2,max}$) (Orupõld *et al.*, 2001). OUR_{max} can be related to the initial substrate concentration (S_0) with the aid of equations (14) and (15) as follows (Orupõld *et al.*, 2001):

$$OUR_{max} = v \cdot V_{max} \cdot \frac{S_0}{K_S + S_0} = V_{O_2,max} \cdot \frac{S_0}{K_S + S_0}. \tag{17}$$

In this thesis the degradation of a single substrate system was modelled by the Michaelis-Menten kinetics (equation (17)). However, phenolic compounds may exhibit an inhibitory effect to their own biodegradation. When the substrate inhibition phenomena was observed during the experiments, the Haldane model, as represented in equation (18), was applied (Palmer, 1995):

$$OUR_{max} = V_{O_2,max} \cdot \frac{S}{K_S + S + \frac{S^2}{K_i}} , \tag{18}$$

where K_i is the inhibition coefficient of substrate.

The kinetic parameters, such as the maximum rate of oxygen uptake ($V_{O_2,max}$), the maximum rate of substrate degradation (V_{max}), the half-saturation coefficient (K_S) and the inhibition coefficient (K_i) were determined from the dependence of the values of OUR_{max} on the substrate concentrations by non-linear regression using the method of the least squares.

Concerning the bi-substrate systems, the Michaelis-Menten kinetics (equation (17)), the random-order mechanism (equation (3)) and, to take into account the possible inhibition effect, the Haldane model (equation (18)) were applied. For a binary mixture, the no-interaction sum kinetics model could be described based on Reardon *et al.* (2000) as:

$$OUR_{max} = \frac{V_{O_2,max,1} \cdot S_1}{K_{S,1} + S_1} + \frac{V_{O_2,max,2} \cdot S_2}{K_{S,2} + S_2}, \quad (19)$$

where the subscripts 1 and 2 denotes the parameter for each of the two substrates.

However, there are interactions between these substrates which cannot be described by additive sum kinetics models using only parameters determined in single substrate experiments. For the substrate mixtures (using the values of $V_{O_2,max,i}$, $K_{S,i}$, and $K_{i,i}$ determined from single-substrate experiments), the sum kinetic models on an unspecified type of interaction were used to predict the consumption of the two substrates and to estimate the interaction parameters. Interaction parameters ($I_{1,2}$; $I_{2,1}$) were determined by applying the sum kinetic models' analogues to the equations (9) and (10) wherein the specific growth rate μ and the specific substrate degradation rate q in the original equations were replaced with the maximum oxygen uptake rate OUR_{max}, as represented in the equations (20) and (21) (Paper II; III):

$$OUR_{max} = \frac{V_{O_2 max,1} \cdot S_1}{K_{S,1} + S_1 + I_{2,1}S_2} + \frac{V_{O_2 max,2} \cdot S_2}{K_{S,2} + S_2 + I_{1,2}S_1}, \quad (20)$$

$$OUR_{max} = \frac{V_{O_2 max,1} \cdot S_1}{K_{S,1} + S_1 + \frac{S_1^2}{K_{i,1}} + I_{2,1}S_2} + \frac{V_{O_2 max,2} \cdot S_2}{K_{S,2} + S_2 + \frac{S_2^2}{K_{i,2}} + I_{1,2}S_1}, \quad (21)$$

where the interaction parameter $I_{1,2}$ indicates the degree to which substrate 1 affects the biodegradation of substrate 2. If one component of mixture showed an inhibition effect in the degradation as a single substrate and another did not, the combination of the equations (20) and (21) was used to take into account the inhibition effect, as described by the equation (22):

$$\mathrm{OUR}_{\max} = \frac{V_{O_2\max,1} \cdot S_1}{K_{S,1} + S_1 + \frac{S_1^2}{K_{i,1}} + I_{2,1}S_2} + \frac{V_{O_2\max,2} \cdot S_2}{K_{S,2} + S_2 + I_{1,2}S_1}. \quad (22)$$

The validity and suitability of the models used to describe the biodegradation kinetics was assessed by comparing the theoretical curves obtained with the corresponding experimental data. The best fitting values of kinetic parameters were determined from the experimental data using a non-linear least squares regression method by minimizing the sum of the squares of the differences (residuals) between the measured (y_i) and the estimated values ($\hat{y}_i$) of the $\mathrm{OUR}_{\max}$ from the regression equation for the entire portion of the response. The standard deviation of regression ($s_{y/x}$) is considered to be the best statistical quantity that measures how well a regression equation fits measured data: $s_{y/x} = \sqrt{(\sum_{i=1}^{i=n}(y_i - \hat{y}_i)^2)/df}$, where ($y_i$ - $\hat{y}_i$) is the residual of the point *i* and *df* is the degree of freedom. The better the fit, the smaller is $s_{y/x}$. If the data fit the model equation perfectly then $y_i = \hat{y}_i$, 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 estimated. R^2 is the fraction of the variance in the dependent variable explained by the relationship with the independent variable. A value of R^2 is equal to 1, if all variance explained by the model. The residuals against the initial concentrations of substrate were plotted to allow a good visual assessment of the quality of the fit for the models that may still give a high R^2 or low $s_{y/x}$. A well-behaved model has residuals randomly distributed about zero (Hibbert and Gooding, 2006).

4.3. Results and Discussion

In this thesis the aerobic biodegradability of phenol, resorcinol, 5-methyl-resorcinol, *o*-cresol, *p*-cresol, and 2,4-, 2,6-, 3,4- and 3,5-dimethylphenols by the activated sludge from the Kohtla-Järve WWTP was investigated. The bio-oxidation of the compounds both as single and mixed substrates at different initial concentrations was studied by respirometry. The biodegradability was assessed using the short-term (BOD_{st}) as well as biochemical oxygen demands (BOD_5, BOD_7), OUR and the kinetic parameters estimated from the respirometric data. As the substrates differed in the theoretical oxygen demand (thOD), the average amount of oxygen consumed per unit of thOD was also calculated. The thOD is the stoichiometric amount of oxygen (expressed as mole of O_2 per mole of the substrate) required for the mineralization of a specific organic compound into the end products such as carbon dioxide and water (Baker *et al.*, 1999).

The factors influencing the degradation rate are difficult to quantify and control in degradation experiments on mixed substrate with an undefined

adapted mixed population taken from a wastewater treatment plant. Hereafter, the results and the variations in the measured parameters from the experiment series run over a period of seven years are discussed. The uncertainties of the measurements were estimated 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 (ISO/IEC Guide 99:2007).

4.3.1. Biodegradation of phenolic compounds as single substrates

The measurement of the biochemical oxygen demand (BOD) of an individual phenolic compound (phenol, resorcinol, 5-methylresorcinol, *o*-cresol, *p*-cresol, and dimethylphenols) at 0.0075 mM and at 0.015 mM was carried out according to the international standard method for BOD_n measurement (ISO 5815-1:2003). Biodegradation was followed by determining the O_2 concentrations every day during the incubation period of 7 days. Depletion of the oxygen concentration in the test with the studied substances was corrected with the blank value and divided by the expected thOD to calculate the percentage of biodegradation. Because of the depletion of the carbon source, the BOD reaches a plateau (Figure 11).

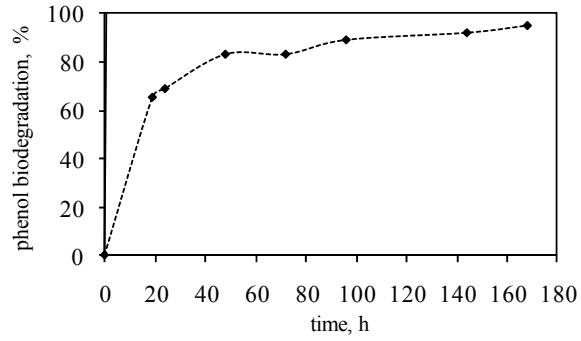

Figure 11. Dependence of the degree (%) of phenol biodegradation on time. Experimental data is gained at a phenol concentration of 0.0075 mM.

The results obtained from the conventional BOD measurements are given in Table 1. BOD_7 values formed 71%–90% of the thOD of the studied phenolic compounds, except in the case of 2,6-dimethylphenol where the BOD_7/thOD ratio did not exceed 25%. The values of the ratio BOD_5/thOD were relatively similar to the respective values of the BOD_7/thOD. The high values of the

measurement uncertainties in Table 1 indicate a relatively high variation in these parameters between the test series. It can be explained by the differences in microbial community of activated sludge in time (Paper I). The obtained BOD_5/thOD values for phenol, *o*- and *p*-cresol and 3,4-dimethylphenol were in the range of those published in the literature (Verschueren, 1983), but in the case of resorcinol, the value obtained in this thesis was somewhat higher. Jiang *et al.* (2002) classified different organic compounds based on the integrated assessment factor $IO = (BOD_5/COD) \times 100$ as readily (IO>45), partially (IO=30–45) and poorly (IO<30) biodegradable organic substances. The inoculum was an activated sludge taken from a wastewater treatment plant. Based on these assessment results, it was concluded that phenol is readily (IO=76) and *o*-cresol is partially (IO=44) biodegradable (Jiang *et al*., 2002). Taking into account that for phenolic compounds the values of COD are approximately equal to the values of thOD of substrates expressed as $COD = 0.98 \times thOD$ (Baker *et al.*, 1999), the results of this thesis, shown in Table 1, indicated that all studied phenolic compounds could be classified as readily biodegradable substances, but 2,6-dimethylphenol is poorly biodegradable.

Table 1. The mean values of the ratios of biochemical oxygen demand (BOD_5, BOD_7) to theoretical oxygen demand (thOD) with the uncertainties of measurement in the case of the phenolic compounds studied (Paper I).

Substrate	thOD mol O_2/mol	BOD_5/thOD	BOD_7/thOD
phenol	7.0	0.76 ± 0.11 (n = 11)	0.76 ± 0.12 (n = 11)
o-cresol	8.5	0.68 ± 0.10 (n = 11)	0.74 ± 0.10 (n = 11)
p-cresol	8.5	0.77 ± 0.08 (n = 11)	0.81 ± 0.08 (n = 11)
resorcinol	6.5	0.78 ± 0.10 (n = 10)	0.84 ± 0.09 (n = 10)
5-methylresorcinol	8.0	0.75 ± 0.09 (n = 11)	0.79 ± 0.09 (n = 11)
3,4-dimethylphenol	10.0	0.67 ± 0.17 (n = 10)	0.71 ± 0.13 (n = 10)
3,5-dimethylphenol	10.0	0.57 ± 0.15 (n = 3)	0.82 ± 0.65 (n = 3)
2,4-dimethylphenol	10.0	0.74 ± 0.09 (n = 3)	0.90 ± 0.25 (n = 3)
2,6-dimethylphenol	10.0	0.22 ± 0.22 (n = 3)	0.25 ± 0.18 (n = 3)

n – the number of experimental series

When the oxygen demand of the substrate in the conventional BOD_n measurements was studied as a function of time, it was found that oxygen uptake proceeded rapidly within the first two or three days and then slowed down (Figure 12). The maximum OUR (expressed in mg O_2/l·h) was achieved within 50 hours, varying in different tests and showing higher values for phenol and *p*-cresol. In the case of *o*-cresol and dimethylphenols, the maximum values were achieved by the 74th hour, and a lag period of approximately one day was observed before the oxidation occurred (Paper I). For the biodegradation of *o*-

cresol a 1-day lag period has also been reported in the literature (Verschueren, 1983).

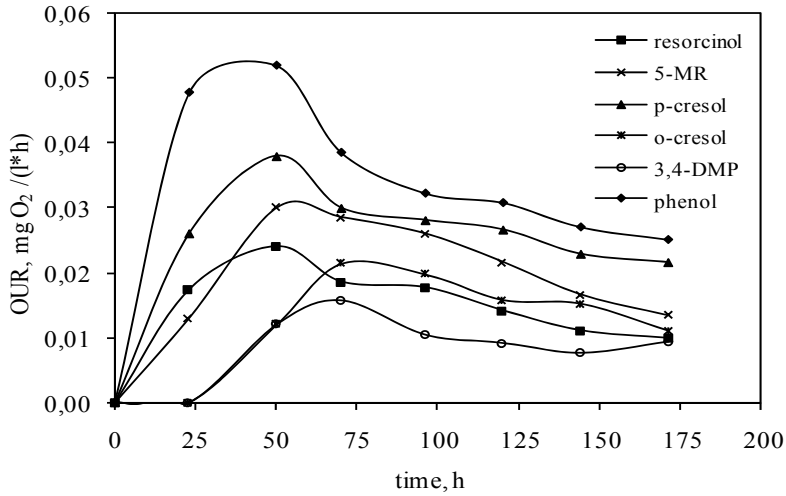

Figure 12. OUR values as a function of time for different phenolic compounds: MR – methylresorcinol and DMP – dimethylphenols (Paper I).

In the case of phenol, resorcinol, 5-methylresorcinol, *o*- and *p*-cresol, the dependence of the BOD_5 and BOD_7 values on the initial concentration of substrate was investigated at the concentrations of 0.0075 mM and 0.015 mM. Comparing the values of the ratio BOD_5/thOD and BOD_7/thOD at both initial concentrations and taking into account the uncertainties of measurement, it could not be concluded that there were any significant variation in dependences of the BOD_5 and BOD_7 values on the initial concentrations of substrates studied in the experiments. However, in the case of all studied compounds the values of the oxygen uptake rate at given time-moment at the concentration of 0.0075 mM were slightly lower than those for 0.015 mM. Concerning the biodegradation of *o*-cresol and 5-methylresorcinol at 0.0075 mM a 1-day lag period was observed more frequently than at a concentration of 0.015 mM.

To estimate the biodegradability of individual phenolic compounds, the short-term oxygen uptake measurement was also used. From the measured data the oxygen uptake rates (OUR) as a function of time and the oxygen demand caused by substrate degradation (ΔC_{O_2}) were calculated (see Figure 10). The ΔC_{O_2} gives an estimate of the short-term oxygen demand (BOD_{st}) caused by the addition of a substrate during the test. It was possible to calculate the ΔC_{O_2} values only if the oxygen uptake rate was approximately equal to the endogenous oxygen uptake rate at the end of the experiment (Paper III). If the concentration of substrate is too high, the oxygen uptake rates decrease due to the oxygen limitation, and the determination of the ΔC_{O_2} values does not give

the desired information (Orupõld *et al.*, 2001). The linear dependences of the measured ΔC_{O_2} values on the added substrate concentrations in short-term tests for different compounds are presented in Figure 13.

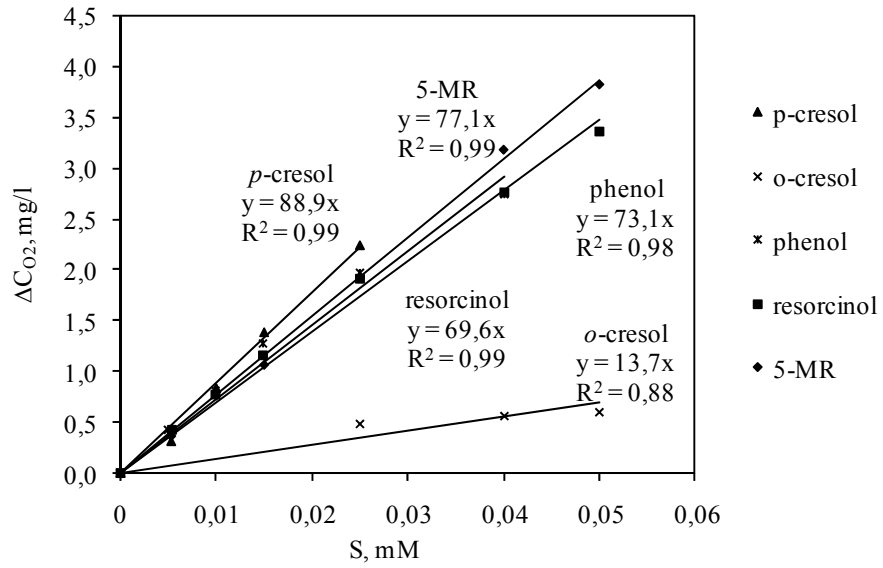

Figure 13. The dependence of the measured ΔC_{O_2} values on added substrate concentrations (*S*) for different studied compounds. The slopes of the figure's lines characterize the average amount of oxygen in mg utilized per mmole of the substrate. 5-MR – 5-methylresorcinol (Paper II; III).

It was shown that the activated sludge from the Kohtla-Järve WWTP was able to oxidize all phenolic compounds tested, but differences existed in the maximum oxygen uptake rates as well as in the amounts of oxygen consumed per mole of substrate. The results of the short-term oxygen demand measurement showed that microorganisms in the activated sludge quickly consume resorcinol, phenol, *p*-cresol, and 5-methylresorcinol. The BOD_{st} values of these compounds were 21%–28% of the theoretical oxygen demand (thOD), showing the highest value for phenol (Paper I; II). It is worth to mention that in the earlier period of study (Paper I) about 25%–43% of thOD was consumed by activated sludge during the short-term measurements, showing the highest value for resorcinol. In the case of *o*-cresol the BOD_{st}/thOD value was only 10%–16%, because the activation and induction of enzymes required for degradation of *o*-cresol could take longer time than for other studied phenolic compounds. Mean values of the ratio of BOD_{st} to thOD with accompanying uncertainties of measurement are summarized in Table 2 (Paper II; III). The results indicated a relatively high variation in these parameters between the series of test over the period of study. When phenol was used as a substrate, approximately 2.0 mole of oxygen were consumed per mole of phenol supplied, but some experiments indicated the uptake of 1.5 or 3.0 mole

of oxygen per mole of phenol (Papers I–III). The differences in the BOD_{st} values obtained with various activated sludge samples might be caused by variations in the concentration and the properties of the influents to the WWTP and in the operating conditions of wastewater treatment, which could influence the properties of activated sludge including biomass concentration, the composition and the metabolic potential of the microbial community, thereby affecting the rate and extent of biodegradation (Paper II; III). The variations in the inoculum quality and quantity can be critical for efficiency and reproducibility of test results and variability of estimated parameters are expected when activated sludge is used as a source of inoculum (Vazquez-Rodriguez *et al.*, 1999 and 2003). The BOD_{st}/thOD values achieved in this thesis for resorcinol, 5-methylresorcinol and phenol were compared and correlate well with the results obtained by Orupõld *et al*. (2001) from the activated sludge of the Kohtla-Järve WWTP formerly.

Table 2. The mean values of the ratio of short-term oxygen demand (BOD_{st}) to theoretical oxygen demand (thOD) and the maximum oxygen uptake rate (OUR_{max}) at 0.015 mM with the uncertainties of measurement for studied phenolic compounds (Paper II; III).

Substrate	BOD_{st}/thOD	OUR_{max}, mg O_2/(mmol·min·MLSS)
phenol	0.28 ± 0.03 (n = 29)	7.27 ± 1.26 (n = 29)
o-cresol	0.10 ± 0.04 (n = 9)	1.93 ± 0.50 (n = 9)
p-cresol	0.24 ± 0.04 (n = 9)	6.09 ± 2.42 (n = 9)
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

The short-term oxygen demands formed 26%–50% of the BOD_7 of different phenolic compounds except in the case of *o*-cresol where the BOD_{st}/BOD_7 value was 13%–22%. The ratio of BOD_{st}/BOD_7 illustrates how big part of the BOD of a certain compound was consumed by the activated sludge in the short-term.

The best-degrading dimethylphenol was 3,4-dimethylphenol, in which case the measured short-term oxygen demand was up 16% from the thOD and the BOD_{st}/BOD_7 value was 22% (Paper I). 3,5-, 2,4- and 2,6-dimethylphenols did not degrade as single substrates in the time-frame of the short-term BOD measurement. The activation and induction of enzymes required for degradation of these phenols could take a longer time than the duration of the short-term oxygen demand measurement. However, the results of the BOD_5 and BOD_7 measurement show a long-term degradation of dimethylphenols. While resistant to the biodegradation as single substrates, dimethylphenols may degrade in mixture with other phenols or aromatic compounds, as enzymes can be induced by the more readily degradable substrates (Paper I). The results of short- and long-term measurements showed that 2,6-dimethylphenol was the worst-degrading phenolic

compound investigated in this thesis. The ring cleavage retardation of that compound may be explained by the blocked hydroxylation step because both of the two methyl groups are in the *ortho* position relative to the hydroxyl group and another hydroxyl group cannot be inserted next to the initial hydroxyl group (Paper I). It is known that in the case of *ortho*-methylsubstituted phenols, hydroxylation is directed at the position of 6; both 2- and 6-hydroxylation seem to be possible in *meta*- and *para*-substituted phenols such as 3,4-dimethylphenol (Pieper *et al.*, 1995).

To assess the biodegradability of phenolic compounds, the maximum OUR corresponding to the initial rate of degradation reaction for a certain amount of substrate was also used. Since OUR_{max} depends on the biomass concentration, it was estimated per dry weight of suspended solids (MLSS) of activated sludge. The OUR_{max} values for various substrates were compared at the concentration of 0.015 mM, data is shown in Table 2. Among the studied substrates, phenol had the highest value of OUR_{max}. OUR_{max} values for *p*-cresol, 5-methylresorcinol and resorcinol exceeded those for *o*-cresol and dimethylphenols. 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 (MLSS, 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 (Papers I–III).

It should be noted that the mean value of OUR_{max} at 0.015 mM was three times higher for *p*-cresol compared with that for *o*-cresol. The curves of the OUR obtained, resulting from the addition of the same amount of *p*-cresol and *o*-cresol, are illustrated in Paper I (see Figure 7a and 7b in Paper I). The shapes of the OUR curves demonstrate the differences in the substrate degradation processes and in the rates of degradation of *o*- and *p*-cresol. The value of the BOD_{st}/thOD ratio of *o*-cresol was ca 2 times lower compared to that of *p*-cresol (see Table 2). The reason could be that in activated sludge containing a mixture of different microorganisms, which have a wider spectrum of metabolic properties, the different catabolic pathways may be induced and the degradation of *p*-cresol may occur through two distinct catabolic pathways at the same time (Paper I). In one case the methyl group of *p*-cresol is oxidized by the *p*-cresol methylhydroxylase (PCMH) to a corresponding carboxyl group (Dagley and Patel, 1957; Hopper and Taylor, 1977; Cunane *et al*., 2000). The following ring-cleavage of protocatechuate can occur via both *ortho* and *meta* pathways (Müller *et al.*, 1996a; Heinaru *et al.*, 2001; Viggor *et al.*, 2002). In the other route, the methyl group of *p*-cresol remains intact and 4-methylcatechol is formed, which is further catabolized via catechol *meta* and/or *ortho* pathways (Ampe and Lindley, 1996; Kolomytseva *et al.*, 2007). But with respect to the *ortho* cleavage of methylcatechols, methylmuconolactones are usually formed as dead-end products (Knackmuss *et al.*, 1976). The degradation of *o*-cresol occurs via the *meta* cleavage of 3-methylcatechol (Bayly *et al*., 1966).

More specific respirometric analyses were carried out with five substrates, i.e. phenol, resorcinol, 5-methylresorcinol, and *o*- and *p*-cresol at initial

concentration of 0.005 mM, 0.01 mM, 0.015 mM, 0.025 mM, 0.04 mM and 0.05 mM. The dependence of the OUR_{max} values on substrate concentrations was investigated (shown in Figure 14) and modelled by the Michaelis-Menten kinetics (equation (17)); when the inhibition effect was observed, the Haldane model (equation (18)) was used. The best fit parameters were determined using a non-linear least squares regression method by minimizing the sum of the square of the residuals between the measured and predicted values of OUR_{max} for the entire portion of the response. The statistical parameters such as the standard deviation of regression ($s_{y/x}$) and the coefficient of determination (R^2) were calculated for the data sets to indicate the goodness of fit of model. Models' suitability was confirmed also by the residuals plots. The values of the parameters associated with the minimum $s_{y/x}$ and the maximum R^2 constituted their best estimates. If the data fit the regression equation perfectly then $s_{y/x} = 0$ and $R^2 = 1$. Low values of $s_{y/x}$ and the near unity values of the coefficient of regression indicated that the models' predictions agreed reasonably well with the experimental data and the kinetics models used were able to properly interpret the experimental results in the activated sludge medium.

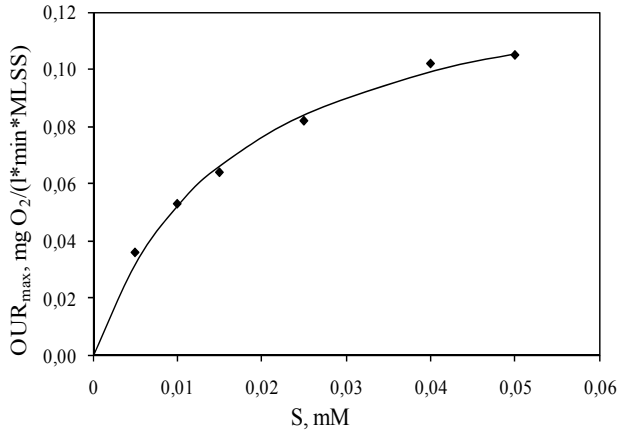

Figure 14. The relationship between the OUR_{max} values and added 5-methylresorcinol concentrations. The symbols correspond to the measured data, whereas the solid line corresponds to the Michaelis-Menten kinetics.

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. The results are summarized in Tables 3 and 4.

Table 3. The mean values of estimated kinetic parameters $V_{O_2,max}$ and V_{max} with the uncertainties of measurements (u_c), describing the biodegradation for different substrates (Paper II; III).

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
o-cresol	0.068 ± 0.027	2.4 ± 0.5	1.3 – 2.7
p-cresol	0.134 ± 0.050	2.1 ± 0.7	0.8 – 4.2
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 4. The mean values of the half-saturation coefficient K_S and the ratio of $V_{O_2,max}/K_S$ with the uncertainties of measurements (u_c), describing the biodegradation for different substrates (Paper II; III).

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
o-cresol	7.5 ± 3.0	2.7 – 12.0	13.3 ± 6.4
p-cresol	1.5 ± 0.6	1.0 – 3.5	99.1 ± 34.9
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 and *p*-cresol had the highest values of $V_{O_2,max}$, followed by 5-methylresorcinol (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, thus, it cannot be increased by using still higher substrate concentrations (Palmer, 1995). 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 (Palmer, 1995). As oxygen uptake profiles yield the same information as substrate depletion profiles owing to the stoichiometric link between these two processes (Riefler *et al.*, 1998), it was also possible to estimate V_{max} by dividing the $V_{O_2,max}$ values by the stoichiometric coefficient v (equation (17)). Coefficient v expresses the quantity of oxygen consumed per quantity of substrate in the degradation process. 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 in the earlier period of study (Table 3 in Paper I). While $V_{O_2,max}$ varies with the total concentration of enzymes present in biomass, K_S is independent of enzyme concentration and is characteristic to the system being investigated. K_S is the value of S_0 at which the specific oxygen uptake as an initial rate is equal to the half of $V_{O_2,max}$. 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 (Palmer, 1995). $V_{O_2,max}$ and K_S are influenced by temperature, type of carbon source and other factors (Bitton, 1994). Considering that K_S is related to the affinity of the microbial community for specific substrate, the diminution of K_S corresponds to an increase of affinity of the bacterial ensemble. The activated sludge had the highest affinity to *p*-cresol and phenol, but the lowest affinity to 5-MR. The mean values of K_S for these compounds were 1.5 μM, 3.7 μM and 12 μM, respectively (Paper II; III). The small magnitude of K_S values indicates that for microbial species utilizing *p*-cresol and phenol, the maximum reaction rate could be reached quickly, if substrate inhibition has not been a factor. 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 *p*-cresol with the average value of 99.1 mg O_2/(mmol·min·MLSS), followed by phenol. The results are given in Table 4. The ratio of $V_{O_2,max}/K_S$ refers to specific affinity and was proposed by Healey (1980) as a more suitable parameter that should reflect both the affinity and catalytic activity.

The K_S values for phenol from this study are in agreement with the findings of earlier studies using mixed microbial cultures (Čech *et al*., 1984, Brown *et al*., 1990; Magbanua *et al.*, 1994). However, comparison of the achieved K_S values for phenol with the data reported by authors other than the above shows that some studies have revealed K_S values to be higher (Nakhla and Al-Harazin, 1993; Páca and Martius, 1996) and some lower (Ellis *et al.*, 1996; Watanabe *et al.*, 1996) than those obtained in this thesis. The K_S values ranging from 8.3 μM to 16.9 μM for phenol, corresponding to an adapted activated sludge on phenolic compounds (Orupõld *et al.*, 2001), and ranging from 6.2 μM to 13.3 μM for phenol uptake rate by *P. putida* (Sokol, 1987) were similar or slightly higher to the values obtained in this study. The value of K_S for both phenol and *p*-cresol was found to be less than 1.0 mg/l (10.6 μM) by Hutchinson and Robinson (1988). It has been reported in literature (Nakhla and Al-Harazin, 1993) that the rate of *o*-cresol biodegradation was lower than that of phenol; average values of the parameters clearly emphasized that phenol (K_S for phenol ranging from 5.9 to 18 mg/l) was more readily biodegradable than *o*-cresol (K_S for *o*-cresol ranging from 9.3 to 40.7 mg/l). A similar trend was observed by Brown *et al.* (1990), who reported higher growth rate for phenol compared to the substituted phenolic compounds. Despite its slower rate of biodegradability, *o*-cresol offered more energy to the microbes than phenol as attested by its higher yield coefficient (Nakhla and Al-Harazin, 1993). The K_S values for 5-methylresorcinol and resorcinol reported in this thesis were similar or slightly lower, respectively, than those reported by Orupõld *et al.* (2001) and Ohta *et al.* (1975). It should be noted that the K_S values obtained using techniques other than respirometry, such as phenol consumption or biomass production, are higher. The differences between the coefficients obtained using respirometry and batch growth techniques 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 of phenol (Ellis *et al*., 2006).

The results of this thesis for various phenolic compounds gained with the same activated sludge (Figure 5 in Paper I) suggested that considerable differences exist in their metabolism. The time-dependent variations of the dependence of OUR_{max} values on added substrate concentrations in the case of the studied compounds are also illustrated in Figure 15a and 15b (Paper II; III) and in Figure 6 of Paper I. 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 microorganisms of the activated sludge samples taken at different times. The specific endogenous OUR expressed in mg of O_2 consumed per hour per gram of suspended solids of the sludge varied between 2.1 and 11.3 $mgO_2/g{\cdot}h$. Variations in the composition of the influents to the WWTP and seasonal changes in activated sludge processes can influence the microbial community and the metabolic potential of the biomass and thereby causing the time-dependent differences in the estimated kinetic parameters.

As shown in Figure 15a, at the concentration of 0.025 mM the OUR_{max} value for *p*-cresol was approximately 5.5 times higher than that of *o*-cresol. The possible reason could be that in activated sludge containing a mixture of different microorganisms, which have a wider spectrum of metabolic properties, the different catabolic pathways may be activated and the degradation of *p*-cresol may occur through two distinct catabolic pathways at the same time (Paper III). The bacterial degradation of methyl-substituted phenols starts with the oxidation of a methyl substituent or with the hydroxylation of aromatic ring by adding a second hydroxyl group in the *ortho* position to the one already present. In the case of *ortho*-methylsubstituted phenols, hydroxylation is directed at the position of 6; both 2- and 6-hydroxylation is possible in *meta*- and *para*-substituted phenols (Pieper *et al.*, 1995). The aerobic biodegradation of *p*-cresol may occur via *meta* and/or *ortho* cleavage of 4-methylcatechol or via *ortho* and/or *meta* cleavage of protocatechuate (Dagley and Patel, 1957; Hopper and Taylor, 1977; van der Meer, 1997). The degradation of *o*-cresol occurs via a *meta* pathway of 3-methylcatechol (Bayly *et al*., 1966).

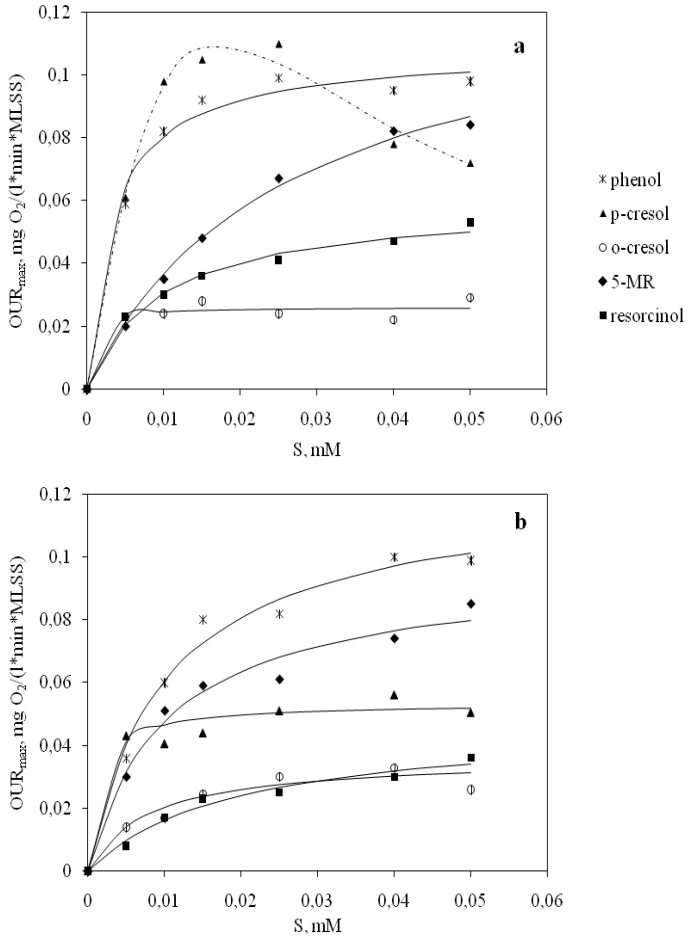

Figure 15. The relationship between the OUR_{max} values and added substrate concentrations (*S*) for different phenolic compounds. The symbols correspond to the measured data, whereas the solid line corresponds to the Michaelis-Menten kinetics and the dotted line to the Haldane kinetics. MLSS of specific activated sludge was (a) 6.9 g/l and (b) 6.5 g/l. The samples were taken at the time-interval of 4 months (Paper II; III).

The value of OUR_{max} for *p*-cresol at a substrate concentration of 0.025 mM in Figure 15a compared to the OUR_{max} value at the same concentration in Figure 15b is approximately 2 times higher. Moreover, the Michaelis-Menten model did not adequately describe the kinetic behaviour of the biodegradation process of *p*-cresol. The maximum value of the OUR_{max} was achieved at a concentration range of 0.015 mM–0.025 mM for *p*-cresol, but at higher concentrations the OUR_{max} values tended to decrease (shown in Figure 15a). The deviation of the dependence of the OUR_{max} on a substrate concentration from the hyperbolic function at higher substrate concentrations can probably be explained by the

substrate inhibition effect. A similar tendency in some tests was also observed for phenol (Paper III). In some instances the observations may be explained on the basis of interaction between the detecting system and excess substrate, but in other cases it appears that the substrate really can inhibit its own conversion to product. In general, it can be seen that substrate inhibition occurs when a molecule of substrate binds to one site on the enzyme and then another molecule of substrate binds to a separate site on the enzyme to form a dead-end complex. This can be regarded as a form of uncompetitive inhibition, the extra substrate molecule being the inhibitor (Palmer, 1995). If the inhibition effect occurred in this thesis, the Haldane kinetics equation (18) was used to describe the dependence $OUR_{max} = f(S)$, and the inhibition coefficient (K_i) was determined. The value of K_i is equal to the concentration of substrate which causes a decrease in reaction rate to one half of the maximum reaction rate ($V_{O_2,max}$). K_i characterizes a loss of the cell ability to oxidize a substrate due to its inhibitory effect. Inhibition concentration is in fact not a constant but is closely related to cultivation conditions (Grady *et al.*, 1999). The degree of inhibition was determined also by the K_S/K_i ratio.

In this thesis the obtained values of K_i for phenol varied widely and were in the range of 7 µM–69 µM with the mean value being 40 µM. The values of the ratio of K_S/K_i were between 0.02–5.85 with a mean value of 1.8. In the case of *p*-cresol the values of K_i were in the range of 22 µM–123 µM with a mean value of 65 µM. The values of K_S/K_i were between 0.03–0.87 with the mean value being 0.37 (Paper III). The larger is the K_S/K_i, the greater the degree of inhibition (Grady *et al*., 1999). Inhibition by phenol depends on a toxic effect, which would result from the formation of chemical bonds between phenol and cellular components (Edwards, 1970) and it has been suggested that the inhibitory effect of phenol is most probably caused by a biochemical phenomenon modifying the *in vivo* enzyme activity (Léonard and Lindley, 1999).

The examination of the values of kinetic parameters showed that the uncertainty of measurement associated with K_S was larger than the uncertainty of measurement associated with $V_{O_2,max}$. This was because the respiration rate associated with a high substrate concentration was close to the maximum, making $V_{O_2,max}$ well defined. K_S, on the other hand, was determined in the region of a low substrate concentration. In general, all three parameters ($V_{O_2,max}$, K_S and K_i) were considered to be accurately assessed at a S_0/K_i ratio above 0.5 and for a S_0/K_S ratio of 1.0, and as the S_0/K_S ratio increased the accuracy of the parameters' estimates was enhanced (Ellis *et al*., 1996). Ellis *et al*. (1996) and McAvoy *et al.* (1998) have pointed out that the estimation of the microbial numbers can be a major source of variation in determining the kinetic parameters. According to Orupõld *et al*. (2001) and Contreras *et al.* (2008), the dry weight of suspended solids (MLSS) have also been used for estimation of the amount of biomass in this thesis; consequently, the obtained results may underestimate the values of the specific oxygen uptake and substrate degra-

dation rate because of the overestimation of the phenol-consuming bacteria that were present in the tested activated sludge samples.

It is difficult to order the studied phenolic compounds with respect to their biodegradability. It appears that phenolic compounds can be ranked based on the values of their different parameters as follows:

BOD_7/thOD: resorcinol > 5-methylresorcinol > phenol > *p*-cresol > *o*-cresol, 3,4-dimethylphenol > 2,4-dimethylphenol > 3,5-dimethylphenol > 2,3-dimethylphenol > 2,6-dimethylphenol;

BOD_{st}/BOD_7: phenol > resorcinol > *p*-cresol > 5-methylresorcinol > *o*-cresol, 3,4-dimethylphenol;

BOD_{st}/thOD: phenol > *p*-cresol > resorcinol, 5-methylresorcinol > *o*-cresol, 3,4-dimethylphenol;

OUR_{max} at 0.015 mM: phenol > *p*-cresol > resorcinol, 5-methylresorcinol > *o*-cresol, 3,4-dimethylphenol;

$V_{O_2,max}$: phenol > *p*-cresol > 5-methylresorcinol > resorcinol > *o*-cresol;

K_S: *p*-cresol > phenol > resorcinol, *o*-cresol > 5-methylresorcinol.

4.3.2. Biodegradation of phenolic compounds as mixed substrates

The biodegradability of 5 different bi-substrate systems of phenolic compounds was also studied: phenol – *p*-cresol, phenol – resorcinol, phenol – 5-methylresorcinol, phenol – *o*-cresol and resorcinol – 5-methylresorcinol. Each contained both components at equal concentrations from 0.005 mM to 0.05 mM (hereafter referred to also as (1:1)).

The Michaelis-Menten kinetics (equation (17)), the Haldane model (equation (18)), and the random-order mechanism (equation (3)) were used to model the experimental data. To ascertain the role played by an interaction between two substrates, the interaction parameters ($I_{1,2}$; $I_{2,1}$) were determined using the sum kinetic models of the unspecified type of interaction (equations (20)–(22)). The interaction parameter $I_{1,2}$ represents the effect of substrate 1 on the degradation of substrate 2. A higher value of the interaction parameter indicates a stronger inhibition on the substrate uptake by the microorganisms (Yoon *et al.*, 1977).

The kinetic parameter $V_{O_2,max}$ estimated by the different simulation models are summarized in Table 5 (Paper II; III). Based on the equation (19), it was also assumed that, if no-interactions between substrates occur, the maximum oxygen uptake rate at a given concentration (OUR_{max}) and $V_{O_2,max}$ for the bi-substrate system is an additive sum of the corresponding parameters of individual substrates. This assumption was proven to be correct only for the mixtures of phenol – 5-methylresorcinol (94% from the additive sum) and resorcinol – 5-methylresorcinol (95% from the additive sum) when the Haldane model was used. It could be explained by that these substrates were degraded by the different enzymes via different metabolic pathways. On the other hand the Haldane model could be taking into account a possible substrate inhibition effect at a higher concentration even though the dependence $OUR_{max} = f(S)$ was

described by the hyperbolic curve (Paper II). The mean values of actual $V_{O_2,max}$ formed 57%–89% of the additive sum of the $V_{O_2,max}$ values of the individual components for the systems of phenol – resorcinol, phenol – *o*-cresol and phenol – *p*-cresol, and resorcinol – 5-methylresorcinol. In the cases when the Michaelis-Menten kinetics or random-order mechanism was used the mean values of $V_{O_2,max}$ for all bi-substrate (1:1) systems were in the range of 40%–88% of the assumed additive values. The values of the interaction parameters $I_{1,2}$ and $I_{2,1}$ determined for the studied bi-substrate systems containing both components at equal concentrations are given in Table 6 (Paper II; III).

In the case of the bi-substrate system containing resorcinol – 5-methylresorcinol, 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 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 value. This finding is up to expectation because it has been shown that the degradation of resorcinol and 5-methylresorcinol in bacteria follows different metabolic pathways; see Figures 7 and 8 (Chapman and Ribbons, 1976a and 1976b; Ohta *et al*., 1975; Ohta and Ribbons, 1976). However, the obtained interaction parameters indicated that 5-methylresorcinol had a greater effect of inhibition on the resorcinol biodegradation than resorcinol had on the biodegradation of 5-methylresorcinol. Chapman and Ribbons (1976a) have shown that resorcinol may be completely catabolized by the enzymes of the orcinol pathway in *P. putida* O1. Orcinol hydroxylase is not very substrate-specific and can also catalyze the hydroxylation of resorcinol. The typical dependence of the OUR_{max} values on added substrate concentrations in the case of the bi-substrate system containing resorcinol and 5-methylresorcinol at equal concentrations is shown in Figure 16. Taking into account the values of $s_{y/x}$ (varied between $7.0 \cdot 10^{-4} \ldots 8.4 \cdot 10^{-3}$ $mgO_2/(l \cdot min \cdot MLSS)$) and the high values of the coefficient of regression ($R^2 = 0.97 \ldots 0.99$) for the data sets, all applied models were fit well and agreed with the experimental data.

Table 5. The mean values of kinetic parameter $V_{O_2,max}$ with the uncertainties of measurements (u_c) and percentage 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 (Paper II; III).

Bi-substrate system (1:1)	Random-order mechanism		Haldane kinetics		Michaelis-Menten kinetics	
	$V_{O_2,max}$, $mgO_2/(l\ min\ MLSS)$	% of additive sum	$V_{O_2,max}$, $mgO_2/(l\ min\ MLSS)$	% of additive sum	$V_{O_2,max}$, $mgO_2/(l\ min\ MLSS)$	% of additive sum
phenol – *p*-cresol	0.139 ± 0.042 (n=10)	40	0.258 ± 0.077 (n=10)	72	0.170 ± 0.068 (n=10)	43
phenol – *o*-cresol	0.143 ± 0.039 (n=10)	52	0.165 ± 0.054 (n=10)	57	0.157 ± 0.035 (n=10)	57
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 the experimental series

Table 6. The values of interaction parameters $I_{1,2}$ and $I_{2,1}$ for the studied bi-substrate systems containing both components at equal concentrations (Paper II; III).

Bi-substrate system (1:1)	$I_{1,2}$ [a)]	$I_{2,1}$
	range	range
phenol – *p*-cresol	0.46...8.67	0.01…1.64
phenol – *o*-cresol	0.60…1.57	0.16…4.51
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 *o*-cresol, *p*-cresol, resorcinol or 5-MR. Concerning the system of resorcinol – 5-MR, subscripts "1" and "2" mean resorcinol and 5-MR, respectively.

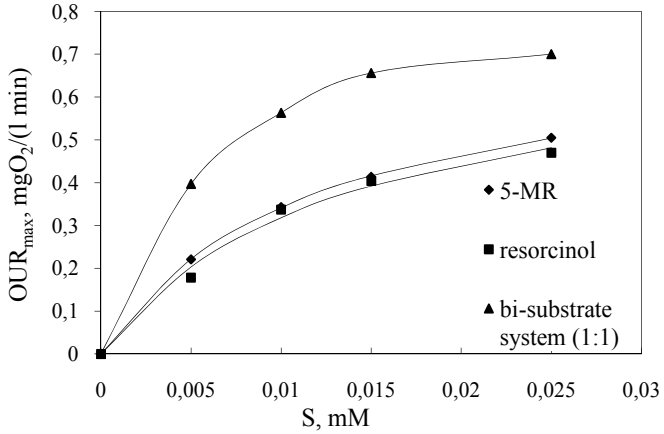

Figure 16. The dependence of the OUR_{max} values on added substrate concentrations for the bi-substrate system containing resorcinol and 5-methylresorcinol (5-MR) at equal concentrations. The symbols correspond to the measured data, whereas the solid line corresponds to the Michaelis-Menten kinetics. MLSS of specific activated sludge was 5.9 g/l (Paper II).

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) systems. The mean value of the actual $V_{O_2,max}$ formed 83%–88% of the additive sum of the $V_{O_2,max}$ values of the individual components when the Michaelis-Menten and the random-order mechanism applied. If the Haldane model was used the actual $V_{O_2,max}$ value was 94% of the assumed additive value (Paper II). However, the determined interaction parameters indicated that

5-methylresorcinol had a greater effect of inhibition on the biodegradation of phenol than *vice versa*, but yet in some experiments these compounds enhanced each other's biodegradation. The typical dependence of the OUR_{max} values on added substrate concentrations in the case of the bi-substrate system containing phenol and 5-methylresorcinol at equal concentrations is shown in Figure 17. The results illustrated that the models proposed adequately described the dynamic behaviours of biodegradation (Paper II).

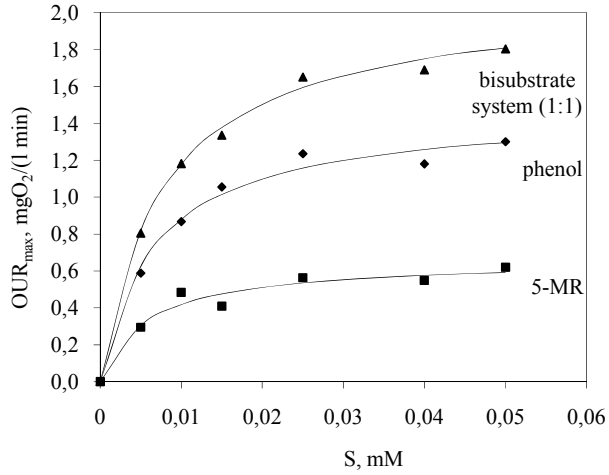

Figure 17. The dependence of OUR_{max} values on added substrate concentrations for the bi-substrate system containing phenol and 5-methylresorcinol (5-MR) at equal concentrations. The symbols correspond to the measured data, whereas the solid line describes the Michaelis-Menten and the random-order mechanism curves. MLSS of specific activated sludge was 5.9 g/l (Paper II).

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 lower values of $V_{O_2,max}$ from 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 (Paper II). The broad substrate specificity of phenol hydroxylase enables this enzyme to convert also resorcinol (Fialová *et al.*, 2003). Furthermore, other enzymes, including resorcinol monooxygenase, could also be involved in the degradation of resorcinol (Chapman and Ribbons, 1976b; Orupõld *et al.*, 2001). The obtained interaction parameters indicated that phenol had a stimulatory effect on the biodegradation of resorcinol, shown by the negative values, and that resorcinol inhibited the biodegradation of phenol. The values of $s_{y/x}$ (varied between $3 \cdot 10^{-3} \ldots 4.3 \cdot 10^{-2}$ $mgO_2/(l \cdot min \cdot MLSS)$ and the values of the 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 (Paper II).

The mean value of the actual $V_{O_2,max}$ formed 40%–72% of the additive sum of the $V_{O_2,max}$ values of the individual components for the system of phenol – *p*-cresol containing both substrates at equal concentrations. Among the 1:1 mixtures the highest $V_{O_2,max}$ value was obtained for the system of phenol – *p*-cresol by the Haldane kinetics equation. The Haldane model ($s_{y/x}$ = $4.1 \cdot 10^{-3}$… $3.9 \cdot 10^{-2}$ $mgO_2/(l \cdot min \cdot MLSS)$; R^2 = 0.92…0.99) appeared to be more suitable than the Michaelis-Menten model ($s_{y/x}$ = $8.6 \cdot 10^{-3}$…$3.9 \cdot 10^{-2}$ $mgO_2/(l \cdot min \cdot MLSS)$; R^2 = 0.74…0.97) or the random-order mechanism ($s_{y/x}$ = $1.2 \cdot 10^{-2}$… $5.4 \cdot 10^{-2}$ $mgO_2/(l \cdot min \cdot MLSS)$; R^2 = 0.67…0.99) to describe the biodegradation process in the bi-substrate system (1:1) of phenol – *p*-cresol due to the substrate inhibition effect at a higher substrate concentration (Paper III). The dependence of OUR_{max} on the substrate concentrations showed the deviation from the hyperbolic function. The maximum value of OUR_{max} was achieved at the concentration range of 0.015 mM–0.025 mM for both components, but at higher concentrations the OUR_{max} values tended to decrease (shown in Figure 18). The obtained interaction parameters indicated that phenol had a stronger inhibition effect on the biodegradation of *p*-cresol than *p*-cresol had on the biodegradation of phenol. When these compounds were studied as single substrates, the results indicated higher values of OUR_{max} and $V_{O_2,max}$ for phenol than those for *p*-cresol; however, activated sludge of the Kohtla-Järve WWTP had shown better affinity to *p*-cresol with the higher value of $V_{O_2,max}/K_S$ for *p*-cresol (Paper III). Clarke and Ornston (1975) have shown that in the case of phenol and cresols the substrates share common steps in their respective metabolic pathways. Under these circumstances, the specific uptake rates depend on the relative concentrations of the two components. The pathways involved in the degradation of phenol and *p*-cresol by *P. putida* are almost identical and many of the same enzymes are utilized in metabolizing both substrates (Sala-Trepat *et al.*, 1972). The broad substrate specificity of phenol hydroxylase enables this enzyme to convert also *p*-cresol (Fialová *et al*., 2003).

Concerning the bi-substrate system (1:1) contained phenol and *o*-cresol, the mean value of the actual $V_{O_2,max}$ formed 52%–57% of the additive sum of the $V_{O_2,max}$ values of the individual components. However, the dependence of the OUR_{max} on the substrate concentrations was mainly described by the hyperbolic function; the curve for the bi-substrate system remained lower than the curve of the dependence $OUR_{max} = f(S)$ for phenol. One explanation could be that there is the competition between substrates for the active site of an initial enzymes such as the phenol monooxygenase or the catechol 2,3-dioxygenase (Paper III). It has been shown in the literature (Bayly *et al*., 1966) that aerobic catabolism of *o*-cresol occurs without oxidation of the methyl group, after the formation of 3-methyl-catechol the ring-cleavage occurs via *meta* pathway. In typical metabolic pathway, phenol is converted by the phenol hydroxylase to catechol, which is further degraded through *ortho* or *meta* cleavage (Fialová *et al*., 2003). The interaction

parameters obtained in this thesis indicated that phenol and *o*-cresol had a similar mild inhibition effect on each other's biodegradation. The values of $s_{y/x}$ (varied between $4.4 \cdot 10^{-3} \ldots 8.6 \cdot 10^{-2}$ $mgO_2/(l \cdot min \cdot MLSS)$) and the values of the coefficient of regression ($R^2 = 0.80 \ldots 0.99$) for the data sets were received from the applied models and yielded an acceptable agreement between the model predictions and the experimental data (Paper III).

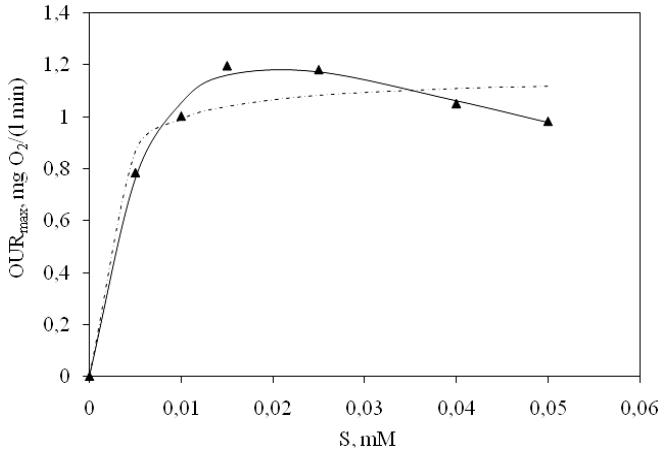

Figure 18. The relationship between OUR_{max} values and added substrate concentrations for the bi-substrate system containing phenol and *p*-cresol at equal concentrations. The symbols correspond to the measured data, whereas the dotted line is the Michaelis-Menten model curve and the solid line corresponds to the Haldane kinetics. MLSS of specific activated sludge was 7.2 g/l (Paper III).

The biodegradability of 4 different bi-substrate systems was also studied: phenol – *p*-cresol, phenol – resorcinol, phenol – 5-methylresorcinol, and phenol – *o*-cresol containing one substrate at a concentration of 0.1 mM and the other varied in the range of 0.005 mM–0.05 mM (Paper II; III). The results are presented in Table 7. The Michaelis-Menten kinetics (equation (17)), the random-order mechanism (equation (3)) and the Haldane model (equation (18)) were used to model the experimental data. To determine the interaction parameters the sum kinetic models of the unspecified type of interaction (equations (20), (21) and (22)) were applied. The Michaelis-Menten kinetics and the Haldane model did not describe adequately the biodegradation process due to the poor agreement between predicted and experimental data in the studied range of substrate concentrations. The Haldane model could be used only in the case of the bi-substrate systems of phenol and *p*-cresol (Paper II; III).

Table 7. 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 (Paper II; III).

Bi-substrate system	Random-order mechanism	Haldane kinetics	Sum kinetics	
	$V_{O_2,max}$, $mgO_2/(l\ min\ MLSS)$	$V_{O_2,max}$, $mgO_2/(l\ min\ MLSS)$	$I_{1,2}$[a)] range	$I_{2,1}$ range
phenol(0.1 mM) – *p*-cresol	0.239 ± 0.056 (n=5)	0.146 ± 0.100 (n=5)	0.47...18.65	0.31...6.54
phenol – *p*-cresol(0.1 mM)	0.222 ± 0.076 (n=5)	0.178 ± 0.096 (n=5)	4.23...38.27	0.01...0.36
phenol(0.1 mM) – *o*-cresol	0.285 ± 0.029 (n=5)	–	–0.18...0.97	1.51...13.13
phenol – *o*-cresol(0.1 mM)	0.297 ± 0.049 (n=5)	–	–0.09...0.76	0.03...8.62
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

[a)] Subscript "1" denotes phenol and subscript "2" denotes *o*-cresol, *p*-cresol, resorcinol or 5-MR;
n – the number of experimental series

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-methylresorcinol, followed by the systems of phenol(0.1mM) – resorcinol, phenol – 5-methylresorcinol(0.1 mM), and phenol – resorcinol(0.1mM). The lowest values of $V_{O_2,max}$ were observed for the mixture containing phenol – *p*-cresol(0.1 mM) by the random-order mechanism and for phenol(0.1 mM) – *p*-cresol by the Haldane kinetics.

The mean value of $V_{O_2,max}$ obtained by the random-order mechanism was higher for system of phenol(0.1 mM) – *p*-cresol than that of the system of phenol – *p*-cresol(0.1 mM). Concerning the Haldane model the opposite trend was observed. However, for the systems of phenol and *p*-cresol containing one substrate at 0.1 mM, the random-order mechanism ($s_{y/x}$ = $1.2 \cdot 10^{-2}$… $7.1 \cdot 10^{-2}$ $mgO_2/(l \cdot min \cdot MLSS)$; R^2 = 0.89…0.96) and the Haldane model ($s_{y/x}$ = $2.1 \cdot 10^{-2}$…$4.0 \cdot 10^{-2}$ $mgO_2/(l \cdot min \cdot MLSS)$; R^2 = 0.78…0.94) appeared to describe the biodegradation process. The lower value of $V_{O_2,max}$ for the system of phenol – *p*-cresol(0.1 mM) could be caused by the inhibition effect characteristic of *p*-cresol, which increased in higher concentrations of *p*-cresol. In higher concentratios of mixture the active sites of enzymes might be saturated with the molecules of substrates and as a result, an inactive complex could be formed. The obtained interaction parameters for both systems indicated that phenol had a stronger inhibition effect on the biodegradation of *p*-cresol than *p*-cresol had on the biodegradation of phenol. A similar trend was also observed in the case of the systems containing both substrates at equal concentrations (Paper III). Okpokwasili and Nweke (2005) observed that in dual-substrate system, sequential substrate utilization is represented by a large value of $I_{1,2}$ and a small value of $I_{2,1}$. Comparing the values of $I_{1,2}$ and $I_{2,1}$ for the studied mixtures of phenol – *p*-cresol presented in Tables 6 and 7, it could be the case also in this thesis. Concerning the different bi-substrate systems of phenol and *p*-cresol the dependence of OUR_{max} on substrate concentrations (depicted in Figure 19) showed a strong deviation from the hyperbolic function. The values of OUR_{max} at the concentration range of 0.005 mM – 0.025 mM were somewhat higher for the system of phenol(0.1 mM) – *p*-cresol than those values of the other mixtures. The reason could be that in lower concentrations of mixture the concentration of phenol was higher compared with the concentration of *p*-cresol, and therefore the biodegradation of phenol could be preferred. The results of the biodegradation of single substrates indicated higher values of OUR_{max} and $V_{O_2,max}$ for phenol than for *p*-cresol (Paper III).

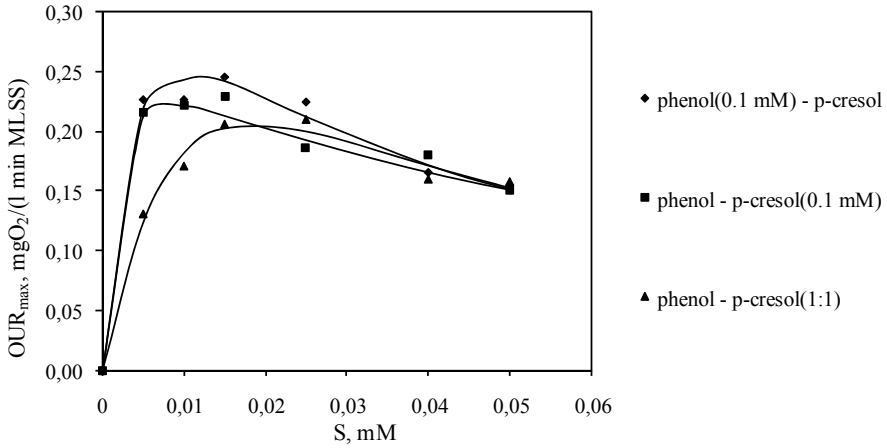

Figure 19. The relationship between OUR_{max} values and added substrate concentrations for the bi-substrate systems containing phenol and *p*-cresol. The symbols correspond to the measured data, whereas the solid line describes the Haldane kinetics. MLSS of specific activated sludge was 4.0 g/l (Paper III).

It was quite unexpected that the mean value of $V_{O_2,max}$ was a little higher for the system of phenol – *o*-cresol(0.1 mM) than that of the system of phenol(0.1 mM) – *o*-cresol, because phenol had higher values of the kinetic parameters ($V_{O_2,max}$, $V_{O_2,max}/K_S$) than did *o*-cresol, when these compounds were investigated as single substrates. However, the obtained interaction parameters for both systems of phenol – *o*-cresol indicated that *o*-cresol had a stronger inhibition effect on the biodegradation of phenol, which in turn had a mild inhibition or even enhancing effect on the biodegradation of *o*-cresol. However, in 1:1 mixture, phenol and *o*-cresol had a similar mild inhibition effect on each other's biodegradation. The values of $s_{y/x}$ varied between $9.5 \cdot 10^{-4} \ldots 4.8 \cdot 10^{-2}$ $mgO_2/(l \cdot min \cdot MLSS)$ and the values of R^2 were 0.73 to 0.99 in the case of the random order mechanism (Paper III).

The mean value of $V_{O_2,max}$ obtained by the random-order mechanism was higher for the system of phenol(0.1 mM) – resorcinol than for the system of phenol – resorcinol(0.1 mM). The obtained interaction parameters (shown in Table 7) for the system of phenol(0.1 mM) – resorcinol indicated that resorcinol had a stronger effect on the biodegradation of phenol than *vice versa*, although the concentration of resorcinol was lower in the mixture. An analogous observation was made for the systems 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 (Paper II). Taking into account the statistical parameters used for estimation of the model's suitability and adequacy, the random order mechanism indicated agreement with the experimental data in the case of the system of phenol(0.1 mM) – resorcinol

($s_{y/x}$ = 1.9·10^{-2}…5.2·10^{-2} mgO_2/(l·min·MLSS); R^2 = 0.94…0.96) and the system of phenol – resorcinol(0.1 mM) ($s_{y/x}$ = 6.9·10^{-3}…8.1·10^{-3} mgO_2/(l·min·MLSS); R^2 = 0.98…0.99). The dependence of the OUR_{max} on substrate concentrations for different bi-substrate systems containing phenol – resorcinol is given in Figure 20. The values of OUR_{max} at the concentration range of 0.005 mM – 0.025 mM were higher for the system of phenol(0.1 mM) – resorcinol than those values for other mixtures shown in Figure 20. In lower concentrations of 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 the OUR_{max} and $V_{O_2,max}$ for phenol than 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 mM – 0.025 mM (Paper II).

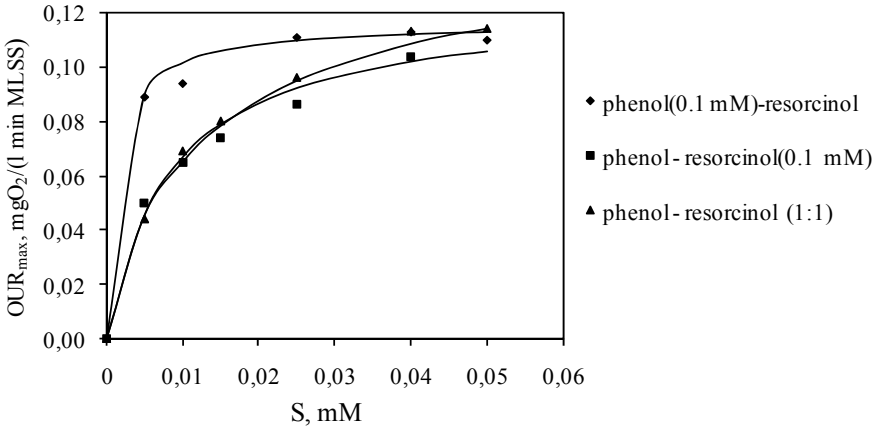

Figure 20. The dependence of OUR_{max} values on added substrate concentrations for different bi-substrate systems containing phenol and resorcinol. The symbols correspond to the measured data, whereas the solid line corresponds to the random order mechanism. MLSS of specific activated sludge was 6.5 g/l (Paper II).

The mean value of the $V_{O_2,max}$ was higher for the system of phenol(0.1 mM) – 5-methylresorcinol (5-MR) than for the 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 (Paper II). The obtained interaction parameters for the system of phenol(0.1 mM) – 5-MR indicated 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 a mild inhibition and in some tests even an enhancing effect on the biodegradation of 5-MR. The values of $s_{y/x}$ (varied between $1.3 \cdot 10^{-3} \ldots 4.3 \cdot 10^{-2}$ $mgO_2/(l \cdot min \cdot MLSS)$ and the values of the coefficient of regression ($R^2 = 0.91 \ldots 0.98$) for the data sets were received by the application of the random order mechanism (Paper II).

The interaction parameters obtained in this thesis indicated that the interaction between substrates depended on the samples and the properties of activated sludge, but also on the concentrations of both substrates in the mixture. Concerning the biodegradation of mixed substrates, several factors could play important roles: the possible interaction between substrates; the microbial community and the metabolic activity of activated sludge; and whether the compounds are degraded simultaneously or sequentially, and the metabolic pathways through which the degradation occurs. 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 mixture, the active site of enzymes may become saturated with the molecules of substrates and an inactive complex could be formed (Paper II; III). The uptake of one substrate does not affect the uptake of the other substitutable (simultaneously degraded substrates which can be separately transformed into products) substrate as long as their binding probabilities are independent (Brandt *et al*., 2003). In addition, substrate utilization rates cannot be assumed to be independent of one another, especially if there were a rate controlling enzymatic reaction at the convergence of the catabolic pathways (Hutchinson and Robinson, 1988). The specific uptake rates depend on the relative concentrations of both components. Although certain mixtures are degraded more rapidly than compounds present individually (Jahnke *et al.*, 1993), the biodegradation rate of aromatic mixtures may be much lower under natural conditions, mostly due to catabolite repression, but also due to insufficient nutrient concentrations or a limited bioavailability of the contaminant (Holtel *et al.*, 1994; Duetz *et al.*, 1996; Müller *et al.*, 1996b).

5. CONCLUSIONS

Pollution of the environment with anthropogenic compounds is a worldwide problem. The designation of certain organic chemicals as priority pollutants with specific effluent limits has created many challenges in modelling and operating of biotreatment processes. The biodegradability of different hydroxyl-, methyl- and dimethyl-substituted phenolic compounds characteristic to the wastewater of the oil-shale industry was studied both as single substrate and as bi-substrate systems at different initial concentrations in this thesis. The degradation behaviour of phenolic compounds was investigated by respirometry using activated sludge sampled from the Kohtla-Järve wastewater treatment plant (WWTP).

Based on the results presented in this thesis, the following conclusions can be made:

I. Activated sludge of the Kohtla-Järve WWTP was able to oxidize monohydric as well as dihydric phenolic compounds without an adaptation before performing the tests. However, differences existed in the maximum oxygen uptake rates as well as in the amounts of oxygen consumed per mole of substrate. The BOD_{st} values of the studied phenolic compounds formed 21%–28% of the theoretical oxygen demand (thOD) and 26%–50% of the BOD_7, showing the highest values for phenol. In the case of *o*-cresol the values of BOD_{st}/thOD and BOD_{st}/BOD_7 was only 10%–16% and 13%–22%, respectively. Among the studied substrates *o*-cresol had also the lowest value of maximum rate of oxygen uptake ($V_{O_2,max}$). The activated sludge of the Kohtla-Järve WWTP had better affinity to *p*-cresol with the lowest value of the half-saturation coefficient (K_S) and the highest value of $V_{O_2,max}/K_S$ for *p*-cresol occurring when these compounds were studied as single substrates. Knowing both the rate and extent of degradation is essential for an understanding of the behaviour of a compound and its persistence.

II. The biodegradability of phenolic compounds is related to their structures. It depended on the nature and the number of substituents of the aromatic nucleus. The results obtained showed that phenol, resorcinols and cresols were degraded by activated sludge faster than dimethylphenols. Phenol and cresols were shown to be easily biodegradable compared to dimethylphenols. However, in some experiments phenol and *p*-cresol exihibited an inhibitory behaviour to the microorganisms at the higher concentrations. The substrate inhibition effect started at 0.025 mM. In these cases, the Michaelis-Menten kinetics was not valid in the studied range of substrate concentrations and the degradation kinetics of phenol and *p*-cresol could be correlated well by the Haldane kinetics model.

III. It appeared that both the number and the position of the methyl group influenced the rate at which methylphenols were removed from the media. The *p*-substituted phenols are more readily degraded than the *m*- or the *o*-substituted ones. The best-degrading dimethylphenol was 3,4-dimethyl-

phenol, in which case the measured short-term oxygen demand was up 16% from the thOD and the BOD_{st}/BOD_7 value was 22%. 3,5-, 2,4- and 2,6-dimethylphenols did not degrade as single substrates in the time-frame of the short-term BOD measurement. However, the results of the BOD_5 and BOD_7 measurement showed a long-term degradation of dimethylphenols. The results of the short- and long-term measurements showed that 2,6-dimethylphenol was the worst-degrading phenolic compound investigated in this thesis and can be classified as poorly degradable compound. The ring cleavage retardation of that compound may be caused by the blocked hydroxylation step because both of the two methyl groups are in the *ortho* position relative to the hydroxyl group and another hydroxyl group cannot be inserted next to the initial hydroxyl group.

IV. The biodegradability of different bi-substrate systems: phenol – resorcinol; phenol – 5-methylresorcinol; resorcinol – 5-methylresorcinol; phenol – *o*-cresol; and phenol – *p*-cresol was also studied. Each contained both components at equal concentrations in the range of 0.005 mM – 0.05 mM, and additionally, contained one substrate at a concentration of 0.1 mM and the other varied in the above-mentioned range. The specific oxygen uptake rates depended on the relative concentrations of both components. 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) systems. 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, showing 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), and phenol – resorcinol(0.1mM). Concerning these bi-substrate systems the results showed that the Michaelis-Menten kinetics and the Haldane model did not fit the measured data in the studied range of substrate concentrations, the Haldane model could be used only in the case of bi-substrate systems of phenol and *p*-cresol.

V. 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. It's not the rule that the component with the higher concentration has the greater effect on the biodegradation of the component with lower concentration, but this is also related to the nature of the substrates. For example, in the both cases of the systems of phenol – *o*-cresol containing one substrate at 0.1 mM, *o*-cresol showed stronger inhibition effect on the biodegradation of phenol, which in turn had a mild inhibition or even enhancing effect on the biodegradation of *o*-cresol. In 1:1 mixture, phenol and *o*-cresol had a similar mild inhibition effect on each other's biodegradation, however, the curve of the dependence of $OUR_{max} = f(S)$ for the bi-substrate system remained lower than that of phenol measured individually. Based on the interaction parameters obtained for all studied bi-substrate systems, purely competitive inhibition

($I_{i,j} = K_{S,j}/K_{S,i}$) or purely additive (no-interaction) effect between these substrates was not observed.

VI. The values of the biodegradation parameters obtained with various activated sludge samples varied considerably. The time-dependent differences between these values could be explained by the variations in the properties of activated sludge, which in turn are conditioned by the variations in the operating conditions and changes in the concentration and the properties of the influents to the WWTP.

Data obtained in this thesis could be used for controlling and optimization of the operation of the WWTP to ensure stable wastewater treatment even under shock loading, because BOD_{st}, $V_{O_2,max}$, V_{max} and K_S, as functions of the compound undergoing biodegradation and the microbial community performing the degradation, yield information about activated sludge processes and the biodegradability of substrate in the WWTP. As oxygen uptake profiles yield the same information as substrate removal profiles, the determination of the oxygen uptake rate makes possible to assess the ability of a bacterial population to remove substances from the WWTP, to determine the effect of the compounds on the bacteria and also to quantify substrate removal. During the treatment of the various kinds of substrates in industrial wastewaters and because of different loads, oxygen uptake may vary considerably. To achieve efficient aerobic treatment, a sufficient amount of oxygen should be supplied to the aeration tank of the biological treatment plant. Therefore, the maximum rate of oxygen uptake is of great importance as a parameter indicating the allowable substrate addition into the aeration tank. Biodegradation in mixed substrate systems, such as in wastewater treatment systems, could be more extensive than the estimated parameters would indicate.

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7. SUMMARY IN ESTONIAN

Fenoolsete ühendite biodegradeeritavus üksik- ja segasubstraatidena aktiivmuda toimel

Keskkonna üha suurem saastumine erinevate antropogeense päritoluga ühenditega on osutunud probleemiks kogu maailmas. Kuna fenoolid on toksilised enamikule elusorganismidele ning mõjuvad inhibeerivalt mitmesugustele biokeemilistele protsessidele, on fenoolsete ühendite lagundatavust uurinud varasemalt mitmed autorid. Kuid reostusküsimused on sageli väga spetsiifilised ning vajavad seetõttu sõltuvalt probleemi olemusest ja tekkepiirkonnast eriviisilist käsitlemist. Veeseaduse alusel on ühealuselised fenoolid kantud veekeskkonnale ohtlike ainete nimistusse kui ühendid, mille veeheidet või sattumist vette muul viisil peab piirama, kuna need võivad oma mürgisuse, püsivuse või bioakumulatsiooni tõttu põhjustada ohtu inimese tervisele ning kahjustada teisi elusorganisme või ökosüsteeme. Ühealuseliste fenoolide heide või mingil muul moel veekeskkonda sattumine on Eestis tingitud peamiselt põlevkiviõli tootmisest, põlevkivi tootmisjäätmete ladestamisest Ida-Virumaal ja põlevkiviõli ettevaatamatust kasutamisest aastakümnete jooksul. Aastal 2009 oli veekeskkonda suunatud ühe- ja kahealuseliste fenoolide summaarne heide kogu Eestis 2780 kg, sellest 2260 kg Ida-Virumaal. 2002. aasta summaarsed heited olid vastavalt 7413 kg ja 7246 kg (Estonian Environment Information Centre, 2001 and 2010). Fenooliheite piiramiseks ja kontrolliks on ettevõtetel vee erikasutuslubades kohustus seirata heitvee fenoolisisaldust. Vette suunatavate fenoolide heidete vähendamise riiklik programm aastani 2014 näeb perioodi lõpuks ette fenooliheidete vähendamise ühe tonnini aastas (Minister of Environment of Estonia, 2010).

Käesolevas doktoritöös uuriti põlevkivitööstuse reoveele iseloomulike fenoolsete ühendite – fenooli, hüdroksü-, metüül- ja dimetüülfenoolide - biolagundatavust nii üksik- kui segasubstraatidena Kohtla-Järve biopuhastusseadme aktiivmuda mikroobikoosluse toimel. Fenoolsete ühendite biodegradeeritavuse hindamiseks kasutati nii lühiajalist kui ka biokeemilist hapnikutarvet ning biodegradatsiooniprotsessi iseloomustavate kineetiliste parameetrite väärtusi. Biodegradatsiooni iseloomustavate parameetrite väärtused varieerusid erinevate aktiivmuda proovide korral märgatavalt, kuna fenoolsete ühendite lagunemise kiirus sõltub oluliselt aktiivmuda omadustest ja kontsentratsioonist, mis omakorda on mõjutatud puhasti sissevoolust ja töörežiimist.

Tulemused näitasid, et fenoolide biodegradeeritavus sõltub ühendi struktuurist: aromaatse tuuma asendajate arvust, nende asendist ja omadustest. *p*-asendatud fenoolid on kergemini lagundatavad kui *o*- ja *m*-asendatud. Kohtla-Järve aktiivmuda lagundas kergesti fenooli, *p*-kresooli, resortsinooli ja 5-metüülresortsinooli, näidates suurimat afiinsust *p*-kresooli suhtes. Uuritud dimetüülfenoolidest lagunes lühiajalisel hapnikutarbe (BOD_{st}) meetodil kõige paremini 3,4-dimetüülfenool, kuid 3,5-, 2,4- ja 2,6-dimetüülfenool ei lagunenud katse läbiviimisaja jooksul. Tõenäoliselt võib dimetüülfenoolide degradat-

siooniks vajalike ensüümide aktiveerimine ja/või indutseerimine toimuda pikema aja jooksul, kui kestab lühiajalise hapnikutarbe (BOD_{st}) määramine, kuna biokeemilise hapnikutarbe (BOD_5 ja BOD_7) määramisel täheldati nende lagunemist. Nii lühiajalise kui ka biokeemilise hapnikutarbe mõõtmise tulemused näitasid, et uuritavatest fenoolidest oli kõige raskemini lagundatav 2,6-dimetüülfenool. Selle põhjuseks võib olla aromaatse tuuma lõhustumise aeglustumine tuuma hüdroksüülimise etapi blokeerumise tõttu. 2,6-dimetüülfenoolis paiknevad mõlemad metüülrühmad algse hüdroksüülrühma suhtes *orto*-asendis ja seetõttu ei saa hüdroksüülimise käigus viia teist hüdroksüülrühma esialgse hüdroksüülrühma kõrvale.

Varasemalt on fenoolsete ühendite biodegradatsiooni ja kataboolseid radu uuritud valdavalt üksiksubstraatidena puhaskultuurides või nende segudes, kuid keskkonna-alaselt on samuti vajalik teave ühendite lagunemise kohta süsteemides, mis sisaldavad ühendite segusid ja erinevaid mikroobikooslusi. Kuna looduslikes keskkondades ja reovees on tegemist mitme substraadi ja ainete segudega, siis on oluline hinnata uuritavate segude komponentide vastastikust mõju erinevatel kontsentratsioonidel üksteise biodegradatsioonile. Sega-substraatide biodegradatsioonil on tähtis roll substraatide vahelistel mõjudel ja sellel, milline on mikroobikooslus ning kas ühendeid lagundatakse samaaegselt või järjestikuliselt ja milliseid metaboolseid radu mööda biodegradatsioon toimub. Substraatide vaheliste vastastikmõjude korral on samuti oluline, kas need ühendid konkureerivad teineteisega nende lagundamiseks vajalike ensüümidega seondumisel või nende lagundamist initsieerivad erinevad substraadispetsiifilised ensüümid. Samuti võivad segu kõrgematel summarsetel kontsentratsioonidel ensüümi aktiivtsentrid küllastuda substraatide molekulidega ja selle tagajärjel moodustuda mitteaktiivne kompleks. Uurides fenoolide biodegradeeritavust bisubstraatsetes segudes osutus, et fenooli ja *p*-kresooli ning fenooli ja *o*-kresooli korral esines substraatne inhibeerimine, mis suurenes kontsentratsiooni kasvades. Erinevate bisubstraatsete segude $V_{O_2,max}$ väärtuste võrdlemisel saadi kõrgemad väärtused fenooli ja resortsinoole sisaldavate segude korral. Bisubstraatsete segude biodegradatsiooni kirjeldamiseks ei sobinud Michaelis-Menteni kineetika, katseandmete kirjeldamisel andsid paremaid tulemusi juhuslik mehhanismitüüp ja summakineetika ning fenooli ja *p*-kresooli segude korral ka Haldane kineetika. Substraatide vahelised vastastikmõjud varieerusid erinevate aktiivmuda proovide korral ning sõltusid nii mõlema substraadi kontsentratsioonist segus kui ka kumbagi komponendi potentsiaalsest toksilisusest aktiivmuda mikroobikooslusele. Määratud interaktsiooniparameetrite alusel ei täheldatud uuritud substraatide vahel puhast konkureerivat inhibeerimist ($I_{i,j} = K_{S,j}/K_{S,i}$) ega ka täielikku teineteisest sõltumatut aditiivset efekti.

Kohtla-Järve biopuhasti aktiivmuda on võimeline uuritud fenoolseid ühendeid lagundama ilma eelneva adaptatsioonita, kuna fenoolide biodegradatsiooniks vajalik spetsiifiline mikroobikooslus on juba välja kujunenud vastavaid aineid sisaldava reovee töötlemise käigus. Lühiajalise hapnikutarbe määramise

meetodiga saadud parameetrid (BOD_{st}, $V_{O_2,max}$, V_{max}, K_S, K_i) iseloomustavad orgaaniliste reoainete biodegradatsiooni puhastusprotsessis. Lühiajalise meetodi eeliseks traditsioonilise biokeemilise hapnikutarbe (BOD_5 ja BOD_7) määramise ees on kiiresti saadavad tulemused, mida saab rakendada reoveepuhasti töö optimeerimiseks, pideval monitoorimisel ka puhastusprotsessi toimimise hindamiseks ja vajadusel avariiolukordades kiirete meetmete rakendamiseks.

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